Proteolytic Cleavages of Molecules Involved in Antigen Processing and Presentation: A Thesis

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PROTEOLYTIC CLEAVAGES OF MOLECULES INVOLVED IN ANTIGEN PROCESSING AND PRESENTATION

A THESIS PRESENTED

BY

LAWRENCE JAMES THOMAS

Submitted to the Faculty of the University of Massachusetts Medical School in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY IN MEDICAL SCIENCES

August 1989

Pharmacology
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PROTEOLYTIC CLEAVAGES OF MOLECULES INVOLVED IN ANTIGEN PROCESSING AND PRESENTATION

A Thesis
By
LAWRENCE JAMES THOMAS

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August 1989
DEDICATION

This thesis is dedicated to my parents

Virginia R. and Andrew H. Thomas.
ACKNOWLEDGMENTS

The progress of my doctoral education has been aided by more people than I could possibly mention here by name. I am truly grateful to each of them.

I would, however, particularly like to thank Drs. William Elliott, David Kostyal, Quoc Nguyen, Ms. Lisa Phillips, Hannah Qvistback and JoAnn Buczek, who contributed significantly to the completion of this research. I would also like to thank the secretarial staff of the pharmacology department, Ms. Judy Verdini, Marion Bonin, Lena DeSantis, Patt Downe and Gail Phillips; and Mr. Daniel Mullen who was absolutely essential to the production of the figures included here.

Above all I am greatly indebted to Dr. Robert Humphreys who took the time to be my advisor. In many respects he made the extra effort to provide me with an exceptional learning experience.
The overall goal of my thesis research was to understand better the mechanisms that control antigen processing and presentation by class II MHC molecules. Towards this goal I investigated ways in which the physical structure and post-translational modifications of the class II MHC alpha and beta chains and associated molecules might serve to regulate antigen processing and presentation. Specifically, I investigated (1) a hypothesis that I might aid binding of foreign antigenic peptides to the class II MHC foreign antigen binding site (desetope), and the application of this hypothesis to the prediction of class II-presented peptides; (2) the proteolytic cleavage of I to p25; (3) the proteolytic cleavage of the class II MHC alpha and beta chains, and (4) the phosphorylation of I and the alpha and beta chains.
In exploring the hypothesis that amphipathic alpha helical peptides digested from foreign antigen, bind to the class II MHC desetope, to be presented to T cell receptors, we found such an extended, amphipathic helix in I₁ (Phe₁₄₆-Val₁₆₄). A hypothesis was developed that this amphipathic alpha helix of I₁ bound to the desetope of class II MHC molecules, and remained there from time of synthesis until catalyzing the charging of the desetope with a foreign peptide. This region of I₁ could then be considered to be the prototypic T cell-presented peptide and the "strip-of-helix" algorithm was developed to search the sequences of proteins for similar amphipathic alpha helices. Such peptides might bind to the class II MHC desetope and have a high probability to be presented to the T cell.

The strip-of-helix algorithm calculated the mean hydrophobicity (from Kyte-Doolittle values; Kyte and Doolittle, 1982) of sets of amino acids in axial strips down sides of helices for 3 to 6 turns, at positions n, n+4, n+7, n+11, n+14, and n+18. Peptides correlating well with T cell responsiveness had: (1) 12 to 19 amino acids (4-6 turns of an alpha helix), (2) a strip with highly hydrophobic residues, (3) adjacent, moderately hydrophilic strips, and (4) no prolines to break the helix. This algorithm predicted 10 of 12 T cell-presented peptides in 7 well-studied proteins.

In a study of the post-translational modifications of I₁, an early proteolytic pathway of the destruction of I₁, resulting in the generation of p25, was described. This 25,000 dalton protein, seen in immunoprecipitates with antibodies to class II MHC molecules or to
I\textsubscript{1} was shown to be a C-terminal fragment of a high mannose form of I\textsubscript{1}. The evidence for this conclusion includes the following results. \[^{35}\text{S}]\text{methionine-labeled I}\textsubscript{1} and associated molecules were immunoprecipitated, denatured, resolubilized and subjected to a second immunoprecipitation with various antibodies. Two antisera to C-terminal peptides of I\textsubscript{1} (183-193 and 192-211), but not an antiserum to an N-terminal peptide (12-28), immunoprecipitated p25. A monoclonal antibody (mAb) to I\textsubscript{1} immunoprecipitated \[^{35}\text{S}]\text{methionine-labeled p25 but not [}^{35}\text{S}\text{cysteine-labeled p25, consistent with the loss of a portion of I}\textsubscript{1} containing the only cysteine in I}\textsubscript{1}, Cys\textsubscript{28}. \[^{35}\text{S}]\text{methionine pulse-chase labeling demonstrated the maximal appearance of p25 at 20-40 min chase times. p25 molecules were reduced to about 10.5 kD by treatment with endoglycosidases F and H. p25 was, therefore, generated from a high mannose form of I\textsubscript{1} in the ER or cis-Golgi. This finding could either implicate that site for class II MHC desetope charging with foreign peptides or reflect a mechanism for degradation of "excess" I\textsubscript{1} molecules in the ER. Digestion of class II MHC antigen-I\textsubscript{1} complexes with various proteases yielded fragments, migrating at and near p25 in 2-D electrophoretic gels, which were relatively resistant to further digestion. This observation was consistent with the presence of relatively protease-resistant secondary structures (domains) and a relatively protease-sensitive (IgG hinge-like) region in I\textsubscript{1} near its insertion into the membrane.

In a study of the post-translational modifications of the class II MHC alpha and beta chains, well conserved pairs of basic amino acids
in the sequences of these molecules were observed. It was hypothesized these could be sites for proteolytic cleavage, as preceded in other systems (i.e. proinsulin processing). These potential cleavage sites fall in significant locations with respect to the deduced structure of the class II MHC desetope, supporting the hypothesis that these cleavages might either aid or destroy antigen presenting functions. To test this hypothesis we looked for remnant polypeptides of the alpha and beta chains. Polypeptides were observed in gels of immunoprecipitated class II MHC complexes. To identify if such polypeptides were derived from the alpha and beta chains, immunoblotting to electrotransferred polypeptides was attempted, with antisera made to synthesized peptides that mimicked eight regions of the alpha and beta chains. These antisera were produced and characterized by dot blotting, ELISA, western blotting, and immunoprecipitation of native and denatured material. One antiserum, to an alpha chain peptide (77-88), blotted to a polypeptide immunoprecipitated by anti-class II MHC antiserum. This observation supported the hypothesis that the alpha and beta chains undergo proteolytic cleavages, possibly in the control of antigen presentation.

It was also demonstrated that I, and the alpha and beta chains can be phosphorylated under varying culture conditions, but this project was not pursued.
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ABBREVIATIONS

ABTS, 2,2' -azino-di-(3-ethyl-benzthiazoline-sulfonate)

BDT, bis-diazohtized toldine

BSA, bovine serum albumin

ER, endoplasmic reticulum

EDAC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride

EDTA, ethylenediaminetetraacetic acid

ELISA, enzyme-linked immunosorbent assay

Endo F, endoglycosidase F

Endo H, endoglycosidase H

FCS, fetal calf serum

HRP, horseradish peroxidase

IEF, isoelectric focusing

IgG, immunoglobulin G

I1, the electrophoretically invariant glycoprotein which associates noncovalently with class II MHC alpha and beta chains

I1-CS, chondroitin sulfate form of I1

Ip, sialic acid-derivatized forms of I1

KLH, keyhole limpet hemocyanin

LIP, leupeptin-induced proteins

mAb, monoclonal antibody

mIgM, membrane immunoglobulin M

MHC, major histocompatibility complex

NEPHGE, nonequilibrium pH gradient gel electrophoresis

NRS, normal rabbit serum
PBS, phosphate-buffered saline
SDS, sodium dodecylsulfate
TcR, T cell receptor (for class II MHC antigen-presented foreign antigen)
TBS, Tris-buffered saline
TRIS, tris(hydroxymethyl)aminomethane
CHAPTER I

Introduction

A. Specific Aims.

The overall goal of my thesis research has been to understand better the mechanisms that control antigen processing and presentation by class II MHC molecules. Towards this goal I have investigated the following questions:

1. What is the function of $I_1$? Specifically:
   a. What aspects of the structure of $I_1$ would give a clue to its function and mechanism?
   b. What structural modifications does $I_1$ undergo?

2. Do the class II MHC alpha and beta chains undergo proteolytic cleavage and other structural modifications in the control of antigen processing and presentation?

B. Literature Review.

Antigen processing and presentation in B cells. The presentation of foreign antigen by a B cell to a CD4$^+$ T lymphocyte involves surface immunoglobulin-mediated internalization, as indicated in Fig.
1.B.1. This binding serves to concentrate and focus antigen to B cells which possess immunoglobulin genes rearranged to bind the antigen of interest. This immunoglobulin-mediated internalization limits activation to B cells which have a high probability to produce soluble immunoglobulin specific to the foreign antigen (Rock et al., 1984; Lanzavecchia, 1985; Tony and Parker, 1985).

In many cases antigens have been demonstrated to be altered by the antigen presenting cells, and this alteration can be simulated by limited proteolytic degradation (Shimonkevitz et al., 1983; Buus and Werdelin, 1986a). As is the case with other antigen presenting cells, B cells must process antigen before presentation, (Lanzavecchia, 1985). Although it has been suggested that binding of processed peptide to class II MHC alpha and beta chains could occur on the cell surface (Buus and Werdelin, 1986a), most likely the endosomal compartment where antigen is processed fuses with the class II-containing compartment intracellularly, and binding occurs there. Cresswell (1985) demonstrated that transferrin-neuraminidase conjugates, internalized by means of receptor-mediated endocytosis, can interact with newly synthesized class II MHC proteins and associated molecules, and cause desialylation of Iα and the beta chain. This observation supported the hypothesis that the compartment containing internalized antigen can fuse with the compartment containing the newly synthesized class II MHC alpha and beta chains.

In the compartment containing processed peptide and the class II MHC proteins, certain peptides are selected for binding. Since not all peptides from foreign antigen are presented by the class II complex,
some type of selection criteria probably exists. Attempts to predict which peptides are presented by class II MHC molecules are dealt with in a section below. The process by which class II complexes bind peptide is not understood. When measured with peptides and purified class II MHC antigens, the association rate for binding of class II presented peptides to purified class II MHC antigens (of the respective MHC alleles which restrict presentation of the peptides) has a high affinity at equilibrium \( (k_d = 3 \times 10^{-6} \text{ M}) \), but a slow formation rate \( (k_a = 1 \text{ M}^{-1}\text{sec}^{-1}) \) (Buus et al., 1986b). In vivo, one might expect some chemically definable mechanisms to catalyze desetope charging with digested, foreign peptides. Attempts to define mechanisms which might catalyze the interactions of such peptides in vivo and/or regulate associations of peptides and class II MHC antigens at the time of charging or discharging, has led to the search for accessory proteins or enzymes which might regulate or catalyze desetope charging.

After the class II MHC alpha and beta chains have bound peptide, the complex is moved to the cell surface. Specific T cells that recognize the class II MHC proteins with bound antigen are activated. The activated T cells release factors which activate the B cell and promote proliferation and differentiation. The B cell matures to a plasma cell and produces antibody of the type that was originally on the cell surface.

**Structure of class II-presented peptides.** Of the many peptides
obtained by proteolytic digestion of foreign protein antigens, only a few can be presented to T cells. For example, only two peptides of lysozyme (34-45 and 46-61) have been found to account for recognition by 8 of 10 T cell I-A<sup>k</sup>-restricted clones which were originally selected with intact antigen (Allen et al., 1985). An additional peptide (81-96) is recognized with I-A<sup>b</sup>-restricted clones (Shastri et al., 1985). What primary or secondary structural characteristics define peptides which are finally selected for surface expression in complexes with class II MHC molecules?

Following a structural analysis of the peptides responsible for T cell reactivity to well-studied protein antigens, a hypothesis was developed by DeLisi and Berzofsky (1985), and refined by Margalit et al. (1987). That hypothesis suggested that amphipathicity, the presence of opposing hydrophilic and hydrophobic surfaces, in some nonrandom, organized and stable, secondary structure, is required for a peptide's presentation to the T cell receptor (TcR). They postulated that the hydrophobic surface binds relatively nonspecifically to the desetope of class II MHC antigens, and the hydrophilic surface is recognized by a complementary TcR. This algorithm quantitated the moment of hydrophobicity in 7 amino acid stretches in the periodicities of 100° (alpha helix), 120° (3<sub>10</sub> helix), or 180° (beta-pleated sheet). It selected peptides with an axial strip of aliphatic, hydrophobic amino acids and, otherwise, variably hydrophilic amino acids and ranked them for best fit. Generally, this technique predicted sequences which stimulated T cell clones, especially when alpha helices were assumed to be present (Spouge et
An alternative method for the prediction of T cell-recognized epitopes has been proposed (Rothbard and Taylor, 1988a; Rothbard et al., 1988b). The authors analyzed known cytotoxic and helper T cell epitopes for similarities and noted similar motifs in the sequences which can be summarized as: (charged or glycine) - (hydrophobic) - (hydrophobic) - (polar or glycine) or, (charged or glycine) - (hydrophobic) - (hydrophobic) - (hydrophobic or proline) - (proline or glycine) - (proline or glycine). This method did not rank putative peptides with regard to their probability or efficiency of class II MHC presentation. The result was the identification of peptides similar to those identified by the DeLisi and Berzofsky method, above.

The chemistry of the class II MHC proteins. The class II MHC molecules in the human are derived from the HLA-D region of the MHC on chromosome 6. Although several loci are present in this region, the expressed class II molecules fall into three isotypes, HLA-DR, HLA-DQ and HLA-DP. Each of these isotype loci contain the genes for at least one expressed alpha and beta chain (Trowsdale and Campbell, 1988).

The alpha chain consists of about 233 amino acids (Figueroa and Klein, 1986) and migrates at about 33-34 kD (Shackelford et al., 1982), as shown in Fig. 1.B.2. The class II MHC alpha chain contains two N-linked oligosaccharide chains, one high mannose chain at Asn78, and a complex N-linked carbohydrate at Asn118 (Shackelford and Strominger, 1983; Claesson-Welsh et al., 1986b). Further, there
is evidence that the alpha chain might contain an O-linked carbohydrate chain (Nishikawa et al., 1979; Claesson-Welsh et al., 1986b).

The beta chain consists of about 238 amino acids (Figueroa and Klein, 1986) and migrates at about 27-30 kD (Shackelford et al., 1982). The beta chain contains one N-linked oligosaccharide processed to a complex form and no O-linked chains (Shackelford and Strominger, 1983; Claesson-Welsh et al., 1986b).

It has been shown that both the alpha and beta chains can be sulfated, however that sulfate does not attach to an N-linked carbohydrate (Sant et al., 1988).

The chemistry of the I\textsubscript{a} gene products. A nonpolymorphic chain was found in association with the class II MHC alpha and beta chains in both murine (Jones et al., 1978) and human systems (Charron and McDevitt, 1979). This chain has been called the invariant chain, In chain, I\textsubscript{a}, I\textsubscript{i}, \gamma chain, p33 and p32. The I\textsubscript{a} gene is on chromosome 5 (Claesson-Welsh et al., 1984), while the MHC is located on chromosome 6. The gene has been cloned and sequenced, and has been shown to have its N-terminus on the cytoplasmic side of the membrane (Claesson et al., 1983b; Kudo et al., 1985; O’Sullivan et al., 1986), as seen in Fig. 1B.2. The putative transmembrane region is from position 31-56.

The I\textsubscript{a} gene has several protein products, utilizing alternate initiation sites and an extra exon. The use of the two in phase AUGs
to produce two different forms of the molecule is depicted in Fig. 1.B.3. The major form, p33, commonly known as \( I_1 \), lacks the 16 amino acid region that is included in the alternative forms (p35 or \( \gamma_2/\gamma_3 \)) (Quaranta et al., 1984; Strubin et al., 1986a; Strubin et al., 1986b; O'Sullivan et al., 1987).

The \( I_1 \) gene also contains an exon (termed 6b, Fig. 1.B.3) which, if included in the transcript, will produce the species called p41 (Yamamoto et al., 1985; Strubin et al., 1986b; O'Sullivan et al., 1987). A species called p43, which is only occasionally seen in vivo, is the result of the inclusion of this extra exon and use of the alternate initiation site which adds 16 amino acids (Strubin et al., 1986b; O'Sullivan et al., 1987).

\( I_1 \) has both two O-linked and two N-linked oligosaccharide units (Machamer and Cresswell, 1982; Charron et al., 1983; Machamer and Cresswell, 1984; Rudd et al., 1985; Claesson-Welsh et al., 1986b). N-linked glycosylation sites are at positions Asn\(_{114}\) and Asn\(_{120}\), as predicted from the primary sequence (Claesson et al., 1983b). Both of the N-linked carbohydrate sidechains are processed to a complex form with sialic acid on one or both of these chains (Rudd et al., 1985). One of the O-linked chains might be on Thr\(_{156}\), from experiments presented in this thesis.

A product of the \( I_1 \) gene is also the core protein of the class II associated chondroitin sulfate proteoglycan (CSPG, \( I_1 \)-CS), in both mice and humans (Sant et al., 1983; Sant et al., 1984; Sant et al., 1985a; Sant et al., 1985c; Giacoletto et al., 1986). This molecule migrates in a heterogeneous manner from 40 kD to 180 kD.
(Giaucoletto et al., 1986; Bono et al., 1987; Sorli and Humphreys, unpublished observations). Although a function for this molecule has not been elucidated, it seems that only a small fraction (2-5%) of class II MHC alpha and beta chains contain this species, or that it associates with the class II complex rapidly and transiently (Sant et al., 1985b). Further, Ii-CS seems to be surface-expressed (Sant et al., 1985b) and no class II MHC positive cells have been found that did not also express Ii-CS (Bono et al., 1987).

Ii has been shown to bind palmitic acid at Cys28, located on the cytoplasmic tail, close to the transmembrane region. It is probably added before N-linked glycosylation, since it is present in cells exposed to tunicamycin, which inhibits N-linked glycosylation, and if the addition of the palmitic acid is inhibited with cerulenin, N-linked glycosylation does not occur (Koch and Hämmerling, 1986).

It has also been shown that Ii can be sulfated, however that sulfate does not attach to an N-linked carbohydrate (Sant et al., 1988).

A degradative pathway for a fully processed form of Ii, probably in a post-Golgi compartment, has been described (Blum and Cresswell, 1988; Nguyen et al., 1988). When cells were incubated with leupeptin, two N-terminal remnants of Ii, p21 and p10, were produced. These peptides contain fully processed oligosaccharide chains and were produced maximally 2 to 5 hr after synthesis. The conclusion from these experiments was that these proteins, p21 and p10, were produced from Ii by a leupeptin-insensitive enzyme, and were normally, quickly degraded by a leupeptin-sensitive enzyme to
small undetected peptides.

**Associations of I\(_i\) with the alpha and beta chains.** I\(_i\) is cotranslationally associated with the alpha and beta chains via their extracytoplasmic regions (Marks and Cresswell, 1986) and inserted in the endoplasmic reticulum (Claesson and Peterson, 1983a; Kvist et al., 1982). They are transported together through the Golgi, and most or all of the I\(_i\) dissociates thereafter (Machamer and Cresswell, 1982; Claesson and Peterson, 1983a). There is a large pool of free I\(_i\) in excess of the quantities of the alpha and beta chains (Machamer and Cresswell, 1982; Kvist et al., 1982; Thomas and Humphreys, unpublished observations). Kvist et al. (1982) presented evidence that this pool of free I\(_i\) never leaves the ER, however observations in this lab imply otherwise. In immunoprecipitates of I\(_i\) there are forms of I\(_i\) processed in the Golgi (complex N-linked and O-linked carbohydrate) that are in excess of the alpha and beta chains, implying that some free I\(_i\) does leave the ER (unpublished observations).

The work of Nowell and Quaranta (1985) supported the idea that fully processed I\(_i\) is removed from the class II MHC proteins in an acidic compartment, which might be the same site as that where antigen processing and binding occur. They demonstrated that cells incubated with chloroquine, which disrupts lysosomal function, had class II complexes that contained I\(_i\) in association with alpha and beta chains. In untreated cells a far larger proportion of class II MHC
alpha and beta chains lacked $I_1$. In other experiments, chloroquine has been shown to inhibit antigen presentation \textit{in vitro} in phagocytic and non-phagocytic cells, presumably by inhibiting degradation of antigen (Ziegler and Unanue, 1982; Grey and Chestnut, 1985).

While it has been clearly shown that the class II MHC alpha and beta chains are seen on the cell surface, the question of the surface expression of $I_1$ (either free or in the class II complex) has been less definitive. Koch \textit{et al.} (1982) did an experiment where surface-expressed molecules were labeled with lactoperoxidase-mediated radiiodination, and then immunoprecipitations were attempted. They claimed to be able to immunoprecipitate surface-iodinated murine $I_1$ without alpha or beta chains, with an anti-$I_1$ antibody; but immunoprecipitated radiiodinated alpha and beta chains without $I_1$ with an anti-class II MHC antibody. They concluded $I_1$ was surface-expressed, but only free of class II complexes. Quaranta \textit{et al.} (1984) could not detect any $I_1$ on the cell surface by immunofluorescence with a monoclonal antibody to $I_1$. Accolla \textit{et al.} (1985) could not detect $I_1$ on the Raji B cell line, using lactoperoxidase-mediated radiiodination, tritiated NaBH$_4$ to label the oligosaccharide, or flow microfluorometry. Claesson and Peterson (1983a) addressed the question if $I_1$ was present on the cell surface by binding radiolabeled cells to poly(ethylенимине)-coated beads, rupturing the cells, and eluting the bound components. They saw only terminally processed alpha, beta and $I_1$, indicating that some $I_1$ might be present on the cell surface. Claesson-Welsh \textit{et al.} (1986a)
demonstrated that $I_\text{a}$ was present on the surface of immunocompetent Langerhans cells in human skin (1-3% of normal viable epidermal cells) by immunohistochemical staining and indirect immunofluorescence. Elliott et al. (1989) could not demonstrate $I_\text{a}$ surface expression by radioiodination, biotinylation or immunofluorescence.

It is not entirely clear what are the accessory components of the class II complex and that question is particularly difficult to address since the composition probably changes as the it passes through the cell. Seemingly, no accessory molecules are mandatory members. Kelner and Cresswell (1986) demonstrated that in cells treated with monensin, a carboxylic ionophore which neutralizes acidic vesicles, complexes consisted of alpha/beta/$I_\text{a}$/$I_\text{a}$-CS in a 1:1:1:1 ratio. Presumably this indicated that some of the class II complexes at the point of the Golgi (where monensin has its effect) contained some accessory molecules.

**Hypotheses on the function of $I_\text{a}$**. It has been hypothesized that $I_\text{a}$ is necessary for the assembly, post-translational modifications, and transport of the class II MHC molecules. Several groups have attempted to test this hypothesis with experiments involving the transfer of the $I_\text{a}$ and alpha and beta chain genes.

Claesson-Welsh and Peterson (1985) injected the genes for $I_\text{a}$ and the alpha and beta chains into *X. laevis* oocytes. They found that when the alpha and beta chains were expressed without $I_\text{a}$, the transport of the class II MHC alpha and beta chains was slowed down
and the two chains appeared incompletely glycosylated. Miller and Germain (1986) did transfection experiments, but only looked at the amount of murine class II (Ia) on the cell surface. They found that there were equal amounts of surface-expressed class II MHC antigens whether the I\textsubscript{i} gene was transfected or not. They did not address the kinetics or processing of these molecules. In similar experiments, except in the human system, Sekaly et al. (1986) showed that cells transfected with the genes for HLA-DR, DQ and DP antigens could express these molecules on the cell surface in the absence of I\textsubscript{i}. When the I\textsubscript{i} gene was co-transfected into these cells, the levels of the class II MHC antigens on the cell surface were not increased significantly. These experiments seem to show that I\textsubscript{i} was not necessary for the formation of the alpha-beta complex and transport to the cell surface, but might be necessary for the correct processing.

An alternative hypothesis is that I\textsubscript{i} might retard the class II MHC alpha and beta chains in an endosomal compartment until it is "completed" with a foreign peptide and released to bulk flow transport to the plasma membrane, in a manner similar to p78 retention of mI\textsubscript{G}M heavy chain until a light chain is attached and the receptor is fully assembled (Bole et al., 1986). Consequently in cells without I\textsubscript{i}, class II complexes would be seen on the cell surface, but incomplete complexes would be present.

I\textsubscript{i} might have a more direct role in antigen processing and presentation (Miller and Germain, 1986). Several experiments have been done to address the question whether antigen can be processed and presented in the absence of I\textsubscript{i}.
Sekaly et al. (1988) transfected fibroblasts with the DR alpha and beta chain genes, without the I\textsubscript{1} gene. When these cells were infected with the measles virus, they were able to be lysed by class II-restricted cytotoxic T cells. They concluded that I\textsubscript{1} was not necessary for antigen presentation in this case. To address whether I\textsubscript{1} was necessary for antigen presentation to T helper cells, Stockinger et al. (1989) did an experiment where fibroblasts were transfected with either the genes for alpha and beta chains, or the genes for I\textsubscript{1}, alpha and beta. The pair of cells were then assayed for their ability to present native antigen or peptides of the same antigen. It was seen that only the cells with I\textsubscript{1} were able to efficiently present native antigen while both types of cells could present pre-processed antigen. This indicated some role of I\textsubscript{1} in processing and presentation.

The question as to the function of the various products of the I\textsubscript{1} gene is even more difficult to answer. Rosamond et al. (1987) showed that when the conversion of I\textsubscript{1} to I\textsubscript{1}-CS was inhibited by the incubation of cells with a competitive substrate for proteoglycan synthesis (p-nitrophenyl beta-D-xyloside), class II MHC alpha and beta chains were still detectable on the cell surface but the cells were less effective in antigen processing. Sivak et al. (1987) performed similar experiments in the murine system, with similar results.

One observation about I\textsubscript{1} deserves special mention. Spiro et al. (1980) demonstrated that certain subtypes of hairy cell leukemia expressed large amounts of I\textsubscript{1}. Narni et al. (1986) described that I\textsubscript{1} is highly expressed in chronic lymphocytic leukemia. The level is
high enough that they proposed that $I_1$ could be used as a marker for the disease.
Fig. 1.B.1. Hypothetical scheme of antigen processing and presentation in the B cell. APC, antigen-presenting cell; T_h, T helper cell
MODEL OF THE CLASS II MHC ALPHA AND BETA CHAINS AND \( \text{I}_A \)

![Diagram of class II MHC alpha and beta chains with sites and structures of known and putative glycosylation indicated.](image)

*Fig. 1.B.2. Model of the class II MHC alpha and beta chains and \( \text{I}_A \). Sites and structures of known and putative glycosylation are indicated.*
Fig. 1.B.3. Different splicing and initiation of the $I_i$ gene. (A) The structure of the $I_i$ gene, with black boxes as translated regions. In the p41 mRNA exon 6b is translated. Not drawn to scale. (B) Protein products resulting from the use of the exon 6b and the use of alternate initiation sites. $I_i$ is called p33, and $\gamma_2, \gamma_3$ are called p35 in this diagram. Exons are numbered. Most of exon 8 does not appear in the final proteins. (Strubin et al., 1986b).
CHAPTER II

MATERIALS AND METHODS

A. Cells.

Raji was a Burkitt's lymphoma cell line (Pulvertaft, 1965). The Vavy cell line (WSH9023) was purchased from the American Society for Histocompatibility and Immunogenetics Serum Bank and Repository. Its haplotype was HLA-DR 3, DP 1, DQ 2.

Polyclonally *Staphylococcus aureus*-activated B cells were prepared as follows (Nguyen and Humphreys, 1989). Spleens were obtained from immunocompetent patients undergoing splenectomy for surgical or medical indications. Pieces of the spleens were disaggregated by pushing through a fine steel screen, and suspended in RPMI 1640 medium (GIBCO, Grand Island, NY) with 10% fetal calf serum (FCS). A Ficoll-Hypaque gradient (density 1.078) was used to separate the mononuclear cells, which were washed twice and resuspended in the above media with 10% dimethylsulfoxide at a density of 0.4 to 1.0 x 10^8 cells/ml. Cells were quickly frozen and stored in liquid nitrogen. Upon need, splenocytes were quickly thawed and washed twice with the former medium. Approximately 1 x 10^6 cells/ml were activated with a 1/1250 dilution of a 10% solution of formalinized *S. aureus* (Chemicon) in the standard medium. Cells were cultured for 3 days at 37 °C in 5% CO_2 in air. The cells were then subjected to
the selected protocol below.

Cells were maintained in RPMI 1640 medium supplemented with penicillin (100 IU/ml), streptomycin (100 mg/ml), and 10% FCS. Cultures were used in logarithmic growth phase.

B. Anti-Peptide Antisera.

Selection of peptides. Peptides were selected such that antibodies made to them would both immunoprecipitate and western blot to both native and denatured proteins. Towards this goal, a computer program was written in the lab to search for portions of proteins that would likely be antigenic, based on the work of Hopp and Woods (1981, 1983). Mean Kyte-Doolittle hydrophobicity values for sequential sets of 6 amino acids were computed and reported at the 4th amino acid of each set (Kyte and Doolittle, 1982). These regions were then checked for inclusion of prolines, length, and location in the molecule. Portions of proteins that were hydrophilic and met the other criteria, were selected for synthesis. The exception to this logic was the peptide I\(_1\)(147-169) which was selected as the putative alpha helical region of I\(_1\) that could lie in the class II MHC desetope.

Peptide synthesis. The peptides in Table 2.B.1 were synthesized by the UMMC peptide synthesis facility, with the following exceptions. I needed the peptide I\(_1\)(146-169) before an operator was hired to run the facility. Consequently, I synthesized this peptide (on an Applied
Biosystems peptide synthesizer) and uncoupled the peptide myself. This peptide was difficult to obtain in a purified form, so I synthesized it twice. Also, the peptide, I_{4}(183-193), with addition of a C-terminal tyrosine, was synthesized by Cambridge Research Biochemicals, Ltd., UK.

Syntheses had high coupling efficiencies on each amino acid residue and consequently it was decided that purification was not needed before they were injected into rabbits.

Conjugation of peptide to carrier. The peptides were coupled to keyhole limpet hemocyanin (KLH) with bis-diazotized tolidine (BDT) or with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), as indicated in Table 2.B.1. For BDT conjugations, 50 mg of the carrier protein was dissolved in 5 ml of a 0.25 M borate buffer, 0.2 M NaCl, pH 9.0. Five mg of the peptide was dissolved in 5 ml of the same buffer and the two protein solutions were mixed. Eight mg of BDT was added to the above mixture and put on ice for 2 hr. The peptide-carrier conjugate was dialyzed against PBS for 2 days with frequent solution changes (Bassiri and Utiger, 1972). For EDAC conjugations, 4.8 mg of the peptide in 600 µl H_{2}O (pH 3.0 adjusted with HCl) was incubated with 2.8 mg of EDAC in 5.4 ml H_{2}O, pH 3.0 for 15 min at 0°C. A solution of 24 mg of the carrier protein in 0.5 ml of H_{2}O adjusted to pH 9.0 with (NH_{4})_{2}CO_{3} 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. The peptide I_{4}
(183-193) was purchased conjugated to KLH. Peptides were conjugated to bovine serum albumin (BSA), with EDAC, in the same manner as above for assay purposes.

**Rabbit injection and bleeding protocol.** Rabbits were bled before immunizations began. Peptide conjugates, containing 50 mg 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. Minzhen Xu injected and bleed the rabbits which were immunized with $I_1(147-169)$.

**C. Other Antibodies**

The monoclonal antibody (mAb) VIC-Y1 was the gift of Dr. W. Knapp (University of Vienna, Austria) (Quaranta et al., 1984). Rabbit antisera N350 and C351, from rabbits injected with the N-terminal peptide $I_1(12-28)$ and the C-terminal peptide $I_1(192-211)$, respectively, were the gifts of Dr. V. Quaranta (Scripps Clinic and Research Foundation) (Quaranta and O'Sullivan, 1986; Giacoletto et al., 1986). Normal mouse ascites was the gift of M. Schmidt (University of Massachusetts Medical School). The rabbit anti-p23,30 serum was prepared to purified class II MHC antigens (Humphreys et al., 1976) and did not react with $I_1$ in solubilized membranes of a class II$^-$, $I_1^+$ lymphoblastoid cell line (Spiro et al., 1985).
D. Microsomal Membrane Preparation.

Standard procedures with slight modifications were used (Spiro et al 1985; Kessler, 1975). $1 \times 10^8$ cells for cell lines or $5 \times 10^6$ for S. aureus-activated B cells were labeled for 3 hr in 5 ml methionine-free or cysteine-free RPMI 1640 medium with 0.5 mCi $[^{35}\text{S}]$methionine (Tran $^{35}\text{S}$-Label, ICN Radiochemicals, Irvine, CA or New England Nuclear, Boston, MA, Cat. NEG009T or NEG-009L) or $[^{35}\text{S}]$cysteine (New England Nuclear, Boston, MA, Cat. NEG022T), at $37^\circ\text{C}$ in 5% CO$_2$ in air. Cells were washed with PBS and lysed in 10 mM Tris-HCl buffer, pH 8.1. After removal of nuclear debris by centrifugation, microsomal membranes were prepared by centrifugation at 100,000 x g for 1 hr. Membrane proteins were solubilized in PBS containing 1% Triton X-100. Insoluble material was removed by centrifugation at 100,000 x g for 1 hr.

For the phosphorylation experiments, Raji cells were metabolically labeled for 3 hr with $[^{32}\text{P}]$orthophosphate in phosphate-free RPMI 1640 medium (Irvine Scientific, Irvine CA).

Denatured membranes were prepared as above, except were solubilized in 1.0% Triton, 1.0% SDS, 2.2% 2-mercaptoethanol, 4.3% glycerol, 27 mM Tris. Insoluble material was removed by centrifugation at 100,000 x g for 1 hr, and a tube with the supernatant was put in boiling water for 10 min.
E. Reprecipitation Experiments.

VIC-Y1 mAb-immunoprecipitated complexes were eluted in IEF sample buffer and then were desalted through a Sephadex G-25 column which had been equilibrated with 0.15 M NaCl, 5 mM EDTA, 50 mM Tris-HCl, 0.02% sodium azide, and 0.05% Triton X-100, pH 7.4 (0.05% Triton buffer). Antigens were then subjected to a second immunoprecipitation as described below.

F. Immunoprecipitation.

The detergent-soluble lysates were cleared with formalinized S. aureus after addition of normal rabbit serum (NRS). In the case of denatured membranes (section D), 200 μl of solubilized membranes were cleared with the addition of 20 μl NRS and 1.8 ml 50 mM Tris-HCl buffer, 150 μM NaCl, pH 8.1 and subsequent removal of antibody with formalinized S. aureus. Separately, 100 μl of 10% protein A-Sepharose (Sigma, St. Louis, MO) in 0.05% Triton buffer was reacted with specific antibody, washed, and then incubated with solubilized lysate. The complexes were washed five times with 0.05% Triton buffer, and the immunoprecipitated material was eluted in IEF sample buffer or SDS sample buffer.
G. Protease Digestions.

A standard immunoprecipitation was performed with VIC-Y1 mAb as described above, but complexes were not eluted from the washed antibody-bead matrix. Instead, enzyme solutions (chymotrypsin and trypsin: 10 mg/ml in 0.05 M Tris-HCl, pH 8.0, 30 ml; or proteinase K: 10 mg/ml in 10 mM Tris-HCl, pH 7.4, 30 ml) were added to the pellets for varying times. The digestions were transferred to tubes with 50 mg urea and 50 μl IEF sample buffer, or 50 μl 2X SDS sample buffer, placed into boiling water for 5 min, snap frozen, and stored at -70°C until the electrophoretic gels were run. Proteinase K, trypsin, and chymotrypsin were purchased from Sigma Chemical Co., St. Louis, MO.

H. Endoglycosidase Digestions.

Endoglycosidase solutions were added to immunoprecipitated protein still bound to the antibody/protein A-Sepharose conjugates in tubes which had been in boiling water for 5 min (Pinter and Honnen, 1988). For endoglycosidase H, this solution was 0.06 units (Genzyme, cat. #ENDO-H-1) in 30 μl of 50 mM sodium phosphate buffer, pH 5.7, with 1.5 mM phenylmethylsulfonyl fluoride (Trimble and Maley, 1984). For endoglycosidase F, this solution was 1.5 units (Boehringer Mannheim, cat. #903-329) in 30 μl buffer (Elder and Alexander, 1985). Control samples received no enzyme. Reaction mixtures were incubated
overnight at 37°C for endo H; at 20°C for 18 hr, then 45 min at 37°C for endo F. Reactions were stopped by boiling, and samples were immediately eluted by incubation with 40 µl of IEF sample buffer and 25 mg urea for 30 min at room temperature.

I. Electrophoresis.

For samples which were subjected to two-dimensional gel analysis, the first dimension was run on a nonequilibrium pH gradient electrophoresis tube gel (NEPHGE); the second dimension was run on a 10% SDS slab gel (Spiro et al., 1985; O'Farrell et al., 1977). For accurate determination of low molecular weight proteins, an 8 M urea, 10% SDS polyacrylamide gel electrophoresis system was used (Swank and Munkres, 1971), in a slab form. Molecular weights were calibrated with standards. Gels were autoradiographed and/or stained.

J. Dot Blots.

Peptide conjugated to BSA was deposited on nitrocellulose membrane using an S&S "Minifold". After the membranes were blocked by incubation in 3% BSA/TBS solution, they were incubated overnight with a dilution of the respective antisera. Following washing, the strips were incubated for 2 hr with \( ^{125}\)I(protein A in a 0.5% BSA/TBS solution. The strips were washed with a 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 the walls of each well of a 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 put in 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 and after 10 to 20 min, plates was read in a microtiter plate reader, at 410 nm.

L. WESTERN BLOTTING.

After electrophoresis, by previously described methods, gels and nitrocellulose sheets were equilibrated in blotting buffer (25 mM Tris, 192 mM glycine, 20% methanol) for 15 min. After electrophoretic transfer, the nitrocellulose was blocked with a 3% BSA solution, cut into strips, and incubated in an antibody solution overnight. Following 2 washes in TBS with 0.05% Tween 20, the strips were incubated in a solution of $[^{125}\text{I}]{\text{protein A}}$ in 0.05% BSA in TBS, for 2 hr. The strips were washed 3 times in TBS with 0.05% Tween 20,
dried, and exposed to film.

Alternatively, for colorimetric detection of adherent antibodies the strips were incubated in a solution of goat anti-rabbit antibodies coupled to horseradish peroxidase, after the initial incubation with the primary antibody. After washing, the strips were immersed in a solution of HRP substrate (4-chloro-1-napthol in diethylene glycol). After adequate color development, the strips were rinsed in water, to stop development.

M. STRIP-OF-HELIX HYDROPHOBICITY INDEX.

For the strip-of-helix hydrophobicity index, the mean Kyte-Doolittle hydrophobic values of 3, 4, 5, or 6 amino acids in an axial strip along one side of a putative alpha helix were calculated from amino acid positions n, n+4, n+7, n+11, n+14, and n+18 in a linear sequence. This computation yielded the mean hydrophobicity of residues in 6 successive turns of a helix lying along a strip of amino acids with alpha carbons within 45° of the indexed residue. When presented graphically, the mean hydrophobicity value of a strip was plotted at position n, the N-terminal amino acid of a strip. Highly amphipathic sequences could be found easily by inspection of the plot and identification of hydrophilic strips one or two residues on either side of the hydrophobic strip. While this strip-of-helix index measured hydrophobicity at the frequency for an alpha helix, algorithms for a beta-pleated sheet (n, n+2, n+4, n+6, n+8, n+10) or
for a $3_{10}$ helix ($n, n+3, n+6, n+9, n+12, n+15$) could also be used in connection with visual analysis of the respective graphs.

Sequences of sperm whale myoglobin, hen egg lysozyme, pigeon and beef cytochrome C, hen ovalbumin, pig proinsulin, and foot and mouth disease virus protein VP1 were analyzed with this algorithm, and predictions of antigenic sequences were compared with those of Delisi and Berzofsky (1985), Spouge et al. (1986) and with literature reports as summarized by Spouge et al. (1986).
<table>
<thead>
<tr>
<th>PEPTIDE NAME</th>
<th>SEQUENCE</th>
<th>TYROSINE ADDITION</th>
<th>POSSIBLE ISOTYPE SPECIFICITY</th>
<th>REGION</th>
<th>COUPLER</th>
<th>RABBIT NAME</th>
</tr>
</thead>
<tbody>
<tr>
<td>β1</td>
<td>1-8</td>
<td>DR</td>
<td>N TER M</td>
<td>EDAC</td>
<td>β1</td>
<td></td>
</tr>
<tr>
<td>β2</td>
<td>55-63</td>
<td>DR DP DQ</td>
<td>N TERM</td>
<td>TOLDINE</td>
<td>β2</td>
<td></td>
</tr>
<tr>
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<td>131-138</td>
<td>DR DP DQ</td>
<td>MIDDLE</td>
<td>EDAC</td>
<td>β3</td>
<td></td>
</tr>
<tr>
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<td>163-169</td>
<td>DR</td>
<td>MIDDLE</td>
<td>TOLDINE</td>
<td>β4</td>
<td></td>
</tr>
<tr>
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<td>17-25</td>
<td>DR</td>
<td>N TERM</td>
<td>EDAC</td>
<td>α1</td>
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</tr>
<tr>
<td>α2</td>
<td>39-46</td>
<td>Tyr added C term.</td>
<td>DR DP DQ</td>
<td>N TERM</td>
<td>α2</td>
<td></td>
</tr>
<tr>
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<td>MIDDLE</td>
<td>EDAC</td>
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<td>MIDDLE</td>
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<tr>
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<td>146-169</td>
<td>Tyr sub. for 146</td>
<td>MIDDLE</td>
<td>TOLDINE</td>
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</tr>
<tr>
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<td>Tyr added C term.</td>
<td>C TERM</td>
<td>TOLDINE</td>
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<td>C10</td>
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<td>TOLDINE</td>
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Fig. 2.8.1. Listing of the peptides synthesized and data concerning them.
CHAPTER III

RESULTS

A. The Structural Analysis of \( I_1 \) Leading to a Hypothesis on the Binding of an Amphipathic, Alpha Helical Sequence in \( I_1 \) to the Desetope of Class II Antigens and the Hydrophobic Strip-of-helix Algorithm for Selection of T Cell-presented Peptides.

Periodic hydrophobicity in \( I_1 \). Given the view that peptides presented to T cells had a propensity to form amphipathic alpha helices, we searched various proteins for such structures. In an analysis of the structure of \( I_1 \) a distinctive amphipathic alpha helical sequence with a strip of aliphatic hydrophobic amino acids along one side of the helix, and otherwise generally hydrophilic amino acids was found. A hydrophilicity plot of the \( I_1 \) sequence revealed periodic oscillations in hydrophilicity from Phe\(_{146}\) to His\(_{170}\) (Fig. 3.A.1, Panel a). An Edmundson wheel projection (Schiffer and Edmundson, 1967) along the axis of the cylinder showed hydrophobicity to be centered in quadrant III (Fig. 3.A.2, Panel b). A sheet presentation of the cylinder's surface showed a distinctive, hydrophobic strip in quadrant III, and the generally hydrophilic rest of the cylinder (Fig. 3.A.2, Panel c). It can be suggested that this amphipathic sequence could associate with class II antigens through
the foreign antigen binding site, until dissociation in the endosomes which have received digested foreign antigen. Thereafter, the \( I_\to \) sequences, which could otherwise compete for the foreign amphipathic peptides, might polymerize about their hydrophobic strips, perhaps as tetramers, linked also by ionic and hydrophobic bridges on the outsides of the polymers (Fig. 3.A.3).

*Prototypic amphipathic peptide.* Among a series of protein sequences, the \( I_\to \text{Phe}_{146}-\text{His}_{170} \) polypeptide best fit an idealized, long, amphipathic, alpha helix when sequences were analyzed by the program which computed mean hydrophobic indices of amino acids along one side of an alpha helix (strip-of-helix hydrophobicity index; Fig. 3.A.1, Panel b). The index value of \( I_\to \text{Phe}_{146} \) was the greatest found for any strip-of-helix in analyses of cytochrome C, lysozyme, myoglobin, ovalbumin, tetanus toxin fragment C, HLA-DR 3 alpha chain, HLA-DR3 beta chain, HLA-B27, and clotting factor VIII:C (not shown). Consequently this region of \( I_\to \) could be defined as the "prototypic antigenic amphipathic peptide". Antigenic peptides that most closely approximated this structure would be preferentially presented by class II molecules. Towards the goal of defining such peptides, the strip-of-helix algorithm was produced to compute the hydrophobicity along the side of a helix and to search for peptides that would resemble the structure of \( I_\to (146-170) \).

*Algorithm for hydrophobic strip.* This algorithm started from a
consideration of Berzofsky's more general approach to the analysis of frequencies of periodic hydrophobic moments within 7 amino acid stretches (2 turns of an alpha helix) (DeLisi and Berzofsky, 1985; Margalit et al., 1987). However, our specific model was modified to search for peptides which most closely mimicked the "I1 prototypic amphipathic peptide", which was hypothesized to be complementary to the class II MHC desetope which contains a narrow slit hypothetically accepting hydrophobic, preferably aliphatic, residues of digested, foreign proteins.

An algorithm was created to compute the mean hydrophobicity of amino acid residues in a strip extending axially along an alpha helix by averaging Kyte-Doolittle hydrophilicity values of residues at positions n, n+4, n+7, n+11, n+14, and n+18 in the linear sequence of a protein. Such a strip-of-helix hydrophobicity could be calculated over 3 to 6 cycles of the helix, starting with the position n amino acid. In plotting the position of alpha carbons of amino acids in an alpha helix (in which adjacent amino acids are 100° apart), starting with amino acid n, such a strip ran within ±45° of the chosen starting point through 5 adjacent turns of the helix before the pattern repeated itself (Fig. 3.A.4). These computations could be altered to identify hydrophobic strips on 310 helices (residues n, n+3, n+6, n+9, n+12, n+15) or in beta-pleated sheets (residues n, n+2, n+4, n+6, n+8, n+10).

Note that in Kyte-Doolittle hydrophilicity plots, the mean hydrophilicity value is for a set of 7 sequential residues plotted at the 4th amino acid position in the series, but in the strip-of-helix
plot, the mean hydrophobicity value is for amino acids along a +45° strip from 3 to 6 turns of a helix in the C-terminal direction from the index point (Kyte and Doolittle, 1982).

Strictly speaking, this index measured periodic hydrophobicity and not amphipathicity as defined by Berzofsky, for it evaluated only hydrophobic amino acids in a strip along the side of a hypothetical alpha helix, and ignored contributions to a moment of hydrophobicity from hydrophilic or hydrophobic side chains elsewhere on the cylinder (DeLisi and Berzofsky, 1985).

From the highest scoring hydrophobic strips-of-helix, we identified those with flanking hydrophilicity by finding at least one adjacent strip (within ±2 amino acids from the hydrophobic strip) which had a mean hydrophobicity score of -1 or less. Graphically, the highly hydrophobic index strip was frequently bracketed by multiple, variably hydrophilic strips (Fig. 3.A.5). In contrast, transmembranal regions of a protein had a series of hydrophobic strips without any adjacent hydrophilic strips, reflecting the generally hydrophobic character of all sides of the helical forms of transmembranal peptides. We then excluded from the 5 highest scoring peptides, those which contained a proline which could break the alpha helical pattern. For consistency in ranking analyses (in contrast to selections for syntheses, see below), the boundaries of peptides were defined to be the first and last amino acid in the hydrophobic strip.

Our computerized protocol then consisted of: (1) calculating strip-of-helix hydrophobicity values for 3, 4, 5 and 6 turns of a putative helix (8, 12, 15 and 19 amino acids in length), (2) printing
of sequence position, amino acid and index value in tabular form, and index value and sequence position graphically, (3) listing of the 5 most highly ranked peptides, (4) indicating those which did not have at least one adjacent strip (+1 or 2 amino acids from position n with hydrophobicity score -1.0 or less, (5) indicating those peptides which contained a proline and (6) indicating peptides which contained a lysine +1 or +3 positions after the C-terminal hydrophobic residue.

The design of this algorithm was based upon several observations. For any protein only the five most hydrophobic strip-of-helix peptides were examined because previously studied proteins had a maximum of four antigenic sites (in hen egg lysozyme). Also, one might suspect that competitive binding of peptides to class II antigens would generate a hierarchical order in presentation and thus scoring for 3 or 4 peptides would be sufficient, having allowed for the detection of one transmembrane peptide. In this study, no experimentally antigenic peptide which possessed a hydrophobic strip-of-helix was found to be excluded from the five most highly scoring strips. Selection of -1.0 as the "adjacent hydrophilicity" threshold was based on an examination of sperm whale myoglobin, pigeon cytochrome C, hen egg lysozyme, and hen ovalbumin. This examination showed that all experimentally antigenic peptides had at least one adjacent hydrophilic strip of index -1.0 or less. In addition, few peptides that were not reported to be experimentally antigenic were included at this threshold value. Finally, since the length of experimentally antigenic peptides in the proteins studied varied from 11 to 17 amino acids, analysis of 4 to 6 helical turns (11 to 19 amino acids) was appropriate.
Analysis of classical protein immunogens. Amino acid sequences of sperm whale myoglobin, hen ovalbumin, hen egg lysozyme, beef cytochrome C, pigeon cytochrome C, and foot and mouth disease virus protein VP1 were searched for their highest scoring strip-of-helix hydrophobicity indices at 3, 4, 5 or 6 turns of a helix. The 5 highest scoring peptides in each protein were then tested for an adjacent strip with a hydrophobicity index of -1.0 or less and for the absence of prolines in the hypothesized amphipathic helical region. Strip-of-helix hydrophobicity plots at 5 turns analysis of the highest ranked peptides in sperm whale myoglobin, hen ovalbumin, and beef cytochrome C are presented in Fig. 3.A.5. The peptides selected according to our protocol are listed in Table 3.A.6. We found a close concordance among the peptides chosen with our algorithm, those selected with the DeLisi and Berzofsky procedure, and those found experimentally to be antigenic. The DeLisi and Berzofsky procedure and our selection procedure both included about the same number of "extra" amino acids than those found in experimentally antigenic peptides. The proteases used to obtain experimentally antigenic fragments probably did not cleave out exactly the epitopes needed for binding to the class II desetope. The DeLisi and Berzofsky method and this approach each failed to identify two peptides which experimentally had been found to be T cell-presented. Overall, however, the strip-of-helix hydrophobicity algorithm and the DeLisi and Berzofsky procedure generally predicted the principal peptides which were T cell-presented.

In order to visualize a helical peptide an Edmundson wheel (Fig.
3.1.2. Panel b) can be used to present amino acid residues along the axis of an alpha helix with successive residues 100° apart (Schiffer and Edmundson, 1967). This is the simplest approach to judge clustering of hydrophobic residues about one side of a helix, but it is limited because the axial orientation of residues along one side of the helix is not easily visualized, and residues begin overlapping positions in the wheel after 18 amino acids. A sheet projection of the helix (cut at one position in the Edmundson wheel) displays the linear array of amino acids down each of four quadrants along the axis of the helix. This projection was useful in looking for potential interactions between groups in hydrophilic quadrants II and IV of adjacent helices, given a hypothesis that a polymer was formed around hydrophobic quadrant III associations.

Function of the prototypic amphipathic region. Seeing the "prototypic T cell-presented peptide" within I_1 led to the view that this amphipathic helix might bind to the class II MHC desetope. A diagrammatic representation of the putative structure is shown in Fig. 3.A.7. One could speculate that the function of I_1 was, in part, to keep that class II MHC site free of endogenous peptides until reaching endosomes where foreign antigen was digested by proteases. The dissociation of I_1 from the class II complex could be promoted by several means, including acidic pH or proteolytic cleavage, but the released I_1 amphipathic helix, in either small or large fragments of I_1, might compete with foreign peptides for binding to the class II
desetope. However, analysis of the $I_i$ helical peptide's structure indicated that the peptide probably could no longer bind to the class II MHC alpha and beta chains because it would self-associate tightly through: (1) a core of interdigitating, aliphatic side chains, (2) ionic bridges at the external surface of the polymer, and (3) hydrophobic interactions near the C-terminus of the helices. These interactions were demonstrated clearly in scaled, molecular models of two helices (Fig. 3.A.3). While formation of dimers, trimers, pentamers or other aggregates could not be ruled out, association of these $I_i$ peptides as a tetramer would appear to be the sterically favored polymer. In nature, kinetics of release and dimerization of $I_i$ structures might govern what polymeric form would prevail.
Fig. 3.A.1. Hydrophobicity (Kyte-Doolittle values) and periodic hydrophobicity (in an alpha helix) plots of the I\textsubscript{\text{1}} sequence. (A) Mean Kyte-Doolittle hydrophobicity values of 6 sequential amino acids from the position indicated in the abscissa were plotted. (B) Mean Kyte-Doolittle hydrophobicity values of 6 amino acid side chains in a strip along a putative alpha helix, i.e., positions: n, n+4, n+7, n+11, n+14, n+18 were plotted. Arrowheads indicate the very hydrophobic strip beginning at Phe\textsubscript{146} and the four adjacent hydrophilic strips beginning at positions 144,145 and 147,148.
II PEPTIDE: 146 - 175

AMINO ACID SEQUENCE

146 150 155
Phe - Pro - Glu - Asn - Leu - Arg - His - Leu - Lys - Arg

155 157 150
Thr - Met - Glu - Thr - Gln - Asp - Trp - Lys - Val - Phe

170 175
Glu - Ser - Trp - Met - His - His - Trp - Leu - Leu - Phe

EDMUNDSON WHEEL PROJECTION

Fig. 3.A.2. Peptide sequence presented in an Edmundson wheel and in a sheet projection of the cylinder's surface. (A) Amino acid sequence. The amino acid sequence between residues Phe146 and Phe175 is given. (B) Edmundson wheel. This projection placed each residue at increments of 100° about a circle. Hydrophobic values were presented as thick lines from the circumference, and hydrophilic values as thin lines; the lengths were proportional to the absolute Kyte-Doolittle values for each amino acid. Quadrants were arbitrarily assigned. Most of the hydrophobic values fell in quadrant III. (C) Sheet projection. The amino acid placements about the cylinder represented in the Edmundson wheel pattern were displayed as a sheet (cutting the cylinder along the boundary between quadrants I and IV). The hydrophobic strip is seen in quadrant III.
Fig. 3.A.3. Polymerization of the Iᵢ peptide, three views. In the top view, quadrant orientation of peptides aligned with N-termini together in a putative tetramer follows from the association of hydrophobic residues of quadrant III. In the internal view, hydrophobic residues Leu₁₅₀, Met₁₅₇, and Val₁₆₄ interdigitated between residues Phe₁₄₆, Leu₁₅₃, Ile₁₆₀, and Ser₁₆₇, respectively, in a zipper-like fashion which was then repeated clockwise around the tetramer. In the external view, ionic bridges between His₁₅²-Lys₁₅₄ and Lys₁₆₃-Asp₁₆₁ are possible, the His₁₅²-Lys₁₅₄ pair being pH sensitive.
Fig. 3.A.4. Sheet projection of an alpha helix. The positions of alpha carbons in an alpha helix, 100° from each other, are plotted. The heavier dots are the residues within 45° from the first residue in a strip which is centered at 180°. Each amino acid in the strip is a "turn", in the sense that floors are reached in a spiral staircase. Three turns are thus found in two full cycles of the helix.
Fig. 3.A.5. Representative program-selected peptides. Strip-of-helix hydrophobicity values for regions containing highest-ranked peptides are shown for amphipathic regions from sperm whale myoglobin, hen ovalbumin, and bovine cytochrome C. The singly highest-ranked peptide is indicated with a triangle and the amino acid position. The values of the four adjacent, relatively hydrophilic strips (2 on the N-terminal side, 2 on the C-terminal side) are indicated with tapered arrowheads.

<table>
<thead>
<tr>
<th>PEPTIDE</th>
<th>INDEX*</th>
<th>Reported Antigenic Peptides</th>
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<tr>
<td><strong>Sperm whale myoglobin</strong></td>
<td></td>
<td></td>
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<td>Leu&lt;sub&gt;61&lt;/sub&gt; - Ile&lt;sub&gt;75&lt;/sub&gt;</td>
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<td>74-86</td>
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<td>81-96</td>
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<td>A 4-A 14</td>
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<td><strong>Beef cytochrome C</strong></td>
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<td>66-80</td>
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<td></td>
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<td>2.44</td>
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*Strip-of-helix hydrophobicity index at 5 turns is given except for pigeon cytochrome C which is presented at 3 turns because the highest ranking antigenic peptide Leu<sub>94</sub> - Lys<sub>104</sub> is at the C-terminus. Peptides which contained prolines or failed to meet threshold criteria for adjacent hydrophilic strips, are not listed.
Fig. 3.A.7. Hypothetical model of I_{i} and the alpha and beta chains. I_{i} is shown resting in the desetope of the class II MHC proteins, prior to the binding of foreign antigen. The site of the addition of the extra exon to generate p41 and the site of the addition of 16 amino acids at N-terminus to generate γ2 and γ3 are shown. Sites of certain structural features also are indicated. Additional area of interaction between I_{i} and the alpha and beta chains also probably exist.
B. Proteolytic Cleavage of \( I_i \) to p25.

**Introduction.** To pursue the working hypotheses on the function of \( I_i \), we have examined structural changes of the molecule. On two dimensional gels of immunoprecipitates of \( I_i \) and class II molecules a protein called p25 has been observed (Fig. 3.B.1). In this study we have shown that p25 is the C-terminal portion of the \( I_i \) molecule, containing the amphipathic alpha helix (146-170). Since p25 is seen when immunoprecipitates are done in the presence of protease inhibitors, and maximal labeling is seen at 20-40 min in pulse-chase experiments, we feel it is not an artifact of the protocol. Cleavage of \( I_i \) to p25 appeared not to be determined by a specific substrate sequence, but by a domain character in \( I_i \), since three proteases with varying specificities preferentially cleaved about the immediate exomembrane region of \( I_i \). Further, all p25 molecules were sensitive to treatment with endoglycosidases F and H and, therefore, must be high mannose-derived and, thus, generated in the endoplasmic reticulum or cis-Golgi apparatus.

**Re-immunoprecipitations of denatured, \( I_i \)-associated proteins.** Detergent-solubilized, \(^{35}\)S-methionine-labeled, microsomal membranes were subjected to immunoprecipitation with VIC-Y1 mAb, yielding \( I_i \) and associated molecules (Fig. 3.B.2, Panel A). These immunoprecipitated antigens were denatured with 9 M urea, desalted by
passage through a Sephadex G25 column equilibrated with 0.05% Triton buffer and immunoprecipitated a second time with various antisera. Two rabbit antisera to peptides in the carboxy terminal portion of I₁, anti-I₁(183-193) (E1) and anti-I₁(192-211) (C351), immunoprecipitated p25 and I₁ (Fig. 3.B.2, Panels E and C, respectively). Rabbit antiserum to a N-terminal peptide (12-28) of I₁ (N350) failed to immunoprecipitate p25 but recognized I₁ (Fig. 3.B.2, Panel D). A second immunoprecipitation with VIC-Yl mAb also recognized some of the denatured forms of I₁, but not p25. VIC-Yl is a monoclonal antibody and may be to a more native structure. Consequently it is difficult to conclude anything from this particular reprecipitation. An antiserum to irrelevant proteins (E4) failed to immunoprecipitate I₁ or any I₁-related proteins.

Absence of Cys₂₈ from p25. Since only one cysteine was present in I₁, at sequence position 28, [³⁵S]cysteine-labeled proteins were immunoprecipitated and compared to [³⁵S]methionine-labeled and immunoprecipitated proteins (Fig. 3.B.3). While [³⁵S]-labeled I₁ was present in both methionine and cysteine radiolabeled samples, [³⁵S]-labeled p25 was found in methionine- and not in cysteine-radiolabeled cells. Control precipitates with NRS were blank (results not illustrated). The cleavage site to form p25 was judged to occur after Cys₂₈ in the primary sequence of I₁.
N-terminal sequence of p25. In order to confirm that the molecule known as p25 was the C-terminal portion of $I_1$, we intended to do an N-terminal sequence of p25 (detecting $^{3}H$leucine residues) and compare it to the known sequence of $I_1$. The three major obstacles to overcome were (1) purification of p25 from other membrane proteins including class II MHC molecules and $I_1$, (2) production of enough p25 from which to get a sequence and (3) production of p25 with enough $^{3}H$leucine incorporated to allow detection of leucine residues. The experimental strategy was to label Raji cells with $^{3}H$leucine and purify $I_1$ and p25 on an immunoaffinity column with a covalent linkage between the antibody anti-$I_1(183-193)$ and the support. This would eliminate most of the cold protein from the final immunoprecipitate to enable more radioactive protein to be loaded on a gel, which would be sliced to isolate p25. Unfortunately, the incorporation of leucine into p25 was low. This would make the detection of leucine residues difficult, since the signal would not be much over the noise. Consequently, the goal of matching the sequence of leucine residues to the $I_1$ sequence seemed difficult to attain, and this experiment was abandoned.

Maximal Production of p25 20-40 min after synthesis. Polyclonally S. aureus-activated B cells were labeled with $^{35}$S)methionine for 10 min, then chased for 0, 10, 20 and 40 min. Microsomal membranes were immunoprecipitated with anti-$I_1(183-193)$ and separated by 2 dimensional gel electrophoresis (Fig. 3.B.4). p25 appeared maximally
between 20-40 min following synthesis.

**Proteolytic digestions of native I₄.** In order to analyze whether the *in vivo* cleavage of I₄ to p25 reflected great specificity of a protease for a particular I₄ sequence, or the effect of a secondary, structural (domain) restriction (for example, as found about the hinge region of IgG; Porter, 1959), the susceptibility of native I₄ to several proteases with varying substrate sequence specificities was tested (Fig. 3.B.5). Trypsin (EC 3.4.21.4) cleaved at the carboxy side of lysyl or arginyl residues. Chymotrypsin (EC 3.4.21.1) and Proteinase K (EC 3.4.21.14) cleaved at the carboxy side of aromatic residues and sometimes after hydrophobic residues. I₄ and associated molecules, still bound to the antibody VIC-Yl-protein A Sepharose beads, were subjected to digestion with these enzymes (Fig. 3.B.5). In each instance, I₄ was digested to a series of proteins about 25 kD, some of which overlapped naturally occurring p25. The 25 kD proteins were relatively resistant to further digestion, but at longer digestion times were reduced to smaller peptides.

**Glycosylation of p25.** Anti-class II MHC serum immunoprecipitates of [³⁵S]methionine-labeled proteins which were subjected to endoglycosidase F treatment demonstrated a decrease in molecular weight of p25 from 25 to about 10.5 kD (arrows, Fig. 3.B.6). A third
gel of mixed, equal quantities of the control and endoglycosidase F-treated samples confirmed the decrease in weight of p25 and of I\textsubscript{i} and N-linked Ip forms. The identities of alpha and beta chains of class II MHC antigens, of a nonglycosylated I\textsubscript{i} precursor (I\textsubscript{i}), of four I\textsubscript{i} forms with N-linked sugars (IpN) and five I\textsubscript{i} forms with O-linked carbohydrate side chains (IpO), as presented in Fig. 3.B.1, were established previously (Machamer and Cresswell, 1984). The weight of the 10.5 kD product was confirmed by electrophoresis in an urea/SDS polyacrylamide gel (not illustrated).

In order to establish whether the N-linked sidechains were composed of high mannose or complex forms, similar immunoprecipitates with anti-I\textsubscript{i}(183-193) serum, with VIC-Yl mAb, and with anti-class II MHC serum, were digested with endoglycosidase H (Fig. 3.B.7). In each instance, p25 was reduced in weight to 10.5 kD. This fact was confirmed in gels of mixed, equal samples of control and endoglycosidase H-treated samples (not illustrated).

Since all p25 was subject to digestion with endoglycosidase H, it was concluded that the N-linked carbohydrate chains on the I\textsubscript{i} pool degraded to p25 were all high mannose forms. One I\textsubscript{i} form (the most basic of the IpN spots) was not fully subject to digestion with endoglycosidase H but was digested with endoglycosidase F. It therefore represented molecules in which carbohydrates were probably converted to complex forms. Furthermore, while VIC-Yl recognized Ip-O-linked forms, anti-I\textsubscript{i}(183-193) did not. Since p25 was recognized with anti-I\textsubscript{i}(183-193), it was concluded that it did not have the O-linked carbohydrate forms of Ip-O(1-5). p25, therefore, was
derived from relatively "early" forms of $I_1$ with high mannose sidechains and without O-linked sugars.
Fig. 3.B.1. Diagram of the distribution in 2-dimensional (NEPHGE/SDS) gels of class II MHC and associated proteins. Alpha and beta are two polymorphic chains coded by the class II genes of the MHC. \( I_0 \) is the electrophoretically invariant chain noncovalently associated with the alpha and beta chains. \( I_pN \) is a series of \( I_0 \) molecules which have N-linked glycosylations. \( I_pO \) is a series of \( I_0 \) molecules which have additional O-linked glycosylations. \( \gamma_2 \) and \( \gamma_3 \) are \( I_0 \) forms translated from an upstream alternate start site of the \( I_0 \) structural gene, resulting in 16 extra amino acids. \( p41 \) is an \( I_0 \) form transcribed with an extra exon coding for an extra 64 amino acids after Lys\textsubscript{192}. \( p25 \) is a cleavage product of \( I_0 \).
Reprecipitation of I$_i$-associated, denatured proteins.

Detergent-solubilized, [$^{35}$S]methionine-labeled, microsomal membranes of lysed Raji cells were immunoprecipitated with VIC-Y1 mAb (Panel A). VIC-Y1 immunoprecipitated proteins were eluted with 9 M urea and passed over a Sephadex G25 column which was equilibrated in 0.05% Triton, 0.1 M Tris-HCl buffer, pH 8.0 (Panel B). Immunoprecipitates were prepared from the eluted proteins with each of the following antibodies: C351, rabbit heteroantisera to a C-terminal peptide (192-211) of I$_i$ (V. Quaranta, Scripps Clinic and Research Foundation) (Panel C); N350, rabbit heteroantisera to an N-terminal peptide (12-28) of I$_i$ (V. Quaranta) (Panel D); E1, rabbit heteroantisera to a C-terminal peptide (183-193) of I$_i$ (Panel E); VIC-Y1 mAb (Panel F); E4, a rabbit heteroantisierum to p67/69, a molecule not immunoprecipitated by VIC-Y1 (Panel G).
Fig. 3.B.3. Immunoprecipitation of [35S]cysteine-labeled proteins. [35S]Cysteine-labeled (A) or [35S]methionine-labeled (B) proteins were immunoprecipitated with VIC-Y1 anti-I, mAb from polyclonally S. aureus-activated B cells and were subjected to 2-dimensional (nonequilibrium, pH gradient and SDS) electrophoresis. p25 (arrow) was immunoprecipitated from methionine- but not cysteine-labeled cells, while \( \gamma_1 \), \( \gamma_p \), p41, \( \gamma_2 \), and \( \gamma_3 \) were immunoprecipitated from cells with each type of radiolabeling. Illustration courtesy of Dr. Quoc V. Nguyen.
Fig. 3.B.4. Pulse-chase of polyclonally *S. aureus*-activated B cells showing the appearance of p25 maximally 20 min after synthesis. Illustration courtesy of Dr. Quoc V. Nguyen.
PROTEOLYTIC DIGESTIONS OF NATIVE II

Fig. 3.B.5. Proteolytic digestions of native I\textsubscript{d}. Raji cells were metabolically labeled with [\textsuperscript{35}S]methionine, and their membranes were isolated. VIC-Y1, bound to protein A-Sepharose, was used to immunoprecipitate I\textsubscript{d} and associated proteins. Eluted antigens were presented in Panel A. A parallel immunoprecipitate with normal mouse ascites was shown in Panel B. Antigens still bound to the antibody-Sepharose matrix were digested by various proteolytic enzymes: Trypsin (C-F), Proteinase K (G-J), and Chymotrypsin (K-N). Enzymes were incubated for various times with antigens: 0 time (C, G, K), 3.5 min (H), 5 min (D, L), 8 min (I), 20 min (E, M), 80 min (F, N), 120 min (J). With each of the enzymes there is a time-dependent build up of products in the area where p25 migrates in a two dimensional gel.
Fig. 3.B.6. Digestion of p25 with endoglycosidase F. "Control" panel shows undigested eluate, "Endo F" panel shows eluate digested with endoglycosidase F, and "mixed" panel shows mixture of undigested and digested proteins.
Fig. 3.B.7. Digestion of p25 with endoglycosidase H. "Control" panels show undigested eluate from anti-I\textsubscript{4}(183-193) serum, anti-I\textsubscript{4} monoclonal antibody VIC-Y1, and anti-class II MHC serum. "Endo H" panels show the corresponding eluates digested with endoglycosidase H.
C. Structural Analysis of the Class II MHC Alpha and Beta Chains

Leading to a Hypothesis on Their Proteolytic Regulation.

Observation of adjacent of basic amino acids. It was observed that the alpha and beta chains of most HLA-D molecules contained pairs of basic amino acids, lysines and/or arginines, throughout their sequences (Fig. 3.C.1). These pairs of amino acids are well conserved between haplotypes and between species (Figueroa and Klein, 1986).

Arginine and/or lysine pairs have been shown in a variety of systems to be cleaved by a protease. The classic example of such a cleavage being the processing of proinsulin to insulin (Steiner et al., 1969). It can then be hypothesized that proteolytic cleavages of the class II MHC antigens' alpha and beta chains at these sites could be important, in some way, in antigen presentation.

Correlation of cleavage sites to 3 dimensional structure. Brown et al. (1988) predicted the structure of the class II desetope by comparison of its sequence to the sequence of the crystallized class I MHC molecule. This predicted structure is shown in Fig. 3.C.2. The walls of the desetope are shown as two extended alpha helices. The placement of these helices is indicated in Fig. 3.C.1. If one looks at the potential cleavage sites, mentioned above, in relation to the structure of the desetope, one sees that these cleavages fall in critical locations. Specifically, if cleavage did occur at these sites
the walls of the desetope could be removed.

Hypothetical cellular functions of proteolytic cleavages of the class II MHC alpha and beta chains. Cleavages at these sites could regulate the creation and/or destruction of the antigen presenting function of the class II MHC proteins. Specifically, proteolytic cleavage of the class II MHC proteins at these sites would be a very efficient way to destroy the antigen presenting capability of the complex. Proteolytic cleavage of the alpha and beta chains might occur while foreign antigen is present, after the complex has reached the cell surface. This would serve to prevent further antigen presentation to T cells after sufficient presentation might have occurred. Alternatively, upon possible release of the foreign peptide, susceptible sites of the class II alpha and beta chains could be cleaved by extracellular proteases, rendering that peptide binding site unable to adsorb, and thus present, ambient peptides. Proteases are known to be released by activated T cells (Pasternack and Eisen, 1985; Pasternack et al., 1986). After internalization, proteolytic cleavage of the alpha and beta chains could occur to inactivate the class II complex. This could serve as a mechanism to destroy complexes that have presented foreign antigen. Furthermore, if I1 is required to catalyze foreign peptide binding to class II MHC molecules, these recycled surface molecules, void of I1, might bind such peptides inefficiently. Another possibility is that the inactivation of class II MHC molecules could be a mechanism to limit the number of class II
MHC molecules that reach the cell surface.

Alternatively, or in addition, these proteolytic cleavages could facilitate desetope conversion (Guillet et al., 1987). Specifically, excision of the beta chain third hypervariable region between Lys\textsubscript{71}-Arg\textsubscript{72} and Arg\textsubscript{93}-Arg\textsubscript{94} or the alpha chain hypervariable region between Lys\textsubscript{48}-Arg\textsubscript{49} and Lys\textsubscript{79}-Arg\textsubscript{80} could permit the binding of structurally similar foreign peptides in the endosome containing foreign antigen, in a process of desetope conversion.
Fig. 3.C.1. Putative cleavage sites on the class II MHC alpha and beta chains. Pairs of basic amino acids about the class II MHC desetope could lead to cleavages which eliminate antigen-presenting function. The putative walls of the class II MHC desetope are shown as helices [\(\alpha(51-80), \beta(57-86)\)]. The peptides synthesized are also indicated.
Fig. 3.C.2. Hypothetical structure of the class II MHC desetope (Brown et al., 1988).
D. Proteolysis of the HLA Class II MHC Alpha and Beta Chains

Introduction. Our goal in the following experiments was to demonstrate if the class II MHC alpha and beta chains undergo proteolytic cleavage, and if this cleavage is relevant to the control of antigen processing and presentation. Towards that goal we identified pairs of basic amino acids at structurally prominent sites in the Brown-Wiley model of the class II MHC structure (Brown et al., 1988), demonstrated the existence of class II MHC molecule bound polypeptides in immunoprecipitates by anti-class II sera, and presented strong evidence that these polypeptides were derived from the alpha and beta chains. To achieve this last aim we used western blotting with antisera to synthesized peptides whose sequence was from putatively immunodominant sections of the alpha and beta chains.

Finding endogenous polypeptides bound to class II MHC proteins. In immunoprecipitates, with anti-class II MHC serum, of $^{35}$S)methionine-labeled Raji cells, a polypeptide of about 8.4 kD was seen in reduced samples. This band was not seen in the samples that were not reduced or in precipitates with NRS (Fig. 3.D.1). This gel was 10% acrylamide/8 M urea, with an SDS/Tris buffer of pH 6.8. This polypeptide had not been seen previously, however in the 10% gels that are used routinely, polypeptides of about 8.4 kD (about 75 amino acids) are not resolved. This polypeptide is then a portion of some
protein (or a protein) immunoprecipitated with anti-class II MHC serum. It has about 75 amino acids (or less if it has carbohydrate) and is linked to a larger molecule by a disulfide bond. I₁ as a source of this polypeptide, can be ruled out, since it has only one cysteine, and no disulfide linkages. Upon examination of sequences of the alpha and beta chains, this polypeptide could be the N-terminal portion of the beta chain, with a cleavage around Lys₁⁻Arg₂. Under non-reducing conditions it might still be connected to the rest of the molecule through a disulfide bond, through Cys₁₅, which is broken under reducing conditions.

Observation of four polypeptides in [³⁵S]cysteine-labeled Vavy cells. The above experiment was with the Raji cell line. Interpretation of results with this cell line could be difficult because Raji cells are heterozygous at the DR locus (DR 3, 6) and therefore probably at the DP and DQ loci. Further, the DP and DQ haplotypes of Raji have not been reported. Subsequent experiments have been done with the homozygous cell line, Vavy which is DR 3, DQ 1, DP 2, with each of these molecules being of published sequence (Figueroa and Klein, 1986). Also cells have been labeled with [³⁵S]cysteine rather than [³⁵S]methionine since these residues are more conserved, and upon examination of the sequences of the putative polypeptides (portions of the chains between basic amino acid pairs) cysteines are usually present. Further, since I₁ has only one cysteine in its sequence, labeling cells with [³⁵S]cysteine will mask all but
possibly one polypeptide from $I_i$. Consequently, the polypeptides seen with $[\text{35S}]$cysteine-labeling will be predominantly from the chains of interest, alpha and beta.

Figure 3.D.2 shows an anti-class II MHC immunoprecipitate of $[\text{35S}]$cysteine-labeled Vavy cells run in a 11-23% acrylamide gradient gel. The first lane is the eluted material without digestion. The columns to the right are the same material digested with trypsin of constant concentration, but with increasing time. Four polypeptides are resolved, at about 14.4, 11.4, 8.4 and 4.0 kD. Since these cleavages are not enhanced with incubation with trypsin, we conclude that a more specific enzyme is used in vivo, or that addition controlling factors (i.e. desetope full or empty) play a role. The next experimental goal was to determine if these polypeptides were derived from the alpha or beta chains. To do this we attempted to make anti-peptide antibodies to the alpha and beta chains and western blot them to these polypeptides.

**Production of anti-beta chain and anti-alpha chain antibodies.** To produce antibodies made to specific portions of the alpha and beta chains, we selected four peptides from the DR 3 beta chain and four peptides from the DR 3 alpha chain sequences using a Hopp-Woods type of 9 amino acid window hydrophobicity program generated in the lab. The peptides were called $\beta_1, \beta_2, \beta_3, \beta_4$, and $\alpha_1, \alpha_2, \alpha_3$, and $\alpha_4$. Their sequences are shown in Fig. 3.D.3 and 3.D.4, respectively. The peptides were also selected such that (1) the peptides were from
different regions of the molecules so that they might recognize different putatively cleaved polypeptides and (2) antibodies to the different peptides might selectively recognize different isotypes of the HLA-D complexes present in Vavy cells. For example, the peptides to the beta chain consisted of: (1) one to a C-terminal sequence conserved in all HLA-D isotypes, (2) one to a C-terminal sequence common to only HLA-DR molecules, (3) one to a more central sequence that is common to all HLA-D isotypes and (4) one to a central portion of HLA-DR antigens (Fig. 3.D.3). Similarly, a comparison of the alpha chain peptides to the HLA-D isotypes present in Vavy cells is shown in Fig. 3.D.4. The eight peptides were synthesized, coupled to KLH, and injected into rabbits (Fig. 3.D.5). Sera were taken from the animals.

**Characterization of the anti-peptide sera.** To determine if the rabbits injected with the various peptides were making antibodies to the peptide, which reacted with native or denatured proteins, we formulated a multistep testing protocol, detailed below. The results of this regime are summarized in Fig. 3.D.5.

**Dot blots to characterize anti-beta chain sera.** To assay the sera for antibodies to the injected peptides, a dot blot assay was initially used, as shown in Figures 3.D.6 to 3.D.9. Peptide was coupled to BSA and blotted on a sheet of nitrocellulose. Pieces were incubated with various dilutions of the sera, washed, and further
incubated with $^{125}$I]protein A. The assay worked well to screen out non-productive animals (those injected with peptides $\beta2$ and $\beta4$), but was relatively peptide non-specific for the animals that gave a positive result. That is, sera from the animals injected with the peptides $\beta1$ and $\beta3$ bound to the peptide-BSA conjugate in an antibody dose dependent fashion. However, this titration also occurred to an irrelevant peptide-BSA conjugate. To identify if the animals were making specific antibody we decided to use an ELISA, which (1) being more quantitative, could discriminate positive responses above nonspecific background reactions and, (2) could use uncoupled peptide, to eliminate nonspecific binding.

**ELISA to characterize anti-alpha and beta chain sera.** Sera from the rabbits injected with the peptides $\beta1$ and $\beta3$, as well as those rabbits injected with the four alpha chain peptides, were tested by ELISA. In these assays unconjugated peptide was deposited on the wells of a microtiter plate and exposed to the sera from the respective rabbits. A second antibody, a horseradish peroxidase coupled goat anti-rabbit antiserum, was used and specific binding was detected with enzyme-produced colored product [the substrate was ABTS, 2,2'-azino-di-(3-ethyl-benzthiazoline-sulfonate)]. The results were read with a microtiter plate reader. The results of these experiments are shown in Fig. 3.D.10 to 3.D.13. The animals injected with the peptide $\alpha3$ ($\alpha3^a$ and $\alpha3^b$) both showed specific reactivity to the peptide (Fig. 3.D.10 and 3.D.11, respectively). One animal injected
with the peptide \( \alpha_4 \) (\( \alpha_4^a \)) also showed antibody production specific to the injected peptide (Fig. 3.D.12). All of the other animals tested did not show reactivity to the specific peptide greater than to an irrelevant peptide or the preimmunization sera, including the animals injected with \( \beta_2 \) and \( \beta_4 \). An example of these negative results is shown in Fig. 3.D.13.

**Immunoprecipitates to characterize anti-alpha and beta chain sera.** To address the question if the antibodies produced could recognize alpha chains isolated from cells, immunoprecipitations of \[^{35}S\]methionine-labeled Vavy cell microsomal membranes in the native and denatured state were attempted. Known positive controls were also used. The results of these experiments are shown in Fig. 3.D.14. The migration of immunoprecipitated material was identified by molecular weight and by comparison to known alpha and beta chains. Rabbit \( \alpha_3^b \) (to peptide \( \alpha_3 \)) precipitated two bands, at about 35 and 32 kD. The 35 kD band is the expected weight of the alpha chain. The 32 kD band could be the unprocessed form of the alpha chain. Alternatively this 32 kD species could be \( I_1 \) that was in an \( I_1 \)-alpha chain complex that was resistant to the denaturation procedure.

**Western blots to characterize anti-alpha and beta chain sera.** To address whether these antisera could western blot to electrophoresed, electrotransferred alpha chains, as would be necessary for them to be
used in the following experiments, this ability was assayed. Microsomal membranes from both Raji and Vavy cells were produced without radioactive label and electrophoresed in a 10% acrylamide SDS gel. The proteins were transferred to nitrocellulose, and strips were incubated with dilutions of the antisera. The adherent antibodies were detected with $^{125}$I-protein A (Fig. 3.D.15). The blot of the anti-23,30 (anti-class II) serum indicated the position of the beta chain, about 27 kD, as marked by the arrowhead. The $\alpha_3^a$ antiserum blotted to a protein that was about the molecular weight of alpha chain, while the $\alpha_3^b$ did not. In other experiments specific binding was detected colorimetrically, using an HRP-goat-anti-rabbit conjugate. Those experiments, also using preimmunization sera, confirmed $\alpha_3^a$ and $\alpha_3^b$ both blot to a band that is probably the class II MHC alpha chain. That data are not shown, however Fig. 3.D.16, Panel D, shows similar results.

**Immunoblots of $^{35}$S-cysteine labeled polypeptides.** To show definitively that the polypeptides were derived from the alpha chains, we used the anti-alpha chain sera to blot to these polypeptides. Specifically, Vavy cells were labeled with $^{35}$S-cysteine and immunoprecipitated with anti-class II MHC serum. The immunoprecipitated proteins were electrophoresed in a 17-27% gradient gel system (Fig. 3.D.16). One portion of the gel was saturated with fluor and autoradiographed to determine if the polypeptides in question were present. Panel B shows two small polypeptides at about
15.5 and 13.5 kD and probably a third polypeptide at about 12 kD (indicated by arrowheads). Panel A is a lighter exposure of this piece of the gel, to demonstrate the major species and the molecular weight standards. The rest of the gel, containing [35S]cysteine-labeled anti-class II serum immunoprecipitated proteins, was electroblotted to nitrocellulose. After blotting, the gel was autoradiographed (Panel C). The nitrocellulose was probed with antisera and the respective preimmunization sera (Panel D), and specific binding was detected colorimetrically, using an HRP-goat-anti-rabbit conjugate. α3^a and α3^b sera recognize the class II alpha chain (immunoprecipitated previously by an anti-class II MHC serum), as indicated by arrowheads. α3^b antiserum blotted to a band in the range of the observed polypeptides (about 12-16 kD). The problem here is that there was an inadequate separation of the polypeptides in this gel to determine if: 

(1) there were 2 or 3 species and (2) to which specific band the antiserum blotted. Also since this polypeptide is a minor species, and HRP was used to detect antibody binding, there was a problem with adequate colorimetric intensity of the blotted band.

To better visualize the blotted bands, better separate the polypeptides, and to try to resolve the question of comigration of bands, the above experiment was repeated on a large 11-23% gradient gel system, and adherent antibodies were detected with [125I]protein A (Fig. 3.D.17). Also in this experiment [35S]methionine-labeled Vavy cell membranes, immunoprecipitated with anti-class II MHC antibodies, were run. In Panel A, both the [35S]methionine and [35S]cysteine-labeled immunoprecipitates include the alpha and beta
chains. This piece of the gel was exposed to film longer (Panel B) to demonstrate the \[^{35}\text{S}]\text{methionine-labeled} material included p25 and a polypeptide of about 12.5 kD. In comparison, the \[^{35}\text{S}]\text{cysteine-labeled} immunoprecipitate did not show p25, but did show three polypeptides, of about 15.5, 12.5, and 10 kD. The other side of this gel, which had a continuous trough of \[^{35}\text{S}]\text{cysteine-labeled} immunoprecipitate run into the gel, was electroblotted to nitrocellulose. Strips of this nitrocellulose was probed with various antibodies, and preimmunization sera. The bound antibody was detected with \[^{125}\text{I}]\text{protein A} and the nitrocellulose strips were exposed to film (Panel C). As a control, a piece of nitrocellulose with only the blotted proteins was exposed with the immunoblots to film for the same length of time, to determine the extent of exposure from the \[^{35}\text{S}]. The comparison of the autoradiograph of this strip (labeled "No Ab"), to the blotted strips indicated the amount of film exposure from \[^{35}\text{S}], and also the positions of the proteins transferred to the nitrocellulose. In the case of the western blots with \(\alpha3\text{a}\) and \(\alpha3\text{b}\) sera, there is an enhancement of the alpha chain band indicating exact comigration of the blotted band with the \[^{35}\text{S}]\text{methionine-labeled} alpha chain band. This is very good evidence of recognition of the alpha chain with this antisera. The antiserum \(\alpha3\text{b}\) blotted to a band in the range of the polypeptides. In Fig. 3.D.18 the \(\alpha3\text{b}\) blot is shown again with a longer exposure to the film, along with a longer exposure of a strip of unprobed nitrocellulose, containing the \[^{35}\text{S}]\text{cysteine-labeled}, blotted proteins. The band recognized by \(\alpha3\text{b}\) lines up with
[\textsuperscript{35}S]cysteine-labeled, anti-class II MHC immunoprecipitated polypeptide. In this figure the unprobed strip was exposed to film about three times longer than the $\alpha^b$ blotted strip. This indicates that the intensity of the [\textsuperscript{35}S]cysteine-labeled, unprobed band is greatly enhanced by the $\alpha^b$ antibody probing and detection with [\textsuperscript{125}I]protein A. Consequently, there is a specific recognition of the $\alpha^b$ serum for this polypeptide. This band is probably the most intense of the three polypeptides, the 12.5 kD polypeptide. It can be concluded that the $\alpha^b$ antiserum recognizes one of these polypeptides, and presumably this polypeptide contains at least some portion the alpha chain sequence 77-88.
Fig. 3.D.1. Anti-class II MHC serum immunoprecipitates of Raji cell membranes, showing a 8.4 kD band upon reduction.
**Fig. 3.D.2.** Polypeptides associated with class II MHC molecules. Anti-class II MHC serum immunoprecipitates of [35S]cysteine-labeled Vavy cell membranes, electrophoresed on a gradient gel, indicating four polypeptides, which were calibrated at 14.4, 11.4, 8.4 and 4.0 kD.
**BETA CHAIN PEPTIDES**

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Fig. 3.D.3. Sequence of peptides made to mimic portions of the DR 3 beta chain, with a comparison to the respective sequence in the DP and DQ molecules present in Vavy cells.
### ALPHA CHAIN PEPTIDES

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Fig. 3.D.4. Sequence of peptides made to mimic portions of the DR 3 alpha chain, with a comparison to the respective sequence in the DP and DQ molecules present in Vavy cells.
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Fig. 3.D.5. Chart of anti-alpha and anti-beta chain peptide antisera with the results of various characterization methods.
Fig. 3.D.6. Dot blot titers of $\beta_1^a$ and $\beta_1^b$, with preimmunization sera (-P), to the respective and an irrelevant peptide-BSA conjugate.
Fig. 3.D.7. Dot blot titers of $\beta_2^a$ and $\beta_2^b$, with preimmunization sera (-P), to the respective and an irrelevant peptide-BSA conjugate.
Fig. 3.D.8. Dot blot titers of $\beta^3_3$ and $\beta^3_3$, with preimmunization sera (-P), to the respective and an irrelevant peptide-BSA conjugate.
Fig. 3.D.9. Dot blot titers of $\beta_4^a$ and $\beta_4^b$, with preimmunization sera (-P), to the respective and an irrelevant peptide-BSA conjugate.
Fig. 3.D.10. ELISA titer of antiserum $\alpha 3^a$, with preimmunization and postimmunization sera, against the respective and an irrelevant peptide.
Fig. 3.D.11. ELISA titer of antiserum α3b, with preimmunization and postimmunization sera, against the respective and an irrelevant peptide.
Fig. 3.D.12. ELISA titer of antiserum $\alpha_4^a$, with preimmunization and postimmunization sera, against the respective and an irrelevant peptide.
Fig. 3.D.13. ELISA titer of antiserum $\beta_{1a}$, with preimmunization and postimmunization sera, against the respective and an irrelevant peptide.
Fig. 3.D.14. Immunoprecipitates of native and denatured Vavy cell membranes, showing that the α3β antisera immunoprecipitates 35 and 32 kD species from denatured membranes. The 35 kD species is probably the alpha chain while the 32 kD species is probably Iδ or an unprocessed form of the alpha chain. Panels on either end are films that were exposed to the gel for shorter periods of time.
Fig. 3.D.15. Western blot of Raji and Vavy whole cell membrane proteins. Anti-23,30 (anti-class II MHC) recognizes beta chain (arrowhead). α3 serum recognizes a band migrating at about 35 kD, probably the class II MHC alpha chain.
Fig. 3.D.16. Western blot of anti-class II MHC serum immunoprecipitated proteins, detected with goat anti-rabbit antibodies coupled to horseradish peroxidase. Panel A (short exposure) and Panel B (long exposure) show polypeptides at about 15.5, 13.5, and probably one at about 12.0 kD. [35S]cysteine-labeled immunoprecipitated proteins were blotted with various antisera (Panel D), and then autoradiographed (Panel C). α3a and α3b recognized precipitated class II MHC alpha chains, and α3b also recognized a polypeptide in the range of 12 to 16 kD.
Fig. 3. D. 17. Western blot of anti-class II MHC serum immunoprecipitated proteins, detected with $^{125}$I-protein A. The $^{35}$S-methionine-labeled immunoprecipitate contained alpha chain, beta chain, p25, and a polypeptide about 12.5 kD. The $^{35}$S-cysteine-labeled immunoprecipitate contained alpha chain beta chain, and 3 polypeptides about 15.5, 12.5, and 10 kD (arrowheads; Panel A, short exposure; Panel B, long exposure). The $^{35}$S-cysteine-labeled proteins were blotted and probed with various antisera (Panel C). $\alpha_3^a$ and $\alpha_3^b$ sera recognize class II MHC alpha chain, and $\alpha_3^b$ serum recognizes a polypeptide about 12.5 kD (all strips exposed for the same period of time).
Fig. 3.D.18. Detail and enhancement of Fig. 3.D.17. The polypeptide recognized by α3β serum comigrated with an anti-class II MHC serum immunoprecipitated polypeptide. The "No Ab" strip was exposed to the film about 3 times longer than the α3β probed strip.
E. Phosphorylation of \( I_\dagger \) and the Class II MHC Alpha, Beta and Chains

**Introduction.** Phosphorylation is a well recognized mechanism for the regulation of the function of receptor proteins. Many systems demonstrating this fact have been worked out in fine detail. The class II MHC alpha chain was demonstrated to be phosphorylated in a B lymphoblastoid cell line (Kaufman et al., 1979). However this report was incomplete, since only a low resolution one dimensional gel was published, and no further information on the phosphorylation reported. Specific goals were the following:

1. To demonstrate if the class II MHC alpha, beta and the various forms of \( I_\dagger \) were phosphorylated, and on which amino acid(s), in B lymphoblastoid cell lines and activated human splenic B cells.

2. To determine the functional significance of these phosphorylations.

**Demonstration of the phosphorylation of \( I_\dagger \) and the alpha, beta chains.** Raji cells were labeled with \( ^{32}P \)orthophosphate, microsomal membranes were isolated and immunoprecipitated with anti-class II MHC antiserum (Humphreys et al., 1976). Figure 3.E.1, upper panel, shows that both the alpha and beta chains have incorporated radiophosphate. The lower panel of this figure showed that an alternative result of this same protocol could be obtained, \( I_\dagger \) alone could be phosphorylated. This problem of disparate results from one
experimental procedure was addressed in further experiments.

**Phosphorylation of alpha and beta chains in *S. aureus*-activated human B cells.** To address the hypothesis that the phosphorylation patterns seen are a result of the Raji cells being a cell line, further experiments with cells better resembling the *in vivo* situation, *S. aureus*-activated human B cells were done. Small, resting polyclonal human B cells were activated with formalinized, sterilized *S. aureus* for three days, and labeled with $[^{32}P]$orthophosphate in a manner identical to that in the above section. Again a discrepancy in the results were seen. Phosphorylation of the class II MHC alpha and beta chains were seen as was shown in Fig. 3.E.1, upper panel, and at another time phosphate was incorporated only into $I_1$. The amount of $^{32}P$ incorporated in $I_1$ was again low.

**Cell density and phosphorylation.** To address the hypothesis that the differing phosphorylation patterns resulted from different cell densities at the time of cell harvesting, Raji cells were collected at the following cell densities: 0.33, 0.68, 1.0 and $1.7 \times 10^6$ cells/ml. They were labeled and immunoprecipitated by the above protocol and all showed phosphorylated $I_1$, similar to what is shown in Fig. 3.E.1, lower panel. The amount of $^{32}P$ incorporated was again low.
Assessment of experimental problems. The problems of non-reproducible results and low incorporation of \( ^{32}P \) forced me to discontinue further experiments on this project. However, the observation of phosphorylation of \( I_i \), alpha and beta chains is valid. To summarize, under seemingly the same conditions, sometimes phosphorylation of the alpha and beta chains would be seen but not \( I_i \), while other experiments would show \( I_i \) phosphorylated but not the alpha and beta chains. Further, incorporation in most experiments was so low as to make further protocol modifications (as phosphoamino acid analysis) difficult. The current hypothesis to explain these results was that phosphorylation was dependent on the antigen processing and presentation state of the cells. This would explain the strange results seen in cell lines not presenting antigen, or polyclonally activated cell lines. The observation of the phosphorylation pattern being an either/or situation would indicate that somehow these cells are synchronous with respect to the parameter controlled this effect. Since we did not have a clonal human B and T cell, antigen presenting system this hypothesis was not directly testable. Consequently, it was decided not to pursue the project further. Two other labs had been working on the phosphorylation of \( I_i \) and the class II MHC molecules and at the time we was doing this work, early in the course of my thesis work. They reported having similar problems (R. Spiro and V. Quaranta, personal communication).
Fig. 3.E.1. Phosphorylation of I\textsubscript{i} and the class II MHC alpha and beta chains. Two-dimensional NEPHGE and SDS gel of solubilized membranes from \textsuperscript{32}P-labeled Raji cells immunoprecipitated with anti-class II MHC antiserum. Panel A shows the alpha and beta chains with incorporated radiophosphate, while Panel B shows I\textsubscript{i} with incorporated radiophosphate.
CHAPTER IV

DISCUSSION

A. Structural Analysis of I\textsubscript{1} Leading to a Hypothesis on the Binding of an Amphipathic, Alpha Helical Sequence in I\textsubscript{1} to the Desetope of Class II Antigens.

**Amphipathic helical structure of T cell-presented peptides.** An analysis of the strengths and frequencies of periodic hydrophobicities in sequences of well studied protein antigens led to the view that foreign peptides, which are presented to T cells via class II MHC antigens, can be amphipathic alpha helices, i.e., with opposing hydrophobic and hydrophilic sides (DeLisi and Berzofsky, 1985).

**Observation of an amphipathic helical region in I\textsubscript{1}** Inspection of the amino acid sequence of I\textsubscript{1} revealed a striking amphipathic helix running from Glu\textsubscript{148} to His\textsubscript{170}. Various graphical and structural models showed that this 5-cycle helix possessed: (1) a narrow strip of aliphatic amino acids (Leu\textsubscript{150}, Leu\textsubscript{153}, Met\textsubscript{157}, Ile\textsubscript{160}, Val\textsubscript{164}) down one side of the cylinder, and (2) otherwise, various generally hydrophilic amino acids.
Hypothesis that Iₐ amphipathic helix protects the class II MHC desetope. This observation led to the hypothesis that this amphipathic helix of Iₐ fills the antigen-combining site (desetope) of class II MHC antigens until such complexes reach the endosome where dissociation is induced, e.g., by proteolytic cleavage or pH effects.

After such endosomal release, one might fear the "prototypic amphipathic helix" from Iₐ could compete with foreign peptides for binding to class II MHC antigens. Inspection of structural models, however, led to the hypothesis that the cleaved Iₐ peptides polymerize tightly, probably as tetramers. Hydrophobic groups along one side of each peptide cylinder interdigitate. Looking at a putative tetramer in which the N-termini of the 4 helical peptides are at one end and the hydrophilic strips meet at the core of the tetramer, side chains of one peptide, Leu₁₅₀, Met₁₅₇, and Val₁₆₄, fit between Phe₁₄₆, Leu₁₅₃, Ile₁₆₀, and Ser₁₆₇ of the clockwise-adjacent peptide (repeating a zipper-like pattern four times). Also, on the external surface of the tetramer, His₁₅₂ and Lys₁₆₃ from one peptide form ionic bridges to Lys₁₅₄ and Asp₁₆₁, respectively, of the clockwise-adjacent peptide, again as viewed from the N-termini. Finally, at the C-termini, Trp₁₆₈ and Phe₁₆₅ of the adjacent peptides pair hydrophobically. Such polymerization may not occur with the native molecule due to steric inhibition by other parts of the Iₐ molecule. One can expect that such dimeric or tetrameric polymerization could eliminate Iₐ peptide competition for the class II MHC antigen desetope, although some other biological activity of
such polymers could remain.

While supported by a structural analysis of the I\textsubscript{1} peptide Phe\textsubscript{146} to Met\textsubscript{169}, this view has remained only a working hypothesis, that the amphipathic alpha helix Glu\textsubscript{148} to His\textsubscript{170} binds to the foreign antigen-binding site of the class II MHC proteins until release, when it polymerizes tightly, no longer to compete with foreign peptides for that site. Although the amphipathic helix, I\textsubscript{1}(146-170), has many structural peculiarities favorable to this hypothesis, it could still be only a structural helix of I\textsubscript{1} with the hydrophobic strip turned inward to some other relatively hydrophobic region of I\textsubscript{1}. In order to test this hypothesis, others in the laboratory have pursued a number of pathways. One set of experiments have attempted to chemically crosslink I\textsubscript{1} to the class II molecules. The crosslinked polypeptides have been examined to determine the "nearest neighbor" regions of the three chains.

If this hypothesis is true then it could serve as an explanation for the hyperexpression of I\textsubscript{1} in some leukemic cells (Spiro et al., 1980). In such cells the abundance of I\textsubscript{1} could alter the capacity of those cells to bind and present antigen. The net result might be a decreased ability for the host's immune system to mount a response against the neoplastic cells, which may be unable to present novel oncogenic antigens.

If the I\textsubscript{1} helix does lie in the class II MHC desetope, one might hypothesize this structure is a peptide with near maximal affinity for the desetope. Consequently portions of other proteins that would resemble this region of I\textsubscript{1} would have a high affinity for
the desetope, and might be class II presented. Computer programs to search for portions of proteins resembling the structure of this region of I$_1$ were produced, and have since been refined (Reyes et al., 1988; Reyes et al., manuscript in preparation). This method was called the Strip-of-helix algorithm and is covered in detail in the next section.
B. Hydrophobic Strip-of-helix Algorithm for Selection of T Cell-presented Peptides.

The strip-of-helix algorithm. The strip-of-helix hydrophobicity index was the mean hydrophobicity (from Kyte-Doolittle values) of sets of amino acids in axial strips down sides of helices for 3 to 6 turns, at positions n, n+4, n+7, n+11, n+14, and n+18. Our computerized protocol: (1) calculated strip-of-helix hydrophobicity values for 3, 4, 5 and 6 turns of a putative helix (8, 12, 15 and 19 amino acids in length), (2) printed the sequence position, amino acid and index value in tabular form, and index value and sequence position graphically, (3) listed the 5 most highly ranked peptides, (4) indicated those which did not have at least one adjacent strip (±1 or 2 amino acids from position n with hydrophobicity score -1.0 or less, (5) indicated those peptides which contained a proline and (6) indicated peptides which contained a lysine +1 or +3 positions after the C-terminal hydrophobic residue. The peptides correlating well with published T cell responsiveness had: (1) 12 to 19 amino acids (cycles 3 to 5 or 4-6 turns of an alpha helix, where a cycle is a turn plus one amino acid), (2) a strip with highly hydrophobic residues, (3) adjacent, moderately hydrophilic strips, and (4) no prolines. The degree of hydrophilicity of the hydrophilic strips of a putative antigenic helix above a threshold value did not count in the ranking. That is, the magnitude of amphipathicity was not judged to be the principal selecting factor for T cell-presented peptides. This simple algorithm
to quantitate strip-of-helix hydrophobicity in a putative amphipathic alpha helix, allowing otherwise generally hydrophilic residues, predicted 10 of 12 T cell-presented peptides in 7 well-studied proteins (Reyes et al., manuscript in preparation).

**Evaluation of method.** The strip-of-helix hydrophobicity algorithm well predicted those peptides which experimentally had been found to be T cell-presented (Reyes et al., manuscript in preparation). All except one of the sequences predicted by the algorithm of DeLisi and Berzofsky (1985) were identified with this algorithm. It also scored two antigenic sequences, in beef cytochrome C and in foot and mouth disease virus protein VP1, for which there were no reports of antigenicity. The T cell antigenicity of these sequences could be tested experimentally. Our simpler algorithm consequently performs as well as the DeLisi and Berzofsky method, in this regard.

One advantage of this algorithm is that potentially antigenic regions can be ranked according to their strip-of-helix hydrophobicity indices. With complex or relatively unstudied proteins, one might wish to synthesize and test the highest ranking peptides first. We cannot assess now how far down a series of ranked peptides one might actually find T cell responsiveness.

Some observations are consistent with the view that strip-of-helix hydrophobicity by itself was more important than the magnitude of amphipathicity in predicting antigenicity of peptides. For the hen ovalbumin antigenic peptide Ile$_{323}$ - Ala$_{337}$, which had
a strip-of-helix hydrophobicity index of +3.36 for 5 turns, an adjacent strip was quite hydrophilic (-2.22 for the strip following Ser_324). Another peptide in hen ovalbumin, Ala_23-Ala_37, which had not been found to be antigenic, had a strip hydrophobicity of +3.02 for 5 turns, and an adjacent strip with a hydrophilicity of -1.40 (from His_22). Thus, if some "maximal amphipathic moment" were necessary for antigenicity, it might be somewhere between 5.58 (-+3.36-(-2.22)) and 4.42 (+3.02-(-1.40)). However, other antigenic peptides examined in hen egg lysozyme and pig proinsulin had much smaller "moments", as calculated in that manner. Hen egg lysozyme peptide Ser_81-Ala_95 had a moment of 3.20 (+1.78 hydrophobicity and -1.42 adjacent hydrophilicity), and pig proinsulin Cys_6-Cys_20 had a moment of 3.70 (+1.96 hydrophobicity and -1.74 adjacent hydrophilicity). Thus, the factors governing antigenicity appeared to be (1) hydrophobicity of one strip-of-helix being among the highest in a protein, if not the highest, and (2) the hydrophobic strip being flanked by one or more moderately hydrophilic strips.

Prediction of the structure of the desetope. The close fit of predictions by this algorithm to experimental findings leads to a structural model of the class II MHC antigen-binding site. Since sequences of computer-selected peptides overlapped those of reported antigenic peptides usually by ten or more amino acids, it would be suspected that if T cell-recognized peptides coil as amphipathic alpha helices, then such peptides would compose helices of at least 4 1/2
complete turns (from amino acid n to n+12). The corresponding T cell receptor could be hypothesized to hold a linear crevice at least 3 helical cycles in length (about 15 Å) to receive such a peptide. In fact, if the Iα amphipathic helix Leu150 - Val164 fits such a crevice in the class II MHC molecules, the length of that site could be 5 helical cycles or about 27 Å. Loss of the Iα helix might catalyze charging of the crevice with a foreign peptide with a similar hydrophobic strip-of-helix, if removal and insertion were to occur by a concerted mechanism.

Although binding of a peptide's hydrophobic strip of amino acids to class II MHC could be a minimal requirement for its T cell antigenicity, additional structural determinants seem to govern binding and presentation by any given class II antigen allele. Such effects have been documented at both functional and biochemical levels. In studies of I-A^k-restricted T cell clones responding to hen egg lysozyme, Allen et al. (1985) found two peptides, Phe34-Arg45 and Asn46-Arg61, to account for recognition by 8 of 10 clones. In additional studies of fragments of hen egg lysozyme peptide CM-T11, Shastri et al., (1986) found that peptides Asn74-Ser86 and Ser85-Lys96 were restricted by I-A^k and I-E^k, respectively. Hen egg lysozyme peptide has been found to bind to purified I-A^k but not I-A^d molecules (Babbitt et al., 1985). In additional studies, hen egg ovalbumin peptide Ile323-Arg339 was found to bind to I-A^d but not to I-E^d, I-A^k, or I-E^k (Buss et al., 1986a). Similarly, an alpha helical region of sperm whale myoglobin (Phe106-Arg118) has been shown to be presented by I-A^d.
(Cease et al., 1986) and not by I-E\textsuperscript{d} or some other class II MHC alleles (Berkower et al., 1984; Berkower et al., 1985). It is likely that structures on alpha helical peptides, in addition to the axial strip of aliphatic residues, govern the binding to specific alleles of class II MHC molecules.

Since the original publication of this algorithm (Elliott et al., 1987; Stille et al., 1987) the structure of the class I MHC molecule has been solved (Bjorkman et al., 1987a; 1987b). By sequence comparison of class II to this structure, a hypothetical model of the class II MHC desetope has been predicted (Brown et al., 1988). That model well supported the above predictions of the class II MHC desetope structure. The desetope is made up of an alpha helix of the alpha chain on one side and the beta chain on the other, each comprised of hypervariable residues. The walls of the desetope extend for 6 or 7 turns of the helix. The size of the desetope cleft might be thinner than the diameter of an alpha helix (T. Garrett, personal communication). This, however, might reflect the lack of a conformational change that occurs when a foreign peptide binds in the desetope.

**Prediction of class I MHC-presented peptides.** Similar selection of peptides for class I MHC-mediated presentation of antigen to cytotoxic T lymphocytes (CTL) might also be partially governed at a peptide-binding stage. Townsend et al. (1986a) has analyzed class I MHC haplotype-specific presentation to CTL of various peptides derived
from influenza virus nucleoprotein. They demonstrated the selective presentation of nucleoprotein peptides $\text{Ile}_{365}^{\text{Glu}}_{380}$ and $\text{Ser}_{335}^{\text{Gly}}_{349}$ by murine H-2D$^b$ and human HLA-B37 class I MHC molecules, respectively. Since influenza virus nucleoprotein is not expressed on the surface of an infected cell (or on the viral envelope) and since anti-hemagglutinin CTL kill cells transfected with leader sequence-free hemagglutinin (which is not surface-expressed as intact hemagglutinin), Townsend et al. (1986b) hypothesized that class I-restricted antigens are digested to peptides prior to surface expression of the CTL-recognized fragment. Reyes et al. (1988) used the strip-of-helix algorithm to predict class I MHC-presented peptides from four proteins. The selected peptides well matched the portions of the proteins shown experimentally to be class I MHC-presented.

**Similarity to peptide hormones.** Amphipathicity is a physical characteristic required for the function of many peptide hormones (Kaiser and Kézdy, 1984), as well as for recognition of antigenic fragments (DeLisi and Berzofsky, 1985). One might then question whether strip-of-helix hydrophobicity (and its derivative, amphipathicity) represent an evolutionary convergence of forms to fit a general function, or a divergence of forms from an original function. That is, in early multicellular organisms regulated by amphipathic helical peptide hormones, there might have evolved a need to discriminate self-hormones from structurally similar foreign peptides. Primordial T cell receptor function could have diverged from
those receptors which functioned to recognize such homeostatic peptide hormones, or alternatively the two receptor types may have evolved from a common ancestor, possibly a primordial peptide hormone receptor.

**Design of peptides for synthesis.** In selecting class II MHC-presented peptides of a protein for synthesis, one might wish to examine strip-of-helix hydrophobicity plots over 3-, 4-, 5-, and 6-turns of an alpha helix. In such plots many peptides which have been proven to be antigenic appeared as third-, fourth- or fifth-ranked positions in a 3- or 4- turn plot, but in a 6-turn plot they occupied first- or second-ranked positions (Table 4.B.1). Many of the experimentally antigenic peptides, thus, showed up consistently in 3- to 6-turn plots. If one does not see a consistent or evolving ranking in a comparative analysis of 3-, 4-, 5-, and 6-turn plots, we would suggest a 5-turn plot to be preferred for ranking putatively antigenic peptides because it tends to rank highly most peptides which are found experimentally to be antigenic.

For the synthesis of a potential immunogen, we would not feel restricted to the peptide bounded by the first and last hydrophobic amino acids in the hydrophobic strip. A proline beyond the N-terminus (plus a further N-terminal amino acid) would create a hairpin turn of the peptidyl backbone in which the carbonyl function of the amino acid preceding the proline could hydrogen bond to the amido function of the second amino acid following the proline (Dyson et al., 1985) to
stabilize the N-terminus of the helix. Consequently these amino acids, if present in the protein sequence, could be included in the synthesized peptide.

Spouge et al. (1987) found an increased frequency of lysines in the C-terminal regions of experimentally antigenic peptides. They proposed that lysine could bind to the desetope and/or stabilize the C-terminus of an alpha helix. Alternately, one could suggest that tryptic cleavages, which produced many of the experimentally antigenic peptides, would leave a disproportionate number of C-terminal lysines. However, a closer examination of the sequence position of the lysines showed a preponderance (7/12) of the lysines occurred in the position immediately following the last amino acid in the hydrophobic strip-of-helix (+100° with respect to the axis of the cylinder). 0/12 occurred at the second following position (+200°). 2/12 occurred at the third or later positions. This distribution of lysines with respect to the termination of the hydrophobic strip-of-helix, supported the view that a desetope interaction with the lysine occurred, perhaps with a carboxyl function of the desetope, positioned on the side of a trough complementary to the C-terminus and +100° to the last hydrophobic residue in the antigenic peptide’s strip-of-helix. Given that empiric observation, one might consider adding a C-terminal lysine in the synthesis of a peptide immunogen (whether or not it occurred in the sequence of the antigenic protein).

One might also wish either to substitute Phe with Tyr, or to add a terminal Tyr to permit radioiodination of the peptide. Additional hydrophilic N- or C-terminal amino acids could be included to improve
peptide solubility. While this computer program specifies explicitly and ranks potentially antigenic sequences, additional considerations could determine the exact peptide for synthesis and further experiments.
Table 4.B.1. Selection of peptides as a function of turns in a putative alpha helix.

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<td>index</td>
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C. Proteolysis of I

Proteolysis of I to p25. p25 was shown to be an exomembranal, C-terminal fragment of I, by second immunoprecipitations with various antibodies of VIC-Y1 mAb-immunopurified I-containing complexes, that were denatured. Rabbit antisera to two C-terminal I peptides immunoprecipitated the denatured and resolubilized p25, while an antiserum to an N-terminal peptide failed to precipitate p25. In the same gels, each of these three antisera recognized denatured and resolubilized I. A monoclonal antibody to I immunoprecipitated [35S]methionine-labeled p25 but not [35S]cysteine-labeled p25, consistent with the loss of a portion of I containing the only cysteine in I, Cys28. Judging from the 25 kD weight of p25, the lack of reactivity with an antiserum to the N-terminus of I, the absence of Cys28, and positioning of the transmembranal domain (Gly31-Tyr56), one can conclude that p25 represented the C-terminal portion of I. p25 would then contain the amphipathic, alpha helical region I(146-170) which was hypothesized to fit in the class II MHC desetope.

Cleavage of I in vivo appeared to occur while the molecule was associated with class II MHC molecules, since some p25 was found in immunoprecipitates with an anti-class II MHC heteroantiserum which did not recognize I in immunoprecipitates from a class II+, I+ cell line (Spiro et al., 1985). Since immunoprecipitates with anti-I sera demonstrated perhaps 5-fold more p25 than did
precipitates with anti-class II MHC serum, one can suggest that although p25 was cleaved while associated with class II MHC antigens, some p25 might have been generated by proteolytic cleavage of I\textsubscript{i} not associated with class II MHC antigens, or had since dissociated from the class II MHC antigens. I\textsubscript{i} has been shown to have a transmembranal segment cleavage site which is sensitive to a signal peptidase and can be exposed upon deletion of hydrophilic amino acids on the N-terminal side of the transmembranal segment (Lipp and Dobberstein, 1986).

The relationship of p25 to I\textsubscript{i} was also suggested by Giacoletto et al. (1986) who demonstrated that I\textsubscript{i} and p25 derived from SDS electrophoretic gels of immunoprecipitates had similar tryptic peptide maps.

Proteolytic cleavage of class II MHC antigen-associated I\textsubscript{i} to p25 appeared to be determined by secondary structural characteristics which left the most sensitive site for cleavage to p25 external to the transmembranal segment. Since cleavage with three proteases with different specificities all yielded initially fragments about 25 kD, one can suggest that in vivo cleavage of I\textsubscript{i} to p25 is restricted by secondary structural features (domains), as with IgG (Porter 1959), rather than being the result of an exquisite substrate specificity of some endogenous protease. Marks and Cresswell (1986) digested class II MHC antigen-I\textsubscript{i} complexes with proteinase K and identified in immunoprecipitates the principal I\textsubscript{i} fragments to be from the C-terminal portion of I\textsubscript{i} and to be associated with class II MHC antigens. They did not address the origin of in vivo generated p25,
but their data are consistent with the hypothesis that such a C-terminal fragment of \( I_i \) can remain associated with class II MHC antigens after proteolytic cleavage at a site near its transmembranal region. Although alternate translational start sites and exon splicing accounted for the 4 forms: \( I_i, \gamma 2/\gamma 3, p41 \) and \( p43 \), no genomic basis for a \( p25 \)-coding transcript has been found (Strubin et al., 1986b).

Cleavage of \( I_i \) to \( p25 \) occurred in an ER or cis-Golgi compartment. Since \( p25 \) was completely sensitive to treatments with endoglycosidases F and H, its carbohydrate side chains were not processed to complex sugar forms. It was therefore considered to be derived from a high mannose form of \( I_i \). Also, since anti-\( I_i(183-193) \) serum, which immunoprecipitates \( p25 \), did not recognize either O-linked forms of \( I_p \), or the \( I_i-CS \) proteoglycan form of \( I_i \), \( p25 \) was probably not derived from either of those molecules.

Immunoprecipitation of Percoll-density-gradient fractions from Dounce-homogenized, polyclonally activated B lymphocytes, which were \([^{35}S] \)methionine, pulse-chase-labeled, demonstrated the appearance of \( p25 \) at 20-40 min chase times in Golgi-ER fractions, and not in either plasma membrane or lysosome fractions (Nguyen et al., 1988). The placement of the \( I_i \) cleavage to \( p25 \) in the ER or cis-Golgi, during this time frame, is also supported by work with vesicular stomatitis virus which showed that some proteins transit through the Golgi between 13 and 60 minutes (Morrison and Ward, 1984).

The appearance of \( p25 \) in an ER or cis-Golgi compartment is consistent with the hypothetical functions: (1) that there could exist an ER degradative pathway to destroy incomplete complexes of class II
MHC proteins and associated molecules, or (2) that \( I_1 \) could retard the release of class II MHC molecules to the cell surface until they have bound foreign peptide. Such foreign antigen-containing complexes could be carried to the cell surface by bulk flow. Precedent for the former hypothesis includes the description of a pre-Golgi proteolytic pathway for rapid degradation of newly synthesized, T cell receptor subunits (Lippincott-Schwartz et al., 1988). This pathway appears to serve in the degradation of "unassembled" or "incompletely assembled" T cell receptor \( \alpha, \beta, \delta \) proteins, and is not sensitive to inhibition with lysosomotropic agents such as ammonium chloride, chloroquine, and methylamine. This pathway is the sole destructive mechanism followed by T cell receptor \( \alpha \) chains and \( \alpha-\beta \) complexes in transfected fibroblasts. The degradation of \( I_1 \) to p25 has the characteristics of this ER degradative pathway, including: (1) the relative insensitivity to treatments with chloroquine or monensin (Nguyen et al., 1988), (2) time of cleavage of about 20 min, and (3) cleavage of high mannose species consistent with the T cell receptor complex cleavage in an early compartment. Precedent for the latter model can be found in the work on the p78 BiP protein which associates with mIgM heavy chain until release of IgM to the surface (Bole et al., 1986; Hendershot and Kearney, 1988).

Consequently it was concluded that the cleavage of \( I_1 \) to p25 does not occur in a post-Golgi compartment, for example, upon fusion with an endosomal vesicle with digested foreign antigen. This degradation to p25, however, could still be associated with a regulatory event of class II MHC antigen-charging with foreign
peptides. Fusion of endosomes with ER and cis-Golgi compartments has been described (Opresko and Karpf, 1987). It is also possible this cleavage could reflect destruction of $I_i$ molecules which are synthesized in excess of the number required to associate with all nascent class II MHC alpha-beta chain complexes. However, since $I_i$ is processed to complex sugar and O-glycosylated forms, beyond the point at which p25 is generated, additional functions for such mature forms are likely.

Future work of this laboratory will be aimed at defining the cleavage site which generates p25 and to test whether release of $I_i$ or p25 has a catalytic relationship to antigen charging of class II MHC molecules in vitro and in vivo.

Proteolysis of $I_i$ via a late pathway. Another pathway of $I_i$ proteolysis has been described (Blum and Cresswell, 1988; Nguyen et al., 1988). Incubation of cells with leupeptin or antipain, but not with chymostatin or pepstatin, revealed proteolytic intermediates p21 and p10, at 2-5 hr after synthesis of the class II MHC-$I_i$ complex. These two species were determined to be derived from the N-terminal region of $I_i$ (Fig. 4.C.1) by peptide mapping and precipitation with anti-$I_i$ peptide antibodies of denatured molecules. Blum and Cresswell (1988) called this the "LIP" pathway, for Leupeptin-Induced Proteins. Since p21 was immunoprecipitated with anti-class II MHC antibodies which did not recognize $I_i$ (Spiro et al., 1985), at least the initial step in the path of $I_i$ proteolysis occurred while
I\textsubscript{i} still adhered to the alpha and beta class II MHC glycoproteins. Furthermore, digestions with Endo H and Endo F (Blum and Cresswell, 1988) and the sialic acid-induced electrophoretic heterogeneity (Nguyen \textit{et al.}, 1988) of p21 indicated it was derived from a form of I\textsubscript{i} that had N-linked sugars of the complex form. These observations led to the view that the cleavage of I\textsubscript{i} to p21 and p10 occurred in a post-Golgi or endosomal compartment. As further confirmation for this site of cleavage, cells which were incubated with monensin, trapping I\textsubscript{i} in the Golgi, produced no p21 or p10. In pulse-chase experiments, these polypeptides were seen to be produced maximally about 2-5 hr after synthesis, as would be consistent for a site of cleavage in a post-Golgi or endosomal compartment.

The conclusion is that a late form of I\textsubscript{i} was degraded by a leupeptin insensitive enzyme to the products p21 and p10. Normally these species were quickly degraded by a leupeptin sensitive enzyme to smaller products, that would not have been seen in experiments using routine protocols. This would be consistent with the removal of I\textsubscript{i} from the class II MHC molecules during antigen presentation. Possibly p21 and p10 are the remnants of I\textsubscript{i} after removal from the desetope.

\textbf{Comparison of the pathways of degradation of I\textsubscript{i}.} Two distinct pathways of intracellular proteolysis of I\textsubscript{i} have been described. In the first pathway, an early, high mannose form of I\textsubscript{i} was degraded to p25 (the C-terminal portion of the molecule). This cleavage probably took place in the ER or cis-Golgi compartment about 20 min after
synthesis. Its function could be as a pathway for the degradation of incompletely formed complexes of class II MHC molecules and associated proteins, as has been described for the T cell receptor (Lippincott-Schwartz et al., 1988).

In contrast, cleavage of I\textsubscript{i} via a later pathway resulted in two products, p21 and p10, that were derived from the N-terminus of the I\textsubscript{i} molecule. This cleavage took place about 2-5 hr after synthesis, probably in a post-Golgi or endosomal compartment. This pathway might be the more immunologically relevant pathway, with p21 and p10 possibly being the remnants of I\textsubscript{i} cleaved away from the class II MHC molecules as foreign antigen combined, as the class II MHC molecules were being prepared for presentation on the cell surface.
Fig. 4.C.1. Identity of p25, p21, p10. p25 contains the C-terminal portion of $I_i$, while p21 and p10 contain more of the N-terminal region. The position of the $I_i$ amphipathic alpha helix (146-170) is indicated.
D. Proteolysis of the HLA Class II MHC Alpha and Beta Chains.

Putative sites for regulatory proteolytic cleavages. We searched class II MHC alpha and beta chains for conserved, basic amino acid pairs, which in other systems are known to be sensitive targets for physiologically regulatory, proteolytic cleavages, e.g., in proinsulin (Steiner et al., 1969) and the hemagglutinin of influenza virus (Skehel and Waterfield, 1975). In class II MHC antigen alpha chains, we found Arg₄₂ or Lys₄₂-Lys₄₃, Lys₇₉-Arg₈₀ (DR and DQ), Arg₁₅₀-Lys₁₅₁ (DR only), and Arg₂₂₂-Lys₂₂₃ (DR only). In beta chains we found Lys₇₁-Arg₇₂ (in most DR and DQ), and Arg₉₃-Arg₉₄, which is particularly well conserved.

When the positions of these sequences were examined in relation to the predicted structure of the class II MHC molecules (Brown et al., 1988) interesting observations were made. In both chains, the walls of the desetope were bracketed by, or contained, potential cleavage sites. It was hypothesized that the destruction of the desetope would be one of the most efficient ways to control the antigen presenting function of the class II MHC antigens. These cleavage sites seem to be in crucial positions to do just this. Alternatively, one could hypothesize that after removal of one wall of the desetope that helix could be replaced by a similar structure, in a process of "desetope conversion". Furthermore excised fragments could be presented by other, intact desetopes leading to MHC linkage of autoimmune responses to structurally similar self and foreign
Rationalization of putative polypeptides to observed polypeptides. If one assumes complete cleavage at all the proposed sites, nine polypeptides would be produced (Table 4.D.1). Two of these polypeptides might be recognized with the anti-a3 serum (4.0 kD and 7.8 kD). The former polypeptide, α(80-150), contains the site of a complex carbohydrate addition, which could increase the molecular weight of this fragment to about 13.0 kD. This would put it in the range of the observed polypeptides in Fig. 3.D.16 to 3.D.18. If the α(80-150) polypeptide is that band to which α3b blots, then it will not line up with the methionine labeled band in this range, since the sequence contains no methionines. Consequently future experiments will be aimed at determining with which radiolabeled band the blotted band comigrates. Also immunoprecipitates treated with Endo F should drop the molecular weight of the blotted band to about 7.7 kD. Alternatively, if a polypeptide DRα(43-150) was formed, it would contain both methionines and cysteines, and be recognized by the anti-α3 serum.

It was unfortunate that none of the anti-beta chain rabbits produced specific antibodies. However, the goal of determination of a beta chain origin of some of these polypeptides is still possible. As seen in Table 4.D.1 each polypeptide is unique with respect to amino acid content and carbohydrate. Polypeptide β(1-71) will label only with cysteine and contain carbohydrate, polypeptide β(72-80) will
label with cysteine but be under 1.0 kD and might not be resolved, polypeptide \( \beta(81-93) \) will only partially label with methionine, and probably will be too small to resolve (1.3 kD), and polypeptide \( \beta(94-238) \) will label with both cysteine and methionine, and will migrate at about 16.0 kD. Such examination of the polypeptides is possible but the best plan will be to resynthesize the peptides and produce more antisera with the recommendations mentioned below.

If all of the potential cleavage sites are not used, or if others are used, then the situation would become more complex to interpret. If that were the case, one could still draw conclusions if all data (results from blotting, amino acid incorporation, molecular weight, carbohydrate labeling, etc.) was assembled and systematically compared to the known sequences of the alpha and beta chains.

Possible role of class II MHC proteolytic cleavages: desetope inactivation. One possible function of the proteolytic cleavages of the alpha and beta chains could be in the destruction of the antigen presenting capabilities of the surface-expressed class II MHC molecules and consequent attenuation of antigen presentation to T cells. To address this hypothesis future experiments will involve examination of surface-expressed class II MHC molecules for their pattern of alpha and beta chain cleavages. This experiment will be done using a technique that couples biotin to the cell surface molecules (Jasiewicz et al., 1976; Elliott et al., 1989). These labeled molecules can then be detected using \(^{125}\text{I}}\)-avidin. Dr. W.
Elliott, formerly of this laboratory, demonstrated that class II MHC molecules can be labeled well by this technique. Further, pulse-chase experiments in conjunction with the biotinylation could be done to address the question of how long after surface expression are the chains cleaved.

One must rationalize hypotheses about the role of the putative cleavage sites with the observation that some of the pairs of basic residues are not conserved in all isotypes or alleles of the genes. Lack of these putative cleavage sites may be found to be significant in possible isotype-specific functions of the DR, DP, and DQ complexes. Alleles which lack these cleavage sites might be able to be linked to altered antigen presentation functions, or certain diseases.

Possible role of class II MHC proteolytic cleavages: desetope conversion. Another possible function of the proteolytic cleavages of the class II MHC alpha and beta chains could be to aid in antigen presentation by allowing the binding of a peptide similar to one wall of the desetope. Some antigenic peptides have sequence homology to the putatively excised, hypervariable region peptides of the alleles which restrict T cell presentation of the antigenic peptides (Guillet et al., 1987). This finding led Guillet et al. (1987) to suggest that these hypervariable regions might be displaced in some fashion by the binding of the foreign peptides, although excision at the bracketing pairs of basic amino acids was not proposed. We would now suggest, specifically, that endosomal proteolytic cleavage of the beta chain at
Lys\textsubscript{71}-Arg\textsubscript{72} and Arg\textsubscript{93}-Arg\textsubscript{94} or of the alpha chain at Lys\textsubscript{42}-Lys\textsubscript{43} and Lys\textsubscript{79}-Arg\textsubscript{80} with consequent removal of the cleaved peptides could expose a site which could bind peptides structurally similar to the excised peptide.

Closer examination of this region in the beta chain revealed a conserved sequence Cys\textsubscript{79}-Arg\textsubscript{80}-His\textsubscript{81}-Asn\textsubscript{82}-Tyr\textsubscript{83} which was present in all reported DR, DP and DQ alleles (Figueroa and Klein, 1986). While Arg\textsubscript{93}-Arg\textsubscript{94} was present in all reported DR, DP and DQ beta chain alleles, Lys\textsubscript{71} was substituted in some alleles. One might suggest that Arg\textsubscript{80}-His\textsubscript{81} represented a cleavage site more readily identified under acidic conditions and that Cys\textsubscript{79} (site of a disulfide bond) could stabilize the peptide fragment in the N-terminal portion of this hypervariable region. That is, the entire hypervariable region appeared to consist of two subregions with a potential for preferential excision of a 3 1/2 cycle helix (His\textsubscript{81}-Arg\textsubscript{93}) under some conditions.

**Problems of producing anti-peptide antisera.** We had problems producing some anti-peptide antisera. The most likely explanation was that most of the peptides we synthesized were too short. The unsuccessful peptides (β1-4 and α1-2) were between 7 and 9 amino acids long, while the successful peptides were 12 and 13 amino acids long. Many reports seem to favor longer peptides for immunogens (Ziltener et al., 1987) and several rationales support this view. Longer peptides provide a logarithmically increasing number of possible
epitopes, consequently giving a higher probability of activating more clones. Using this logic, the rabbits that were defined as non-producers could actually have responded to the peptide, but only with a few clones. The resulting antibody response might have been too low to detect. Also shorter peptides have a higher probability of having their structure altered by the coupling to the carrier (Briand et al., 1985).

Another explanation of the low success rate is that only two rabbits were used per peptide. It is known that some rabbits do not respond well to some antigens, possibly due to the fact that they are necessarily genetically identical with respect to the MHC. The peptide conjugate could be injected into additional rabbits.

**Future experiments.** There is much work left to be done on this project. The primary goal is to conclusively define the origin of the polypeptides. To achieve this I would: (1) resynthesize the peptides and repeat the antisera production following the recommendations above, (2) repeat blotting experiments with a urea gel system, or another gel system, that would expand the separation of the peptides allowing identification of which bands comigrate, (3) use a blotting system with luminol, a substance which when used in conjunction with HRP will expose a film with light (Amersham Corp.), which would eliminate background bands due to $^{35}\text{S}$ while allowing overexposure, and (4) repeat blotting experiments after digestion with Endo F and Endo H to determine what peptides contain carbohydrate.
Other experiments that will be important to do will be to (1) follow the production of these peptides with \([^{35}S]\)cysteine pulse-chase experiments to determine when these peptides are produced \textit{in vivo}, (2) surface label the class II MHC molecules with biotin and determine the relationship of surface expression and susceptibility to proteolytic cleavage, and (2) repeat the biotin labeling with pulse-chases to determine how long after surface expression does cleavage take place.
<table>
<thead>
<tr>
<th>CHAIN</th>
<th>PORTION</th>
<th>Number of Amino Acids (Approx. M.W.)</th>
<th>Number of Polypeptide (Vav Haplotype)</th>
<th>Amino Acids Present in Putative Polypeptide (Vav Haplotype)</th>
<th>Recognized by Anti-Serum</th>
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</thead>
<tbody>
<tr>
<td>alpha</td>
<td>1-42</td>
<td>42 (4600)</td>
<td>M-27</td>
<td>M-27 M-40 M-35</td>
<td>α1 α2</td>
</tr>
<tr>
<td>alpha</td>
<td>43-79</td>
<td>36 (4000)</td>
<td>Asn&lt;sub&gt;70&lt;/sub&gt; High Mannose</td>
<td>M-77</td>
<td>α2 α3</td>
</tr>
<tr>
<td>alpha</td>
<td>80-150</td>
<td>70 (7700)</td>
<td>Asn&lt;sub&gt;118&lt;/sub&gt; Complex</td>
<td>C-111 C-111 C-111</td>
<td>α3 α4</td>
</tr>
<tr>
<td>alpha</td>
<td>151-222</td>
<td>71 (7800)</td>
<td></td>
<td>C-167 C-199 C-167 M-190 M-190</td>
<td>α4</td>
</tr>
<tr>
<td>alpha</td>
<td>223-233</td>
<td>10 (1100)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>beta</td>
<td>1-71</td>
<td>71 (7800)</td>
<td>Asn&lt;sub&gt;19&lt;/sub&gt; Complex</td>
<td>C-15 C-15 C-15 (M-14 other DQ)</td>
<td>β1 β2</td>
</tr>
<tr>
<td>beta</td>
<td>72-80</td>
<td>8 (880)</td>
<td></td>
<td>C-79 C-79 M-78 M-79</td>
<td></td>
</tr>
<tr>
<td>beta</td>
<td>81-93</td>
<td>12 (1320)</td>
<td></td>
<td>M-99</td>
<td></td>
</tr>
<tr>
<td>beta</td>
<td>94-238</td>
<td>144 (15,800)</td>
<td></td>
<td>C-118 C-118 M-200 (M-182 unique)</td>
<td>β3 β4</td>
</tr>
</tbody>
</table>

Fig. 4.D.1. Characteristics of postulated alpha and beta chain cleavage products. The positions of specific amino acids are indicated with one letter code and sequence number, (M-27 for methionine at position 27).
E. Phosphorylation of I\textsubscript{i} and the Class II MHC Alpha, Beta and Chains

Experimental observations. We have demonstrated that I\textsubscript{i} and the class II MHC alpha and beta chains can be phosphorylated. Further, it seems that either the alpha and beta chains are phosphorylated or I\textsubscript{i} is, not all three chains at the same time. These two types of phenomena were the result of the same experimental protocol. The hypothesis that the inconsistent results was due to using a cell line (Raji) as the model system was tested, however with S. aureus-activated B cells the same results were seen. The hypothesis that the differing results were due to the cell density at the time of labeling was also tested, however 4 cell densities all provided one result, slight labeling of I\textsubscript{i} with radiophosphate.

The production and use of a human antigen presenting system, with matched B and T cells, might be needed to resolve this discrepancy. In that system one would be more closely simulating the \textit{in vivo} situation and consequently could expect more consistent results. Once the phosphorylation of these molecules are consistently seen, the residue to which the phosphate has been attached could be determined by phosphoamino acid analysis using either high voltage chromatography or a thin layer chromatography method we developed. However, since this system was unavailable at the time, I decided to pursue other, more fruitful, pathways.
Sites of phosphorylation. With the observation that phosphorylation of these molecules do occur, and with the known sequences, it is possible to hypothesize what amino acids might be phosphorylated.

The Raji cells have the haplotype of HLA-DR 3,6 (Larhammar et al., 1982a; Larhammar et al., 1982b; Kaufman et al., 1984). The alleles of the DP and DQ molecules have not been published, however Larhammar et al. (1982a) published a beta chain sequence from Raji that is highly homologous (228/229 amino acids) to the DQ 1 beta chain sequence (Figueroa and Klein, 1986). For the sake of argument it will be assumed Raji has the DQ 1 allele, although for the sequence regions discussed all published alleles are homologous, unless otherwise noted.

The sequence of DR 3 alpha chain is published (Larhammar et al., 1982b; Figueroa and Klein, 1986) and is shown in Fig. 4.E.1. Since (1) most phosphorylations occur on the cytoplasmic portion of the molecule; (2) only serines, tyrosines, and threonines are phosphorylated and; (2) there is only one serine in this area of the molecule; it can be hypothesized that Ser\textsubscript{224} is the site of phosphorylation. This sequence area is well conserved among the published DR alpha chain sequences. Consequently, although we do not know the sequence of the DR 6 alpha chain, one can hypothesize it has a similar sequence. The DQ 1 sequence (Fig. 4.E.1) has two serines in its cytoplasmic sequence (positions 223 and 227), that are conserved in most published alleles. Only two DP alleles are published and they are entirely different from each other with respect to the amino acids
in the cytoplasmic domain that can be phosphorylated. Consequently, interpretation of these isotype products is difficult.

Both the sequences of the DR 3 and DR 6 beta chains are published (Larhammar et al., 1982a, Figueroa and Klein, 1986) and the cytoplasmic tails are identical except for the substitution of Phe\textsubscript{236} in DR3 for Leu in DR6. In these sequences (Fig. 4.E.1) there are three sites of possible phosphorylations, two serines (229, 238) and one threonine (234). The DQ sequence from Raji cells (Larhammar et al., 1982a), in Fig. 4.E.1, contains one amino acid in the cytoplasmic tail that can be phosphorylated, Ser\textsubscript{224}. This serine is entirely conserved in all DQ sequences published (Figueroa and Klein, 1986). The DP beta chain sequences (Fig. 4.E.1) contain an entirely conserved serine at position 224, and four of the five published sequences have an additional serine at 231. This fifth sequence, which is very different than the other four, has a serine at position 242.

Obviously, one of the next experiments that needs to be done is to determine if all three class II MHC isotype products are equally phosphorylated. These experiments did not distinguish between the DR, DP and DQ molecules, but simply looked at the phosphorylation of all the class II MHC molecules. It is easy to imagine that these different isotype products might be phosphorylated differently. Hong et al. (1988) demonstrated that, in their murine system, this might be the case, as H-2D\textsuperscript{k} molecules are phosphorylated while H-2K\textsuperscript{k} are not. It is also interesting to speculate that the presence or absence of amino acids in the cytoplasmic tail that could be phosphorylated, in the DR, DQ, or DP molecules, might be significant in different
functions these isotypes could have.

The sequence of $I_\beta$ has been published (Claesson et al., 1983b), and is shown in Fig. 4.E.1. There are three serines in the cytoplasmic tail sequence (positions 9, 26 and 29). Two of these serines (positions 9 and 29) are conserved in the sequence of the murine $I_\beta$ sequence (Zhu and Jones, 1989).

Since this work was done a paper was published by Spiro and Quaranta (1989) which demonstrated the phosphorylation of $I_\beta$. They showed that $I_\beta$, Ip, and p41 were phosphorylated on serine residues, but the $\gamma 2$ and $\gamma 3$ species were not. They were not able to define the specific residues phosphorylated nor establish a function for these modifications. Through personal communication they indicated they had similar problems to those we encountered.

**Class I MHC phosphorylation precedent.** The class I MHC molecule was shown to be phosphorylated on several residues. The cytoplasmic portion of HLA-A2 is shown in Fig. 4.E.1. The positions of the amino acids that can be phosphorylated are well conserved, with most being perfectly conserved. Pober et al. (1978) showed that the class I MHC molecules were phosphorylated in vivo on a serine residue on the cytoplasmic portion of the molecule and Guild and Strominger (1984a) localized that phosphorylation to serine 341, a residue conserved in all the sequences published by Figueroa and Klein (1986). Guild and Strominger (1984b) also demonstrated the phosphorylation of two serine residues in vitro with cAMP-dependent protein kinase, with one of
these residues in the conserved sequence Arg-Arg-Lys-Ser\textsubscript{315}-Ser\textsubscript{316}; the other was at position 325. Guild and Strominger (1983) further demonstrated that a cytoplasmic tyrosine residue (position 326) was phosphorylated \textit{in vitro} by Rous sarcoma virus protein kinase. The above literature was analyzed by Guild and Strominger (1984b) to show that most of the amino acids that could be phosphorylated were phosphorylated by some kinase, (\textit{i.e.} protein kinases \textit{in vivo}, cAMP-dependent protein kinases, or Rous sarcoma virus protein kinase). Consequently, in the class I MHC example it seems that phosphorylations can be the result of different processes, with the specific amino acid derivatized being significant to the function involved.

Hong et al. (1988) demonstrated that in the murine system, only cell surface forms of H-2D\textsuperscript{k} molecules were phosphorylated. R. Mittler (personal communication) has evidence that, in the human system, at least one of the phosphorylations of the class I MHC molecules might be linked to surface expression.

\textbf{Hypothesized function of I\textsubscript{a} phosphorylation.} The phosphorylation of class I MHC molecules can be viewed as a similar system to the phosphorylation of class II MHC molecules. In that system phosphorylation occurs on virtually all residues on which it is possible. Further it seems that phosphorylation could occur as a result of enzymes that are involved in different functional pathways. Consequently we could hypothesize that phosphorylation could occur on
most of the residues mentioned above, and that these phosphorylations might be the result of different processes.

Phosphorylation of the adrenergic receptors (Sibley and Lefkowitz, 1985, Sibley et al., 1987) could also serve as a precedent for phosphorylations of the class II MHC molecules and \( I_1 \). In that system the beta-adrenergic receptor is phosphorylated after agonist binding. The phosphorylated receptor is sequestered intracellularly and the cell is desensitized to agonist binding. Eventually the dephosphorylated receptor is returned to the cell surface and again could bind agonist.

One possible function of the phosphorylation of the class II MHC molecules could be in relation to their surface expression, as with the class I MHC molecules. Further, phosphorylation could be connected with internalization of the class II MHC molecules resulting in the attenuation of antigen presentation.
ALPHA CHAINS

DQ 1  TM-R-G-L-R-S-V-G-A-S-R-H-Q-G-P-L

BETA CHAINS

DR 3  TM-R-N-Q-K-G-H-S-G-L-P-P-T-G-F-L-S
DR 6  TM-R-N-Q-K-G-H-S-G-L-Q-P-T-G-L-L-S
DQ ?  TM-R-S-Q-K-------G-L-L-H
DP 1  TM-R-S-K-K-V-Q-R-G-S-A

<table>
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<th>30</th>
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Fig. 4.E.1. Sequences of various putatively phosphorylated molecules. The sequence numbers are those from Figueroa and Klein (1986).
F. Hypothetical Scheme to Explain the Functional Significance of Structural Changes in Class II MHC Molecules and I$_i$.

Introduction. A scheme for the dynamic associations and cleavages of the class II MHC alpha and beta chains, and associated molecules, in the context of antigen processing and presentation, is presented in Fig. 4.F.1. The ideas of this figure are based on experiments and hypotheses.

Endoplasmic reticulum. The class II MHC alpha and beta chains can associate with I$_i$ in the endoplasmic reticulum at the time of their synthesis, and remain with I$_i$ during transport through the ER and Golgi (Kvist et al., 1982; Claesson and Peterson, 1983a; Machamer and Cresswell, 1983). There is a large pool of I$_i$ that is in excess of the alpha and beta chains and remains free (Kvist et al., 1982; Nguyen and Humphreys, unpublished observations). Kvist et al. (1982) presented evidence that this pool of free I$_i$ never leaves the ER. However, observations in this laboratory imply otherwise. Immunoprecipitates of I$_i$ show forms of I$_i$ processed in the Golgi (containing complex N-linked and O-linked carbohydrates), that are in excess of the alpha and beta chains (Thomas and Humphreys, unpublished observations). This finding implies that some free I$_i$ does leave the ER.

Since all three chains (alpha, beta, I$_i$) have N-linked
oligosaccharide chains (Machamer and Cresswell, 1982; Claesson-Welsh et al., 1986b), they must receive the asparagine-linked precursor for these carbohydrate substituents in the ER and these are trimmed to a high mannose form (M$_{8}$N$_{2}$-Asn) before leaving this compartment.

Structural analysis of $I_{1}$ has shown that a region of the molecule (Phe$_{146}$-His$_{170}$) has a high probability of forming an amphipathic alpha helix. Since class II MHC-presented peptides also have a high probability of forming such amphipathic alpha helices (DeLisi and Berzofsky, 1985), the hypothesis was developed that the $I_{1}$ putative helix (146-170) might bind in the desetope formed by the alpha and beta chains. This $I_{1}$ helix could block the desetope from the binding of extraneous peptides from the time of synthesis until the complex reaches a compartment containing Ig-internalized foreign antigen.

The $I_{1}$ gene is also the source of the products p41, γ2 and γ3. Since these species are immunoprecipitated with antisera to the class II MHC alpha and beta chains, these species associate with the class II complex at some point.

**Golgi apparatus.** In this thesis I have shown that p25, a protein seen in immunoprecipitates with anti-class II MHC sera, is the C-terminal portion of a high mannose form of $I_{1}$. Although $I_{1}$ has both of its N-linked carbohydrates processed to the complex form, and has an O-linked oligosaccharide chain (Machamer and Cresswell, 1982; Charron et al., 1983; Machamer and Cresswell, 1984; Rudd et al.,
1985; Claesson-Welsh et al., 1986b), these are not seen in p25 (although both N- and O-linked addition sites are included in the postulated sequence). Further, since these modifications are generally accepted to occur in the medial- or trans-Golgi (Green et al., 1981), we conclude that p25 is derived from a form of I^ in the ER or cis-Golgi.

At the present time one can only speculate about the function of this cleavage. It could be a method for destruction of incomplete complexes of class II MHC molecules and associated proteins, similar to the pathway described for the degradation of incomplete T cell receptors (Lippincott-Schwartz et al., 1988). Consequently the pool of I^ that undergoes this cleavage might be free I^, dimers of I^ or I^ complexed with either the alpha or the beta chain. Another possibility is that some complexes of class II MHC proteins and associated molecules might not be considered "complete" without other components, such as p41 or I^-CS. Consequently, complexes lacking any one of these other components might be subjected to this destructive pathway.

After some portion of the I^ molecules are degraded to p25, further processing of the oligosaccharide side chains on remaining, intact I^, and alpha and beta chains, take place. In the medial- and trans-Golgi both of the N-linked chains on I^ are processed to complex forms (Rudd et al., 1985) and the O-linked carbohydrate is added (Claesson-Welsh et al., 1986b). One of the N-linked chains on the alpha chain is processed to a complex form while the other remains in the high mannose form (Shackelford and Strominger, 1983). Also, an
O-linked oligosaccharide chain is possibly added to the alpha chain (Nishikawa et al., 1979; Claesson-Welsh et al., 1986b). The beta chain has its N-linked chain processed to a complex form (Shackelford and Strominger, 1983).

One of the products of the \(I_i\) gene is also thought to serve as the core protein of a chondroitin sulfate proteoglycan molecule, called \(I_i\)-CS (Sant et al., 1985). This large proteoglycan (up to 180 kD) is presumably manufactured in the Golgi apparatus. Most or all of these molecules may not be associated with the class II MHC proteins (Sorli and Humphreys, unpublished observations).

After processing is complete, the class II MHC proteins (with \(I_i\), and possibly \(\gamma_2\), \(\gamma_3\), p41, and \(I_i\)-CS), is transported to a post-Golgi compartment.

**Post-Golgi compartments.** It can be hypothesized that a post-Golgi compartment is the site of the removal of \(I_i\) from the class II MHC molecules and the binding of processed antigen to the class II MHC desetope.

It is well accepted that B cells internalize and process antigen. Further, newly synthesized class II MHC molecules must pass through some post-Golgi compartment after their processing, to their eventual appearance on the cell surface. The question is whether these two compartments, the internalized antigen and the post-Golgi compartment containing newly synthesized class II MHC molecules, can fuse. Cresswell (1985) demonstrated that transferrin-neuraminidase
conjugates internalized by means of receptor-mediated endocytosis, can interact with newly synthesized class II molecules and cause desialylation of \( I_1 \) and the beta chain. Consequently one can hypothesize that the compartment containing internalized antigen can fuse with the compartment containing the newly synthesized class II MHC alpha and beta chains is no evidence to indicate whether this fusion occurs before or after the foreign antigen is processed to peptides.

\( I_1 \) dissociates from the class II MHC molecules after carbohydrate processing is complete (Claesson and Peterson, 1983a) and some of the best evidence implies that \( I_1 \) is not present with the surface-expressed class II MHC molecules (Elliott et al., 1989). If this is true, one could conclude that this dissociation takes place in some post-Golgi compartment before reaching the cell surface. Further, there is evidence for a degradative pathway of a fully processed form of \( I_1 \), that probably takes place in a post-Golgi compartment (Blum and Cresswell, 1988; Nguyen et al., 1988). This pathway is detected when cells are incubated with leupeptin, and two N-terminal remnants of \( I_1 \), p21 and p10, are revealed. These peptides contain fully processed oligosaccharide chains and in pulse-chase experiments are produced maximally 2 to 5 hr after synthesis. The conclusion from these experiments is that these proteins, p21 and p10, are produced from \( I_1 \) by a leupeptin-insensitive enzyme, and are normally, quickly degraded by a leupeptin-sensitive enzyme to small peptides. It can be hypothesized that this degradative pathway is significant in the removal of \( I_1 \) from the class II MHC molecules in a post-Golgi
compartment, before class II MHC antigen expression on the cell surface.

It is widely accepted that class II MHC molecules bind processed foreign peptides in some site, a desetope, on their structure. The in vitro association rate of purified class II-presented peptides to isolated class II MHC molecules is very slow (Buus et al., 1986b). This observation lead to the idea that this association might be catalyzed by accessory molecules. When cells are transfected with class II MHC genes in the presence or absence of the I\textsubscript{i} gene, it can be shown that antigen presentation is more efficient in the presence of I\textsubscript{i}. Since I\textsubscript{i} is not on the cell surface, then one can conclude its effect is probably intracellular. It can then be hypothesized that I\textsubscript{i} could be one of the principle catalytic accessory molecules.

We can now build a hypothesis how I\textsubscript{i} might catalyze the antigen-class II MHC association based on the above observations and conclusions. As the antigen containing compartment fuses with the class II MHC containing compartment, I\textsubscript{i} is resting in the desetope to prevent associations with random peptides, or to prevent the closure of the hydrophobic trough. When processed peptide is available, I\textsubscript{i} is removed from the desetope as the structurally related foreign peptide is brought into the desetope in a concerted manner, which prevents closure of the hydrophobic sides of the desetope trough. The free I\textsubscript{i} is first degraded by a leupeptin-insensitive enzyme then by a leupeptin-sensitive enzyme to small peptides that do not compete further for desetope binding. According to this hypothesis one might expect that purified class II
MHC molecules, without the catalytic effect of I\textsubscript{i} leaving, would be only slowly charged with amphipathic foreign peptides, as has been observed experimentally (Buus et al., 1986b). Further this hypothesis is consistent with the observations that chloroquine, which neutralizes intracellular acidic compartments, inhibits class II MHC antigen presentation (Grey and Chestnut, 1985), I\textsubscript{i} dissociation from the alpha and beta chains (Nowell and Quaranta 1985, and the production of these peptides in the presence of leupeptin (Blum and Cresswell, 1988).

Kelner and Cresswell (1986) have found complexes of class II MHC proteins and associated molecules consisting of alpha and beta chains with I\textsubscript{i} and I\textsubscript{i}-CS in a 1:1:1:1 ratio. If I\textsubscript{i} is not present on the cell surface, then it can be concluded that I\textsubscript{i}-CS associates with some class II MHC molecules intracellularly. Sant et al. (1985b) previously demonstrated that only 2-5% of class II complexes had I\textsubscript{i}-CS, including some cell surface molecules, and that this interaction was rapid and short-lived. Consequently it can be hypothesized that after I\textsubscript{i}-CS associates with the class II MHC molecules with bound I\textsubscript{i}, I\textsubscript{i} dissociates, the complex goes to the cell surface and I\textsubscript{i}-CS dissociates. The questions are still unanswered whether there are additional molecules associated with the surface-expressed class II MHC molecules, as γ2, γ3 or p41.

**Surface expression and reinternalization.** Class II MHC molecules containing processed peptide are expressed on the cell surface, for
presentation to T cells. It has been shown that some class I MHC molecules are phosphorylated when surface-expressed. Since I have shown in this thesis that the class II MHC alpha and beta chains, along with $\text{I}_1$, can be phosphorylated, that phosphorylation might occur when the class II complex is surface-expressed. This change could be a signal for the internalization of the complex, or might be a mechanism for attenuation of presentation. Specifically, the phosphorylation might be a feedback signal indicating that effective presentation has occurred and that the surface expression of the complex is no longer needed. Alternatively, binding of soluble factors to the B cell, that initiate proliferation and differentiation, also could cause phosphorylation of the class II MHC molecules and associated proteins and thereby terminate surface expression and antigen presentation.

It has been shown in this thesis that the class II MHC molecules might undergo proteolytic cleavages. The putative sites of these cleavages are located around the desetope and cleavages at these locations could terminate the antigen presenting capability of the class II MHC molecules. It can be imagined that after presentation of antigen to T cells, there would be a point in time when it would be beneficial to terminate any presentation. These proteolytic cleavages could serve in this regard.

Proteolytic cleavages of the alpha and beta chains might occur while foreign antigen is present, after the complex has reached the cell surface. This would serve to prevent further antigen presentation to T cells after sufficient presentation might have occurred.
Alternatively, these sites could be accessible for cleavage by extracellular proteases only after release of foreign peptide. That cleaved peptide binding site would be unable to adsorb, and thus present, ambient peptides. Proteases are known to be released by activated T cells (Pasternack and Eisen, 1985; Pasternack et al., 1986). After internalization, proteolytic cleavage of the alpha and beta chains could also occur to inactivate the class II MHC presentation ability. This could serve as a mechanism to destroy complexes that have presented foreign antigen. Furthermore, if I$\text{I}$ is required to catalyze foreign peptide binding to class II MHC molecules, these recycled surface molecules, void of I$\text{I}$, might bind such peptides inefficiently.

The studies presented in this thesis have characterized some structural changes in I$\text{I}$ and the class II MHC alpha and beta chains, which might be important in the regulation of antigen presentation. With those, and information from other laboratories, I have put together the hypothetical scheme above. Much work is needed to test the hypotheses it contains. However, an understanding of the control of antigen processing and presentation will be valuable in the research of a large number of biological problems.
Fig. 4.F.1. Hypothetical scheme to explain the functional significance of structural changes in class II MHC molecules and $I_i$, and the associations of these molecules, in the context of antigen processing and presentation.


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