**Anil Thankappan. CHARACTERIZATION OF GLYCANS ON MAJOR HISTOCOMPATIBILITY COMPLEX CLASS II MOLECULES IN CHANNEL CATFISH, *ICTALURUS PUNCTATUS.* (Under the direction of Dr.Thomas J. McConnell) Department of Biology, Aug 2004.**

The glycans associated with mammalian Major Histocompatibility Complex (MHC) class II molecules are fairly well characterized. In the present study using enzymatic methods we characterized the glycans associated with MHC class II in channel catfish to understand glycosylation patterns in a teleost. Extensive assays did not reveal any indications of O-linked glycosylation on class II molecules of catfish. Unlike for the mammalian class II molecules, channel catfish derived 28S T cells were found to express high mannose N-glycans on class II molecules as seen from endo H experiments. Whereas our studies on peripheral blood leukocytes revealed that catfish possess the ability to transform high mannose to complex type sugars, but the majority of the class II cell surface glycoproteins were of the high mannose type, perhaps indication of an alternate trafficking pathway. Glycosylation in the channel catfish, a model system for teleost, may have significant differences from the glycosylation patterns characterized in mammalian systems.

# CHARACTERIZATION OF GLYCANS ON MAJOR HISTOCOMPATIBILITY COMPLEX CLASS II MOLECULES IN CHANNEL CATFISH, *ICTALURUS PUNCTATUS.*

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Anil Thankappan

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CHARACTERIZATION OF GLYCANS ON

MAJOR HISTOCOMPATIBILITY COMPLEX CLASS II

MOLECULES IN CHANNEL CATFISH, *ICTALURUS PUNCTATUS.*

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## TABLE OF CONTENTS

## LIST OF FIGURES

## LIST OF ABBREVIATIONS

| | |
|---|---|
| APC | Antigen presenting cell |
| B cells | B lymphocytes |
| CAS | Castanospermine |
| CLIP | Class II associated invariant-chain peptide |
| CNX | Calnexin |
| CD | Cluster of differentiation |
| DMSO | Dimethyl sulfoxide |
| DSS | Disussinimidyl suberate |
| DTT | Dithiothreitol |
| Endo H | Endoglysosidase H |
| ER | Endoplasmic reticulum |
| GlcNAc | Glucose N-acetlyamine |
| Ii | Invariant chain |
| kDa | Kilodalton |
| mAb | Monoclonal antibody |
| MHC | Major Histocompatibility Complex |
| PBL | Peripheral blood leukocyte |
| PICS | Protease inhibitor cocktail |
| PBS | Phosphate buffered saline |

| | |
|---|---|
| PNGase F | Peptide-N-glycosidase F |
| SDS | Sodium dodecyl sulfate |
| SDA-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| T cells | T lymphocyte |

# CHAPTER 1. INTRODUCTION

Major Histocompatibility Complex (MHC) class II molecules are cell surface glycoproteins that present antigenic peptides to $CD4^+$ T lymphocytes which initiate adaptive immune responses in vertebrates (Germain, 1994; Cresswell, 1994). Almost all of the key molecules involved in the adaptive immune responses are glycoproteins and tend to be conserved across species (Rudd *et al.*, 1999). MHC class II molecules also undergo co- and post–translational modifications by the addition of oligosaccharides (Swiedler *et al.,* 1985; Cowing and Chapdelaine, 1983). Oligosaccharide attachment has been found to be essential for folding, subunit assembly, cell-cell and cell-matrix interactions, intracellular and intercellular trafficking, signaling and protection from intracellular degradation by proteases (Varki, 1993; Varki, 1998; Rudd *et al.,* 2001; Magnadottia, *et al.,* 2002; Ou *et al.*, 1993). Oligosaccharides are also important for several chaperone-assisted folding of proteins (Ou *et al.*, 1993). Oligosaccharide attachment to MHC class II imparts structural and modulatory functions (Wei *et al.*, 1991; Nag *et al.*, 1992; Hammond *et al.*, 1994). However, evidence for the involvement of oligosaccharides on T cell activation is still lacking.

Of the different types of glycosylation modifications, N- and O-linked modifications are relatively well understood (Kornfeld and Kornfeld, 1985; Helenius, 1994; Van den Steen *et al.,* 2000). N-linked sugar addition, a co-translational event, occurs at the consensus sequence Asn-X-Ser/Thr whereas O-linked addition occurs in the Golgi apparatus most frequently on Ser/Thr residues; though without a clear consensus motif.

(Van den Steen *et al.,* 2000). It has been demonstrated that mammalian MHC class II molecules undergo N–glycan addition whereas Barrera *et al.,* (2002) suggested that O-linked glycosylation may occur on human MHC class II molecules.

Even though glycosylation machinery is highly conserved, most glycoproteins emerge with characteristics patterns (Rudd *et al.,* 1999). The exposed N-glycans are either of high mannose, hybrid, or complex type, all of which are generated from a common lipid-linked precursor, $Glc_3\ Man_9\ GlcNAc_2$ (Hsieh *et al.,* 1983). The high mannose types are commonly found in lower eukaryotes like yeast whereas complex types are encountered in the higher eukaryotes (Trimble *et al.,* 1983).

The factors leading to the formation of complex sugars or high mannose sugars is not fully understood. The common precursor, $Glc_3\ Man_9\ GlcNAc_2$ core is modified to various forms as the molecules move through the Golgi influenced by various factors such as the availability of precursor oligosaccharides, availability of different processing enzymes, rate of transport of molecules through the Golgi and the structure of the protein backbone as well as the position of the N-linked sugars on the protein (Williams and Lennarz, 1984; Varki, 1998; Suzuki and Lee, 2004; Helenius and Aebi, 2001; Swiedler *et al.,* 1985). In the early secretory pathway the glycans have a common role in promoting quality control and sorting but later processing by the Golgi enzymes creates the diversity displayed in the mature protein (Varki, 1998). This diversity could be the result of selection pressure that might facilitate the glycoprotein to acquire different functions

without any change in peptide sequence (Gagneux and Varki, 1999). The appearance of two different forms of N-linked glycans partially processed high mannose and complex type on surface expressed CD45 was reported by Baldwin *et al.,* (2002). They found CD45 with high mannose sugars were trafficked to the cell surface, by-passing the Golgi complex. Therefore, alternate cellular transport can be assessed by observing the sugar modifications.

Wei *et al.,* (1991) discussed the conserved nature of N-linked acceptor site (Asn-X-Ser/Thr) on MHC class I and class II α and β chains during evolution. All the class II α and β chains studied from different species are found to carry these consensus sequences for the N-linked sugars, the only exception being the channel catfish MHC class II α chain (Godwin *et al.*, 2000). Recently, Fuller *et al.,* (2004) using endoglycosidase digestion demonstrated the N-linked glycosylation patterns of class II α and β chains and confirmed the absence of N-glycan in the α chain, leading to a proposal for an alternate pathway for MHC class II assembly in channel catfish.

Glycans expressed on mammalian class II have been extensively characterized and human class II molecules have been shown to express complex type of N-linked glycans (Swindler *et al.*, 1985; Engering *et al.,* 1998). However, with the exception of work by Fuller *et al.,* (2004) no analysis of glycans associated with teleost MHC class II has been previously described.

The present study describes the first report of oligosaccharides expressed by MHC class II molecules from a teleost. Our results indicate that N-linked sugars are required for efficient expression of MHC class II. The class II molecules expressed by 28S T cell lines are exclusively of high mannose type whereas high mannose and complex forms were seen with peripheral blood leukocytes which suggest alternate pathways of class II processing and transport in different cell populations expressing MHC class II in catfish.

# CHAPTER 2. REVIEW OF LITERATURE

## *2.1. Major histocompatibility complex (MHC) class II structure and function.*

Major histocompatibility complex (MHC) class II molecules are membrane glycoproteins that present exogenous peptides to αβ T-cell receptor (TCR)-bearing $CD4^+$ lymphocytes, to initiate a specific immune response (Germain, 1994; Cresswell, 1994). These molecules have been well studied in humans and mice (Germain, 1995) but not as extensively in non-mammalian vertebrates. Surface expression of these molecules is restricted to professional antigen presenting cells, including dendritic cells, B-lymphocytes, macrophage, and thymic epithelial cells, and activated T cells (Ko *et al.*, 1979; Holling *et al.,* 2004).

Class II molecules are formed by two noncovalently associated chains, α andβ, each of which spans the membrane; the α1 and β1 domains together define the class II binding groove (Kaufman *et al.,* 1984; Germain and Margulies, 1993). In the ER, newly synthesized MHC class II molecules associate with the invariant chain (Ii) (Roche *et al.*, 1991). The class II associated invariant chain peptide (CLIP) in exon 3 associates with the binding cleft of MHC class II (Freisewinkel *et al.,* 1993). Association with Ii chain might facilitate proper folding, efficient transport, and prevent class II molecules from loading peptides in the ER (Claesson-Welsh and Peterson, 1985; Teyton *et al.,* 1990; Anderson and Miller, 1992). Class II molecules acquire a proper folded conformation in the ER by assembling into a nonameric complex of three α, three β chains, and three invariant chains (Roche *et al.*, 1991). In this form, MHC class II molecules are

transported through the Golgi and to the trans-Golgi network (TNG). Endosomal targeting signal found on the cytoplasmic tail of Ii directs the nonameric complex to endosomes (Bremnes *et al.,* 1994). In the endosome, the Ii is degraded by various proteases (Blum and Cresswell, 1988) and CLIP is finally exchanged for antigenic peptides (Pierre *et al.,* 1996) before MHC class II molecules are expressed on the surface for recognition by T helper cells.

### *2.2. Functional roles of glycans*

Glycoproteins are widely distributed in nature and are active in intracellular, cell membrane, and extra-cellular recognition processes (Kornfeld and Kornfeld, 1976; Rao and Mendicino, 1978; Goldberg and Kornfeld, 1981; Kornfeld *et al.*, 1981). Inside the cell, protein glycosylation assist folding and stability, quality control, and protein sorting whereas external cell functions may include recognition and adhesion between cells (Gagneux and Varki, 1999; Helenius, 1994; and Helenius and Aebi, 2001). The evidence for the quality control mechanism was demonstrated when calnexin (CNX) an ER chaperone, was found in association with incompletely folded glycoprotein and such an interaction occurred only with glycoprotein bearing monoglucosylated glycoprotein but not to nonglucosylated proteins (Hammond *et al.*, 1994). In mouse the membrane bound class II antigens which are a class of asparagine-linked glycoproteins encoded by the MHC are the cell surface moieties primarily recognized by responder thymic lymphocytes in the mixed lymphocyte reaction (Bach *et al.*, 1972). In mixed lymphocyte reactions pre-treatment of stimulator cells with tunicamycin, a drug which inhibits the

formation of asparagine-linked glycan, blocked the ability of stimulator cells to induce a primary blastogenic response by thymic lymphocytes (Hart, 1982), suggesting that asparagine-linked saccharides on the class II molecules antigens may play a direct role in mediating cell-cell interactions with thymic lymphocytes or that asparagine linked saccharides might be required for proper conformation, interchain association or the normal positioning of class II molecules in the membrane (Hart, 1982).

### *2.3. N-linked and O-linked Glycans*

Two types of glycosylation patterns have been well documented. The first, N-linked glycosylation involves the transfer of a 14-saccharide core from a lipid carrier to the amide group of asparagine in the context of Asn-X-Ser/Thr facilitated by the oligosaccharyltransferase enzyme complex (Kornfeld and Kornfeld, 1985; Helenius, 1994). The second type of glycosylation is O-linked glycosylation, which most commonly links GalNAc to the hydroxyl group of Serine and Threonine (Van den Steen *et al.,* 2000). The co-translational addition of N-linked oligosaccharide in the form of a 14-saccharide core unit ($Glc_3$ $Man_9$ Glc $NAc_2$) to the consensus sequences Asn-X-Ser/Thr, is a common one, but not all sequences of this type carry carbohydrates. The N-linked oligosaccharides are added to the polypeptide chain in the ER whereas O-linked glycosylation occurs in the Golgi apparatus (Van den Eijnden and Joziasse, 1993). Major features of biosynthesis of N-linked sugars are conserved within eukaryotes in contrast to the varied patterns found in O-glyocosidic linkages and structures (Spiro *et al.*, 2002).

### *2.4. N-linked glycan processing*

The biosynthetic pathways involved in the formation of asparagine-linked oligosaccharide are well characterized (Anderson and Grimes, 1982; Swiedler *et al.,* 1985). The major features include recognition of a tripeptide sequence by a transferase enzyme, which subsequently transfers an oligosaccharide containing $Glc_3\ Man_9\ GlcNAc_2$ from a lipid donor to an accepting asparagine residue in the rough endoplasmic reticulum (Li *et al.*, 1978; Kornfeld *et al.*, 1978; Tabas and Kornfeld 1978). Glucosidase I immediately trims the terminal glucose residue (Varki, *et al.,* 1999) followed by Glucosidase II which trims the final two glucose residues (Trombetta *et al.,* 1996). Although this initial process is well documented in all the eukaryotes studied so far, there exist some differences in the processing of N-linked sugars between lower eukaryotes and higher eukaryotes (Varki and Kornfeld, 1980). Once the newly synthesized glycoproteins exit the ER they enter the Golgi apparatus, which contains various degradative glycosidases and synthetic glycotransferases responsible for the processing of N-linked glycans. In the Golgi substrate molecules are progressively modified as they move from the entry side (cis) to the exit side (trans) (Palade, 1975). Presumably functional heterogeneity occurs at this time (Atkinson *et al.*, 1981). Mature N-linked glycoproteins are of high mannose in lower eukaryotes (Trimble *et al.,* 1983) whereas in higher eukaryotes they are either high mannose or complex type based on their component sugars and both forms are derived from a common lipid linked precursor (Hubbard and Ivatt, 1981; Kornfeld and Kornfeld 1985).

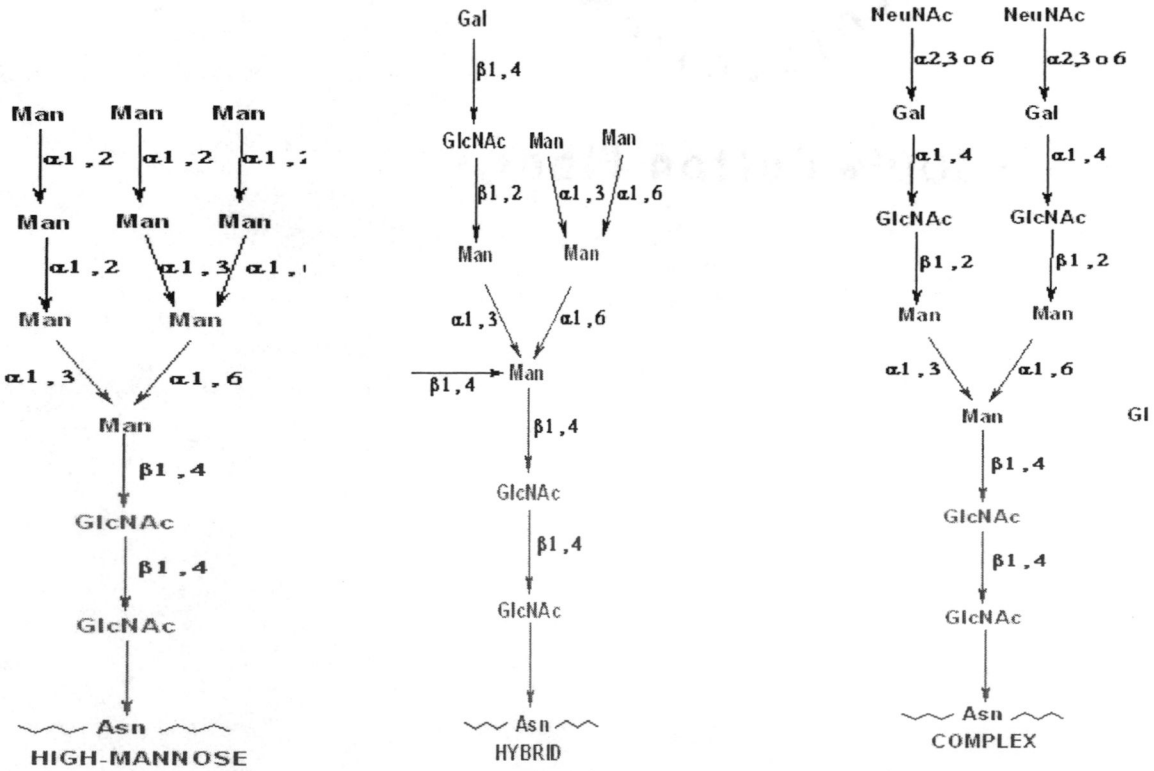



**Figure A: Different forms of mature N-linked glycans**

Adapted from BIOCHEMISTRY, 2nd Ed. By Garrett and Grisham,

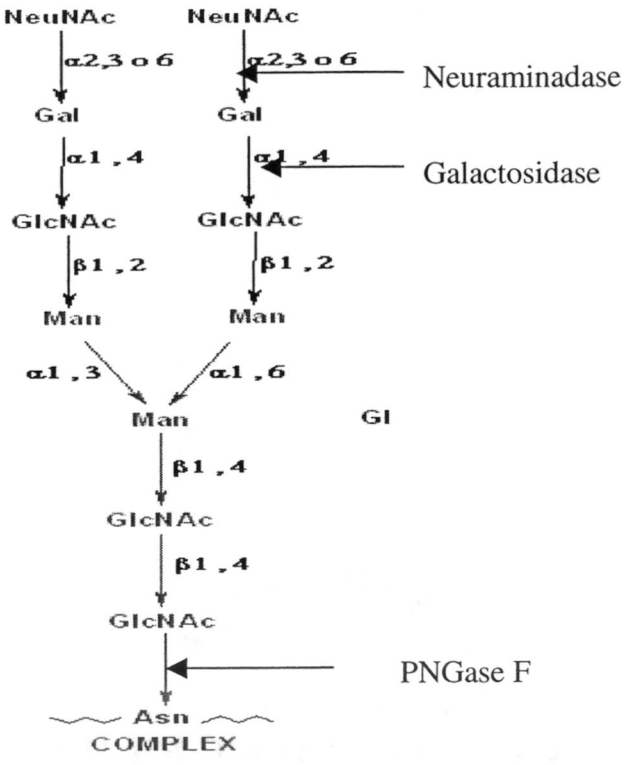



**Figure B: Cleavage site for various enzymes**

Adapted from BIOCHEMISTRY, 2nd Ed. By Garrett and Grisham,

### *2.5. Factors affecting the N-linked glycan complexity*

The factors leading to the formation of complex sugars or high mannose sugars is not fully understood. Even when a cell contains all the enzymes required for processing the N-linked sugar to a complex type, some cells will express a mature glycoprotein with a high mannose configuration (Trimble *et al.,* 1983). One of the factors that regulate the processing of N-linked sugars is the protein structure (Williams and Lennarz, 1984; Neel *et al.*, 1987). During processing, folding of the nascent polypeptide renders the oligosaccharide chains in some domains inaccessible to both the smooth endoplasmic reticulum and the Golgi mannosidase required for the conversion of high mannose to complex type (Hubbard and Ivatt, 1981). Hsieh *et al.*, (1983) and Trimble *et al.*, (1983) showed that the primary factor that affects the processing is the three-dimensional structure and its steric accessibility to the processing enzymes and not the primary amino acid structure. Williams and Lennarz (1984) supported these findings through an *in vitro* study on RNase B. They found that the disruption of the native structure by reduction and alkylation of RNase B resulted in a modest increase in the rate of complex chain formation whereas the same molecule in the native form remained completely in a form sensitive to digestion by endoglycosidase H (endo H). High mannose and complex type can usually be differentiated by treatment with the endo H. High mannose sugars are often released by endo H treatments, but once the tertiary structure of the molecule has been perturbed almost all the high mannose sugars become sensitive to endo H. It was noted in carboxypeptidase Y and invertase, high mannose sugars are released by endo H treatment only if the protein is completely denatured (Trimble and Maley, 1977).

Another aspect regulating the occurrence of high mannose or complex type sugars is the cellular processing machinery. In one study a single strain of virus was grown in a number of different host cells and the presence of both complex and high mannose oligosaccharide led to the conclusion that cell processing machinery may also influence the extent of oligosaccharide processing (Hsieh *et al.*, 1983).

The occurrence of high mannose or complex sugar is also related to the position of the acceptor sequence (Asn-X-Ser/Thr) in the polypeptide chain. It is estimated that a minimum of 30 amino acids must be added beyond an acceptor site for the glycosylation to occur (Glabe *et al.*, 1980). In mouse IgM Asn563 appeared to be only partially glycosylated, possibly because Asn is only 13 amino acids from the COOH terminus (Anderson and Grimes, 1982). This evidence supported the previous indications that complex structures are generally found towards the $NH_2$ terminal region and high mannose oligosaccharide towards the COOH- terminal region. The extent of processing of N-linked sugars seems to increase with distance from COOH- terminus (Anderson and Grimes, 1982).

In mature glycoproteins, N-linked glycan moieties are structurally diverse (Helenius and Aebi, 2001). In addition to the occurrence of high mannose and complex type of sugars there is a great diversity in the types of sugar residues found in the complex type sugars (Atkinson *et al.,* 1981 and Anderson and Grimes, 1982). Structural diversity observed

within a species and between different organisms is generated as the glycoprotein passes through the Golgi complex (Helenius and Aebi, 2001). Presumably functional heterogeneity occurs at this time (Atkinson *et al.*, 1981). The switch from structural uniformity in the ER to diversification in the Golgi complex coincides with a marked change in glycan function (Helenius and Aebi, 2001). In the early secretory pathway, the glycans have a common role in promoting protein folding, quality control and sorting events but later processing by Golgi enzymes prepare them for the diverse functions that the sugars display in the mature proteins (Varki *et al.*, 1999). The single most important function of N-linked sugars appears to be in the promotion of proper folding of newly synthesized polypeptide in the ER (Paulson, 1989; Helenius, 1994). Supporting this proposition is the fact that that addition of N-linked glycans must occur co-translationally in the ER (Helenius and Aebi, 2001).

Heterogeneity in carbohydrates has been observed in number of systems (Anderson and Grimes, 1982). The diversity in the sugar residues has often been considered to be responsible for the phenomenon of microheterogenity. At any given glycosylation site on a given protein synthesized by a particular cell type, a range of variation can be found in the precise structure of the glycan. The extent of this heterogeneity could lead to array of different oligosaccharide structures depending on the site of glycosylation, protein structure, cell type, and developmental stages. Nevertheless, the functions and the diversity with regards to the sugar residues that form the N-linked sugars are poorly understood (Williams and Lennarz, 1984). Several studies have produced dramatic

examples of the complexity of glycoprotein carbohydrates (Anderson and Grimes, 1982). In human α1 acid glycoprotein, there are 15 to 20 different oligosaccharides attached to four of five glycosylation sites (Fournet *et al.,* 1978; Van Halbeek *et al.,* 1981) whereas ovalbumin has 10 different oligosaccharide structures on a single glycosylation site (Atkinson *et al.,* 1981; Iwase *et al.,* 1981; Carver *et al.,* 1981). The differences in ability of antigen-presenting cells to activate T lymphocytes in an antigen specific manner were attributed to heterogeneity of carbohydrate components in MHC encoded molecules (Cullen *et al.*, 1981; Cowing and Chapdelaine, 1983; Frohman and Cowing, 1985). But a study involving disruption of N-linked acceptor site either by site directed mutagenesis or by enzymatic removal of N-linked sugars of murine MHC class II molecules did not reveal any evidence of involvement of N-linked sugars in T cell activation. Nag *et al.,* (1992) concluded that N-linked sugars may play an important role in stabilizing MHC class II-peptide complexes on the surface of APC.

MHC class II α and β chains show differences in oligosaccharide sialylation and branching patterns (Swiedler *et al.,* 1985; Neel *et al.*, 1987). This heterogeneity of class II molecules expressed on different APC subsets has been associated with differences in the capacity of these APC subsets to activate T-lymphocytes in Ag-specific manner (Cullen *et al.,* 1981; Cowing and Chapdelaine, 1983; Frohman and Cowing, 1985; Krieger *et al.,* 1988). Wei *et al.,* (1991) argued these experiments might not be fully accurate because the alteration of oligosaccharides on the other surface molecules may affect other non-MHC restricted interactions that contribute to effective T-lymphocyte activation. They

also used site directed mutagenesis of N-linked sites to reveal the potential role of N-linked sugars in class II expression and function. Whereas the deletion of N-linked sugars definitely affected the secondary structure of class II molecules, there was no obvious explanation for the functional consequence of N-linked sugars in T cell activation (Wei *et al.,* 1991). Glycans are essential for maintaining the overall folded structure once glycoprotein has been folded (Helenius, 1994; Imperiali and O'Connor, 1999). Anderson and Grimes (1982) initiated studies on mouse IgM, to understand the relationship between heterogeneity, carbohydrate biosynthesis and protein structure and concluded that both three-dimensional structure of the nascent polypeptide and availability of processing enzymes are responsible for the heterogeneity. Further to study the effect of cell lines on glycosylation, Sheares & Robbins (1986), transfected different cell lines with the same gene that encoded ovalbumin and identified differences in glycosylation patterns on ovalbumin where as the influence of protein structure has been documented for mouse MHC (Swiedler *at al.*, 1985).

Swindler *et al.,* (1985) studied the influence of peptide structure on the detailed branching and sialylation of the complex-type oligosaccharides found at a particular glycosylation site. When human MHC class II molecules were expressed in two different cell lines, the 430 and TF 22.9 overall protein glycosylation patterns varied extensively between different cell types, which could indicate differences in specific glycotransferase in each cell line (Neel *et al.,* 1987). The efficiency of glycosylation process also varies with the cell type (Hsieh *et al.,* 1983).

### *2.6. Adaptive immunity and teleost*

Teleosts represent one of the most ancient lineages of vertebrates, possessing both innate and acquired immunities analogous to those in the mammals (Magor and Vasta, 1998). *In vivo* and *in vitro* studies have shown proliferation of T-cell clones derived from teleost, but whether stimulation of T cells in teleosts is through the engagement of TCR and MHC has not been demonstrated (Stuge *et al.,* 2000). However, the presence of components required for specific immune responses such as TCR, MHC class I and class II and other immunoregulatory gene suggest that teleosts are able to mount specific immune responses (Wilson *et al.*, 1998; Scapigliati *et al.,* 2000; McConnell *et al.*, 1998; Hashimoto *et al.,* 1990; Sangrador-Vegas *et al.,* 2000). The identification of LMP2, LMP2/d, TAP1A, and TAP2B in class Ia locus in rainbow trout suggest that class I dependent Ag processing and presentation could follow the same pathway as in mammals (Hensen *et al.,* 1999). However, relatively little is known about T-cell mediated immunity in fish species (Stuge *et al.,* 2000). Recently, Boudinot *et al.,* (2001) using an immunoscope technique showed modifications of the T cell repertoire by examining changes in CDR3 length distribution induced by VHSV viral infection in rainbow trout, which would indicate that diversity and dynamics of immune repertoire in lower vertebrates and mammals are comparable.

The catfish, *Ictalurus punctatus*, has been a very productive model system for study of key molecules involved in initiating specific immune response in teleost. The availability of functionally distinct clonal long-term leukocytes from channel catfish makes this

species an excellent candidate for immunological studies. Initially, the genes encoding MHC class II molecules were characterized and recombinant proteins were made and used to develop antibodies to MHC class II α and β chains. Other molecules such as calnexin and invariant chain (Ii) essential for MHC assembly were also identified (Fuller *et al.*, 2004). Recently, Fuller *et al.*, (2004) addressed some basic aspects of MHC class II assembly. Their data assumes special significance in the light of absence of N-linked glycosylation on the α chain, which has been considered to be essential to MHC class II assembly in all other species from which it has been described (Anderson & Cresswell 1994).

The present study examines the glycosylation patterns of MHC class II α and β chain using enzymatic treatments. The studies were conducted on 28S T cell lines and peripheral blood leukocytes derived from channel catfish.

## CHAPTER 3. MATERIALS AND METHODS

### *3.1. Cell lines:*

The channel catfish clonal cell lines 28S (T cell lines) graciously provided by Norman Miller of the University of Mississippi Medical Center were maintained at 27°C in AL-5 culture media (AIM-V/L-15; Life Technologies, Rockville, MD) with 5% catfish serum as described previously (Miller *et al.,* 1994). The rat cell line R1 T cells was graciously provided by Dr. Mark Mannie, Brody School of Medicine, East Carolina University, and was maintained at 37°C maintained in RPMI medium supplemented with 10% heat-inactivated FBS (Summit, Boulder, CO) as described previously (Patel *et al.,* 1999).

### *3.2. Antibodies:*

The monoclonal antibodies A3 (anti-MHC class II α), Bc1.1, Bc2.1, and Bs1.1 (anti-MHC class II β) were previously described (Fuller *et al.,* 2004). Anti-KLH monoclonal antibodies developed in the hybridoma laboratory facility at East Carolina University were used as a negative control. The rat anti-MHC class II antibody OX6 was graciously provided by Dr. Mark Mannie, Brody school of Medicine, East Carolina University.

### *3.3. Preparation of denatured lysates:*

Fifty µl of denaturing lysis buffer (1% w/v SDS, 50 mM Tris HCl pH 7.4, 10 mM dithiothreitol, 2 µl/ml Protease inhibitor: PICS) were added to 1x $10^7$ cells (28S T cells or RI T cells) and vortexed briefly for 5 s. The samples were heated for 5 min at 95°C. To neutralize SDS, 950 µl of non-denaturing lysis buffer (1% w/v NP-40, 10 mM Tris HCl

pH 7.4, 150 mM NaCl, 10 mM Iodoacetamide, 2 µl/ml PICS was added to each sample. This 20-fold dilution was sufficient to sequester the SDS. The released DNA was sheared by passing the lysates five to 10 times through a 25G needle attached to a one ml syringe. The samples were incubated for 30 min on ice and lysates were cleared by centrifuging at 14000 rpm for 20 min at 4°C. Using Bradford assay the protein concentration was determined and found to be 1000 µg/ml. The lysates were used for various enzymatic treatments.

### *3.4. Enzymatic protein deglycosylation:*

Enzymatic protein deglycosylation kit (Sigma, Saint Louis, MO) was used and contained following enzymes: PNGase F (cleaves all asparagine linked complex, hybrid or high mannose oligosaccharide unless α (1-3) core fucosylated), O-Glycosidase (cleaves serine or threonine linked unsubstituted Gal-β(1-3)- Gal NAc- α), Neuraminidase (cleaves all terminal branched and unbranched sialic acids), β (1-4) Galactosidase (release only β (1-4)-linked non-reducing terminal galactose), and β-N- Acetylglucosaminidase (cleaves all non-reducing terminal β-linked N-acetylglucosamine residues). To deglycosylate the protein sample 10 µg of denatured lysates (28S T cells or R1 T cells or PBLs) were treated with or without enzymes. To each reaction, 1 µl of enzyme was added either individually or in combination with other enzymes in a total volume of 20 µl which has 4 µl of 5x reaction buffer supplied along with the kit. After overnight incubation the reaction the reactions were stopped by heating the samples for 5 min at 95 °C after adding

5 µl of 5x reducing sample buffer (Pierce). The samples were analyzed by SDS-PAGE (4-20% gradient SDS-PAGE) and detected by immunoblotting.

### *3.5. Endoglycosidase H treatment:*

Ten µl of denatured 28S T cell or RI T cell lysates were added to tubes containing 4 µl of 0.5 M Sodium citrate, pH 5.5, 2 µl of 1% Phenyl methylsulfonate (PMSF) in isopropanol, with or without 2 µl of 0.5 U/ml Endoglycosidase-H (Boehringer-Mannheim). After 16h of incubation at 37 °C, the reaction was stopped by heating the samples for 5 min at 95 °C after adding 5 µl of 5x reducing sample buffer (Pierce). The samples were analyzed by SDS-PAGE (12% acrylamide) and detected by immunoblotting.

### *3.6. Peripheral Blood leukocyte isolation:*

Catfish blood was collected by cardiac puncture using 22G needle into heparinized vacutainer, and PBLs were isolated by centrifugation of 4 ml diluted blood (1Blood: 2 catfishRPMI) over 3 ml of Accu-Paque (Accurate Chemicals & Scientific Corp.) at 800 x g for 20 min at room temperature. The PBLs collected were washed in cRPMI and centrifuged at 350xg for 10 min. Cell lysates were prepared using 1x $10^7$ cells as described above.

### *3.7. Cell surface biotinylation and pull down assay:*

3x$10^7$ cells (28S T cells) were rinsed 3x times with ice-cold PBS, pH 8.0 and incubated with 1 ml of PBS, pH 8.0 containing 0.5 mg of Sulfo-NHS-LC Biotin (Pierce, Rockford,

IL) for an hour in the cold room (4 °C) on an orbital shaker. The reaction was quenched by washing cells twice with 10 ml of ice-cold PBS containing 50mM TrisHCl, pH 7.5. Denaturation lysis was performed as described above. Biotinylated cell surface proteins were separated by incubating with Immunopure immobilized streptavidin (Pierce, Rockford, IL) for 3 h in cold room with constant mixing. Proteins bound to streptavidin beads were recovered by adding 40 µl of IgG Elution buffer (Pierce) to streptavidin beads using Handee Spin column (Pierce) into a tube containing 12 µl of 500 mM TrisHCl (pH 7.5). The samples were analyzed by 12% SDS-PAGE after deglycosylation with endo-H and immunoblotting was performed as described above.

***3.8. Immunoprecipitation and elution:***

28S T cells ($2x10^7$) were extracted in 150 mM NaCl, 10 mM TrisHCl pH 7.4, containing 1% NP-40 (Sigma), 10mM iodoacetamide and 2 µl/ml PICS and precleared with nonspecific anti-KLH mAb (IgG) bound to Protein A agarose (Pierce) for 2 h. The precleared extracts were incubated with Bs1.1 (anti catfish-MHC class II β mAb) conjugated to Protein A agarose (Pierce Rockford, IL) overnight and pellets were washed with 0.1% NP-40 wash solution containing 150 mM NaCl and 10 mM TrisHCl pH 7.4. Proteins bound to the beads were recovered by adding 60 µl of IgG Elution buffer (Pierce Rockford, IL) to the beads using Handee Spin column (Pierce) into a tube containing 16 µl of 500 mM TrisHCl (pH 7.5). The samples were resolved by 12% SDS –PAGE and O- linked western blotting was performed according to manufacturer's protocol (Pierce).

### *3.9. Gel-electrophoretic analysis.*

4-20% SDS-PAGE gradient gels were purchased from Pierce and electrophoresed according to the manufacturer's protocol. SDS-PAGE was carried out according to Laemmli (1970). Sample preparation and analyses were carried out as described previously (Fuller *et al.*, 2004).

### *3.10. 2D gel electrophoresis:*

For isoelectric focusing, Immobiline$^{TM}$ Dry Strip (Amersham Biosciences Corp) pH gradient 3-10 non-linear 7 cm long were rehydrated in a Dry Strip reswelling tray (Pharmacia Biotech). Each dry strip was rehydrated overnight in 125 µl of IPG rehydration solution (8M urea, 2% w/v NP-40, 2% w/v pharmalyte, 0.002% Bromophenol Blue) at room temperature. Thirty microgram of denatured 28S T cell lysate/ (PNGase F treated) following desalting were loaded to the rehydrated strips by cup loading at the cathodic end. Focusing was performed in a step up mode (150 V for 30min, 300 V for 1 h, 1500 V for 1 h and 3500 V for 2 h) with an Amersham Pharmacia Multiphor II apparatus with plates maintained at 15$^{o}$ C.

For the second dimension run, the focused IPG strips were equilibrated first in 10 ml equilibration solution (2% w/v SDS, 50 mM TrisHCl, pH 8.8, 6M urea, 30% v/v glycerol, 0.002% bromophenol blue and 100 mg DDT) for 15 minutes, then in another 10 ml equilibration solution with the following composition (2% w/v SDS, 50mM TrisHCl, pH 8.8, 6 M urea, 30% v/v glycerol, 0.002% bromophenol blue and 250 mg iodoacetamide)

for 15 minutes. The strips were washed with deionized water and placed over a Laemmli SDS-PAGE (12% acrylamide) without a stacking gel. To achieve a good contact between the gel and the IPG strip, 0.5% agarose solution made with gel running buffer was used for the overlay. Gel electrophoresis was performed as described previously (Fuller *et al.,* 2004) and detected by immunoblotting.

### *3.11. Castanospermine treatment and flow cytometric analysis*

To assess the effect of inhibition of glucose trimming on the expression of MHC class II molecules in catfish, 5x $10^5$ cells (28S T) were incubated in 5 ml of culture media with and without 200 µg/ml castanospermine (Calbiochem). After three days of culture, 5x$10^5$ cells were harvested and washed 3x with 5 ml of HBSS. The washed cells were treated with 100 µl of anti-MHC class II mAb (Bs 1.1) for 45 min. After incubation the cells were washed 3x with 5 ml HBSS and treated with 1:20 diluted stock of 1mg/ml FITC-conjugated goat anti-mouse Ig (H+L) (Southern Biotechnology corp., USA) for 45 min. These cells were again washed 3x with 5 ml HBSS and finally diluted in 500 µl of PBS. Subsequently flow cytometric analysis was performed. A negative control was also prepared and analyzed by treating 28S T with FITC- conjugated goat anti-mouse Ig (H+L) to assess the background.

# CHAPTER 4. RESULTS

## *4.1. Enzymatic deglycosylation*

To determine the contribution of N-linked and O-linked glycans and other residues such as sialic acids, and galactose associated with these glycans on MHC class II molecules denatured 28S T cell lysates were subjected to enzymatic treatments either individually or in combinations as indicated in the Fig. 1 and Fig. 2, followed by SDS-PAGE. MHC class II α and β chains were then visualized by western blotting using monoclonal antibodies A3 and Bc 1.1 respectively. Electrophoresis patterns indicated a reduction in apparent molecular weight by about 3.5 kD for class II β chain which has PNGase F in the reaction mixture. This result confirms the previous observation that the catfish β chain carries one N-linked sugar and the α chain lacks an N-linked glycan (Fuller *et al.,* 2004). However, other treatments with sialidase, galactosidase, O-glycanase did not produce any appreciable shift in molecular weight on SDS-PAGE. These results indicate that sialic acids, galactose and O-linked sugars may be absent or contribute very little to the modifications of class II molecules. Fetuin was used as a positive control for neuraminidase (data not shown).

A sensitive method to probe the O-linked glycans with a mAb to one of the types of O-linked modification (O-GlcNAc modification) was done on class II α and β chain recovered from immunoprecipitation of 28S T cell lysates using mAb to class II β (Bs 1.1) (Fig. 3). Monoclonal antibody Bs1.1 immunoprecipitated both β chain and αβ complex. Denatured immunoprecipitate when probed with anti-class II α (A3) and anti-

class II β (Bc 1.1) detected both α and β chain (Fig. 3, lane 4). However, lane 3 probed with mAb to O-linked sugars could not detect O-linked sugars on either the α chain or on the β chain, although this mAb detected O-linked glycans on the control (lane 1).

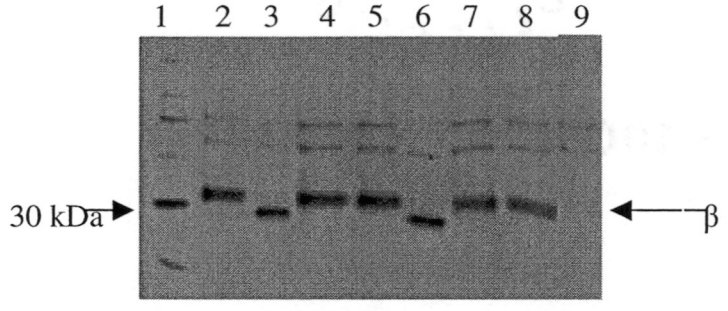



**Fig. 1. Western blot analysis of deglycosylated cell lysates for catfish class II β chain of 28S T cells.** Cell lysates obtained from 28S T cells were deglycosylated with enzymes either individually or in combinations under conditions outlined in Materials and Method and then resolved by 4-20% gradient SDS-gel under reducing conditions, followed by immunoblotting with anti-MHC class II β antibody. Lane 1. Marker, 2. Untreated, 3. PNGase F, 4. Neuraminidase, 5. O-Glycosidase, 6. PNGase F + Neuraminidase + O-Glycosidase, 7. β (1-4) Galactosidase, 8. β (1-4) Galactosidase + β –N-Acetlyglucosaminidase, 9. Isotype matched irrelevant control

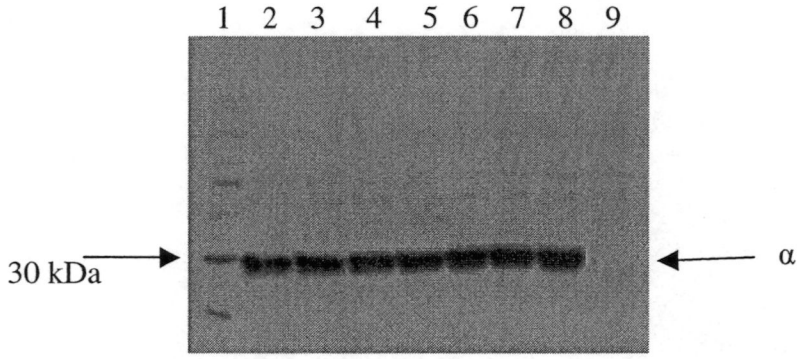



**Fig. 2. Western blot analysis of deglycosylated cell lysates for catfish class II α chain of 28S T cells.** Cell lysates obtained from 28S T cells were deglycosylated with different enzymes individually or in combination under conditions outlined in Materials and Method section and then resolved by 4-20% gradient SDS-gel under reducing conditions followed by immunoblotting with anti-MHC class II α antibody. Lane 1. Marker, 2.Untreated, 3. PNGase F 4. Neuraminidase 5. O-Glycosidase 6. PNGase F + Neuraminidase + O-Glycosidase 7. β (1-4) Galactosidase 8 β (1-4) Galactosidase + β – N-Acetlyglucosaminidase 9. Isotype matched irrelevant control.

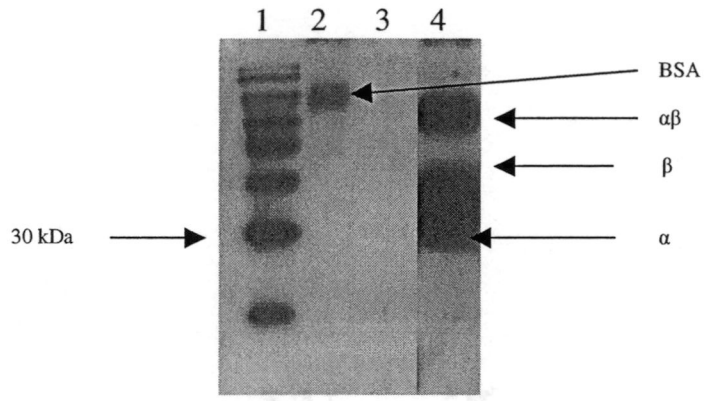



**Fig. 3. Western blotting for O-linked sugars (O-GlcNAc modification):**

Immunoprecipitation of non-denatured 28S T cell lysates with anti –MHC class II β (Bs 1.1) antibody was performed as described under Materials and Methods. Immunoprecipitates were denatured with gel loading buffer and probed for O-linked sugar in both α and β chains using O-GlcNAc western blot kit. Lane 1. Marker, 2. Positive contol for O-linked sugar (Bovine serum Albumin), 3. Immunoprecipitate probed with O-linked western blotting reagents, 4.Western blot of immunoprecipitates probed with anti-MHC class II α and β mAbs to show the presence of α and β in the immunoprecipitates.

### *4.2. Endoglycosidase H digestion*

Digestion by endo H was used to reveal the type of N-linked sugar carried by MHC class II β chain. N-linked sugars added initially inside the ER remain sensitive to endo H but acquire endo H resistance upon movement from the ER to the medial Golgi resulting in the transformation of N-linked glycans from high mannose type to complex form (Kornfeld and Kornfeld , 1985). This is facilitated by various enzymes present in the Golgi complex. We observed from the endo H digestion experiments that catfish class II β did not acquire endo H resistance (Fig. 4 A) signifying a lack of processing of high mannose sugar to complex forms in 28S T cells. This result was again confirmed by cell surface biotinylation experiments on 28S T cells (Fig. 5). Cell surface expressed class II β obtained after streptavidin pull-down assay and subjected to endo H digestion gave the same results seen with whole cell lysate obtained from 28S T cells.

The same experimental protocol performed on rat T cells (R1 T cells) gave results consistent with the previous observations. Mammalian MHC class II molecules are known to acquire endo H resistance, ie., high mannose glycans are processed to complex type (Pieters *et al.,* 1991). After endo H digestion the MHC class II molecules from rat T cells acquired endo H resistance (Fig. 4 B & C). Thus, we conclude that the N-linked glycan of MHC class II molecules on 28S T cells are exclusively high mannose.

In order to test glycosylation status under in vivo conditions, peripheral blood leukocytes were isolated from catfish blood and subjected to endo H digestion (Fig. 6 A). It was

interesting to note that the endo H digestion of PBLs revealed the presence of both endo H resistant and sensitive forms of class II β chain. This result remained the same when endo H digestion was performed for expressed MHC class II by surface biotinylated experiments (Fig. 7). Thus catfish possess the machinery to process high mannose forms to complex forms. The endo H resistant forms could be associated with other cell types in the PBLs other than the T cells that express MHC class II molecules. But it is also possible that 28S T cells may have an altered processing capability after long-term culture, losing the ability to process high mannose to complex forms.

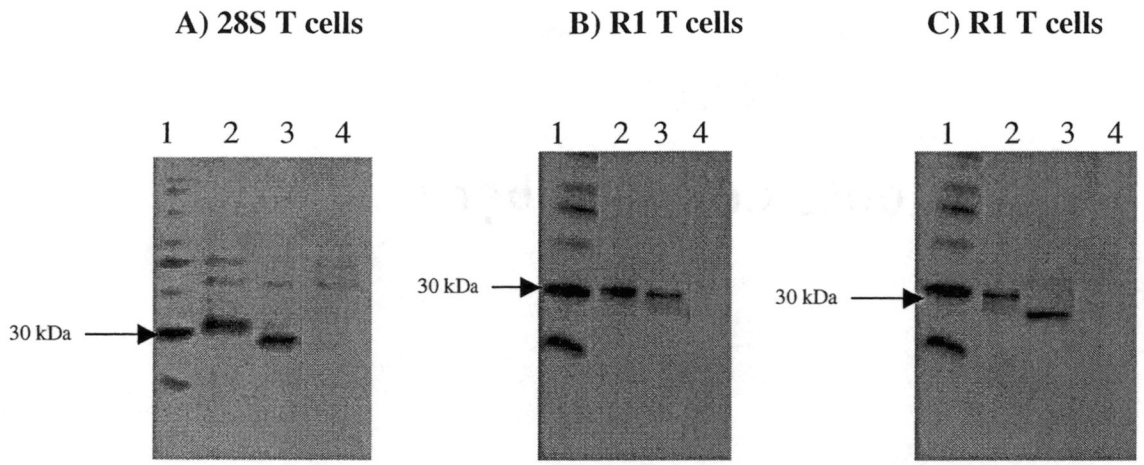



**Fig. 4. Western blots of endo H digested of 28S T cells and rat T cells.** A ) Denatured 28S T cell lysates were digested with or without endo H and resolved by 4-20% SDS-PAGE, followed by immunoblotting with anti-MHC class II β antibody ( Bc1.1). Lane 1. Marker, 2. Untreated 3.endo H treated, 4. Isotype matched irrelevant control.

B) Denatured rat T cells (RI T cells) lysates were digested with or without endo H and resolved by 4-20% SDS-PAGE, followed by immunoblotting with anti-MHC class II antibody OX6. Lane 1. Marker 2. Untreated 3. endo H treated. 4. Isotype matched irrelevant control.

C) Rat T cells (RI T cells) were subjected to PNGase F digestion to show the presence of N-linked sugars on rat MHC class II molecules. Lane 1. Marker, 2. Untreated, 3.PNGase F treated, 4. Isotype matched irrelevant control.

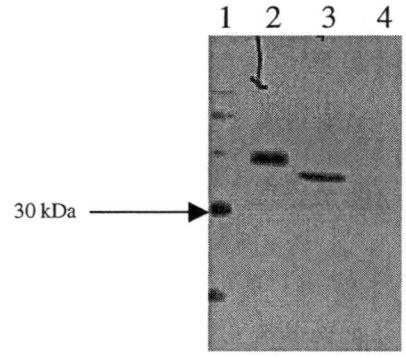



**Fig. 5. Western blot of endo H digested cell-surface MHC class II β proteins from 28S T cells revealed high mannose type N-linked glycans on MHC class II β chain:** 28S T cells were biotinylated for 1 h to label the surface proteins prior to lysis and a streptavidin pull-down assay was performed. Bound proteins were eluted to isolate the surface biotinylated protein. The eluted samples were treated with endo H and resolved by 12% SDS-PAGE and followed by immunobloting with anti-MHC class II β antibody (Bc 1.1). Lane 1. Marker, 2. Untreated, 3. endo H treated, 4. Isotype matched irrelevant control.

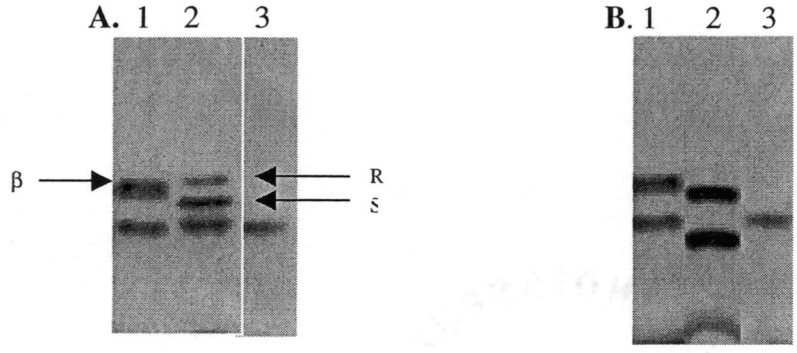



**Fig. 6. Western blot of endo H and PNGase F digested MHC class II β from peripheral blood leukocytes (PBLs).**

A). Denatured PBLs lysates were digested with or without endo H and resolved by 12 % SDS-PAGE, followed by immunoblotting with mAb Bc1.1. Lane 1. Untreated lysate, 2. endo H treated lysate, 3 Isotype matched irrelevant control. Endo H sensitive form is indicated by S and resistant form by R.

B) Denatured PBLs were subjected to PNGase F digestion to show the complete denaturation of the lysate, PNGase F completely cleaved the N-linked sugar as indicated by the gel shift. Lane 1. Untreated lysate 2. PNGase F treated lysate 3. Non-specific control.

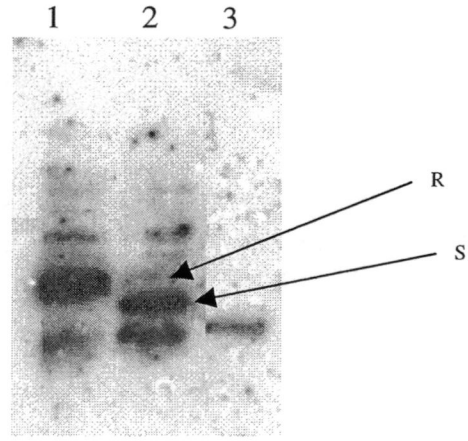



**Fig. 7. Western blot of endo H digested cell-surface MHC class II β proteins peripheral blood leukocytes.** PBLs were biotinylated for 1 h to label the surface proteins prior to lysis and a streptavidin pull-down assay was performed. Bound proteins were eluted from the beads to isolate the surface biotinylated protein. The eluted samples were treated with endo H and resolved by 12% SDS-PAGE and followed by immunobloting with anti-MHC class II β antibody (Bc 1.1). 1. Untreated, 2. endo H treated, 3. Isotype matched irrelevant control. Endo H sensitive form is indicated by S and resistant form by R.

### *4.3. 2D Gel analysis of catfish MHC class II molecules*

We examined the fine structure of catfish MHC class II molecules by two-dimensional (2D) gel electrophoresis. Since the oligosaccharide processing could result in different structural products, 2D gels can be used to differentiate these intermediate immature components. The whole cell lysates obtained from 28S T cells were subjected to first dimension isoelectric focusing on an immobilized pH gradient 3-10 for about 4 hrs to separate proteins according to their pI, followed by second dimension vertical SDS-PAGE. Immunoblotting with monoclonal antibodies A3 and Bc 1.1 showed the presence of class II α and β respectively Fig. 8. From the amino acid sequence the pI of class II α and β chain were found to be 4.81 and 8.10 respectively. It was seen from our results that class II α and β chain could be resolved based on acid and basic nature. The α chain was found to be relatively acidic compared to the β chain. This is in agreement with the pI values calculated using amino acid sequences.

It was interesting to note that α chain focused as single spot whereas β appeared as cluster of spots. However, it is known that in the case of mammalian MHC class II when analyzed by 2D gels both α chain and β chain appeared as cluster of spots (Nag et *al.*, 1994). To test whether this multiple banding pattern is due to the presence of N-linked sugars carried by the β chain, PNGase F treated and untreated samples were analyzed by 2D gel (Fig. 9). PNGase F treatment did not alter the banding pattern much. So N-linked glycans cannot account fully for the multiple banding of the β chain.

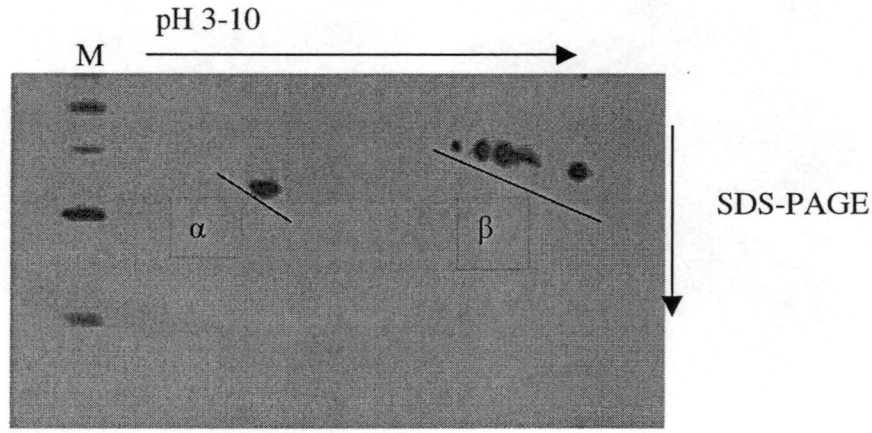



**Fig. 8. 2D gel profile of catfish MHC class II α and β chain.** The horizontal axis shows the isoelectric focusing performed under a pH gradient 3to 10. The vertical axis represents the second dimension SDS-PAGE. Two-dimensional gel electrophoresis of class II MHC complex from 28ST cells followed by immunoblotting with anti-MHC class II β (Bc 1.1) and anti-MHC class II α (A3) demonstrates the microheterogenity of the class II β chains whereas the α chain focused as a single spot.

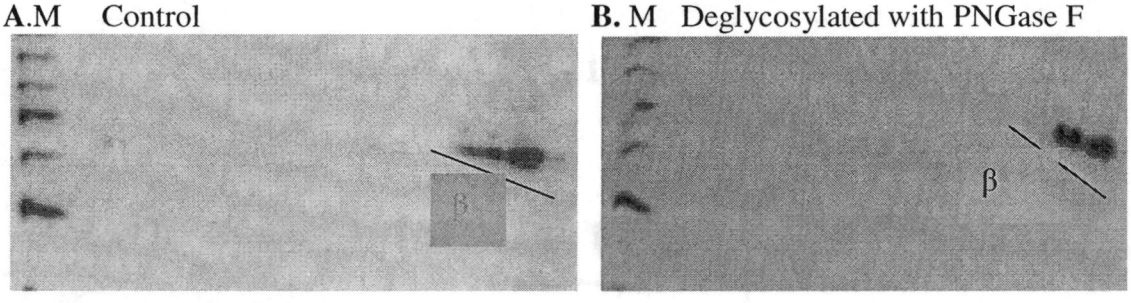
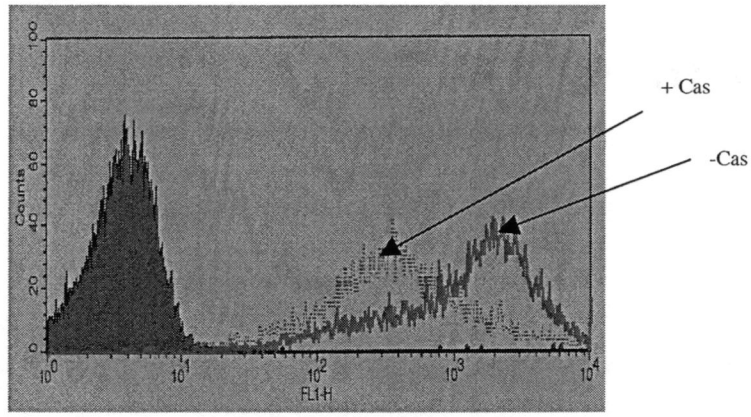



**Fig. 9. 2D gel profile of deglycosylated catfish MHC class II β molecules.** 28S T cell lysates were deglycosylated with PNGase F followed by immunoblotting with anti-MHC class II β. (A). Control , (B). PNGase F treated .

### *4.4. Effects of Castanospermine on surface expression of MHC class II*

To investigate the importance of N-linked sugars on the surface expression of MHC class II, castanospermine (CAS) was used to disrupt the processing of N-linked glycan. After 72h incubation CAS treated and untreated 28S T cells were analyzed by flow cytometry. As shown in Fig. 10, MHC class II surface expression was significantly reduced in CAS-treated cells compared with untreated cells. However, this reduction in surface class II does not appear to have been due to any negative effects of CAS on 28S T cells as CAS treatment did not show any negative effects on the cell viability as seen from the cell counts done during the treatment periods. It has been reported earlier that castanospermine treatment of monocytes from human PBMC did not affect the expression of CD80 whereas expression of glycoproteins such as CD1b and MHC class I were reduced (Huttinger *et al.*, 1999).

**A.**




**B.**

| Molecule | Mean Fluorescence Intensity (MFI) | | |
|---|---|---|---|
| | - cas | + cas | %Change |
| MHC class II | 1860 | 685 | -63% |

**Fig. 10. Effect of castanospermine on MHC class II expression.** 28S T cells were incubated for 72 h in medium containing with or without 200 μg/ml castanospermine and treated with primary antibody (Bs 4.1). The cells were washed, followed by incubation with FITC-conjugated goat anti-mouse IgG, and then analyzed by flow cytometry. (A) The fluorescence profiles of the negative control (shaded area), untreated cells (solid line), and the castanospermine treated (dashed line). The mean fluorescence intensities (MFI) for treated and untreated were 1863.2 and 686.7. (B) The MFI values after subtracting the MFI value of the negative control are shown.

# CHAPTER 5. DISCUSSION

The glycosylation status of catfish MHC class II molecules and the significance of N-linked sugars for surface expression are reported in the present study.

Deglycosylation experiments with various enzymes revealed catfish MHC class II β chain has one N-linked sugar and constituted about 10% of the total mass of the glycoprotein whereas α chain is devoid of an N-linked modification. This finding is in agreement with earlier reports on catfish MHC class II molecules (Godwin *et al.,* 1997; Godwin *et al.,* 2000; Fuller *et al.,* 2004). The absence of N-linked sugars on the α chain is unique to catfish as the consensus sequence for the addition of N-linked sugars, has been conserved on MHC class II α studied from other species such as humans, mice, zebrafish, carps, and striped bass (Sultmann *et al.*, 1993; Van Erp *et al.*, 1996; Hardee *et al.*, 1995; Wei *at al.,* 1991) which illustrates the importance of an N-linked glycan. The evolutionary significance of elimination of the N-glycan consensus sequence on the MHC class II α chain remains to be evaluated. Even though it is well known that glycans have a positive role on the protein folding process (Fabienne *et al.,* 2000) evolution of protein into better folders can also be achieved by the elimination of glycosylation consensus sequence in the primary amino acid sequence (Helenius & Aebi, 2001).

In mature glycoproteins, N-linked glycans are structurally diverse (Helenius and Aebi, 2001). The switch from the structurally uniform N-linked glycans found in the ER to

diverse forms found on surface is generated by glycosyltransferases activities as the glycoprotein pass through the Golgi complex (Varki, 1998; Helenius and Aebi, 2001). During this process, high mannose N-linked glycans are converted to complex forms, but this is not true for all high mannose N-linked glycans. The conversion of high mannose to complex forms of glycans results in the addition of residues such as N-acetylglucosamines, galactose, and sialic acids to the modified Glc3 $Man_9$ $GlcNAc_2$ core. The complex forms cannot be cleaved by endo H due to steric hindrance that develops when high mannose forms are transformed into complex forms in the Golgi. Endo H hydrolysis has shown that N-linked glycans on human MHC class II molecules are processed to complex forms after transport from the Golgi in dentritic cells and in B cells. (Pieters *et al.,* 1991; Engering *et al.,* 1998). Here our results with rat T cells indicate that MHC class II molecules acquire endo H resistance (Fig. 4B) suggesting that expressed MHC class II molecules are of complex type in rat similar to humans. Unlike mammalian MHC class II molecules, catfish class II from 28S T cells carried a high mannose type (Fig. 4A). Additional experiments on 28S T cells using surface biotinylation and endo H treatments (Fig. 5) revealed that even the cell surface MHC class II molecules are exclusively of high mannose type. However, endo H digestion of whole peripheral blood leukocytes (PBLs) and surface expressed MHC class II suggests a more complicated picture. Most of the MHC class II molecules remained endo H sensitive but a small percentage were resistant to endo H. This would suggest that catfish possess the machinery to process high mannose to complex forms. This pattern held even after extended period of incubation and at high concentration of endo H. In carboxypeptidase

Y and invertase, all the high mannose sugars were not released by endo H if the protein was not completely denatured (Trimble and Maley, 1977; Trimble *et al.,* 1983). The appearance of endo H resistant forms could also not be the result of any steric hindrance as digestion with PNGase F cleaved all the N-linked sugars, which suggest that complete denaturation of the proteins occurred in the PBLs lysates.

The conversion of high mannose to complex N-linked sugars is influenced by various other factors such as cell processing machinery, position of the acceptor sequence, three-dimensional structure, and cell types (Glabe *et al.,* 1980; Hsieh *et al.,* 1983; Williams and Lennarz, 1984; Yasuda *et al.,* 1999). For example, cathepsin E from rat carried both forms of N-linked sugars, i.e., high mannose and complex depending on the type of the cell from which they are isolated (Yasuda *et al.,* 1999).

Taken together, the results from 28S T cells and PBLs indicate that in catfish cellular processing of MHC class II molecules could be different in T cells and in other antigen presenting cells like dentritic cells, macrophages and B cells in channel catfish. Even inside a cell, molecules like CD45 get transported to the surface by two different pathways (Baldwin and Ostergaard, 2002), by a Golgi independent pathway, by the fusion of ER vesicles to plasma membrane and the other through the Golgi. Baldwin and Ostergaard, (2002) demonstrated that N-linked glycans on CD45 molecules that by-passed the Golgi remained high mannose while CD45 transported through the through the Golgi carried a complex type as they were processed by the Golgi enzymes from high

mannose to complex forms. So from endo H hydrolysis studies it is tempting to propose a Golgi independent transport of MHC class II in T cells whereas in other cell types like macrophages, dentritic cells and B cells class II might pass through the Golgi complex.

But, what was observed with 28S T could also be due to some oligosaccharide processing defects as seen with cell lines under culture. The defect could be an absence of one or more of enzymes glycosidases or glycotransferases responsible for processing the N-linked sugars from high mannose to complex forms in the golgi complex. It has been observed that efficiency of the conversion of high mannose into complex type structures and glycotransferase varied with cell type (Neel *et al.,* 1987).

Two-dimensional gels revealed the occurrence of polymorphic β chain whereas α chain focused as a single spot. Similar results were observed on 2D gels for HLA-DR molecules from two different cell lines (Neel *et al.*, 1987; Nag *et al.*, 1994) and in rat MHC class II antigens (Nag *et al.*, 1994). The appearance of β chains as cluster of spots could not be the result of artifactual modification because the α chain focused as single spot. With regards to glycosylation of MHC class II molecules in channel catfish it is known that the α chain has no N-linked whereas the β chain has one N-linked sugar and it is presumed that sequential glycosylation reaction that take place on N-linked glycan in the glogi to be one of the reason for the observed heterogeneity. To correlate the source of this heterogeneity of β chain on 2D gels to the N-linked sugars, PNGase F treated samples, which cleave all N-linked glycans were analyzed on 2D gels. The N-linked post

translational modifications cannot totally account for the multiple banding pattern of β chain. The β chains are known to be highly polymorphic whereas the α chain is structurally monomorphic in other human and mice (Charron & McDevit, 1979; Neel *et al.*, 1987). It has already been shown at the genome level that there are two MHC class II B loci with encoded β chains differing by at least 33 nucleotides in channel catfish (Godwin *et al.*, 1997). The isoelectric point (pI) calculated from the two gene sequence data gave distinct values, 8.10 and 8.48. These results may be the first demonstration of existence of multiple genes for MHC class II β chains from the teleost. The possible presence of more MHC class II B loci cannot be ruled out. Nag *et al.,* (1994) observed that cluster of spots could be the result of occurrence of multiconformational forms of α and β chains even under strong denaturation conditions. In teleost whether MHC class II molecules carry the processed antigenic peptide has not been shown unequivocally, so it is difficult to correlate the heterogeneity to the presence of strongly attached antigenic peptide of different pI on the β chain. The absence of a visible gel shift on one dimensional vertical SDS-PAGE when compared to control for the α and β chains (Fig.1 and Fig. 2) in treatments with neuraminidase or galactosidase indicate either an absence of these sugars or that these methods may not be sensitive enough to detect the changes. 2D gel analysis would be a valuable tool that can be employed to study the presence of these components.

Other post–translational events like phosphorylation, O-linked sugar addition may also contribute to the observed heterogeneity (Shackelford and Strominger, 1980; Nishikawa

*et al.,* 1979; Barrera 2002). Our results indicate that both the α and β chains are likely lacking any O-linked modifications of O-GlcNAC type.

Finally, studies on cells treated with the glucosidase inhibitor, castanospermine (CAS) gave some valuable insight into the role of N-linked sugars on the surface expression of MHC class II molecules in channel catfish. Unlike tunicanmycin, which was found to be cytotoxic to 28S T cells even at very low concentration of 0.1 μg/ml, castanospermine treatments did not affect the cell viability as assayed by cell counts. Castanospermine, a plant alkaloid preferentially inhibits α-glucosidase I activity (Elbein, 1991). Glucosidase I is involved in the initial processing of $Glc3Man_9GlcNAc_2$ N-glycan. Inhibition of Glucosidase I block the further processing of Glc3 $Man_9$ $GlcNAc_2$ to monoglucosylated form $Glc_1$ $Man_9$ $GlcNAc_2$. It has been shown that monoglucosylated form of N- glycan is required for the productive folding of many newly synthesised glycoproteins by transiently interacting with the ER chaperone, calnexin (CNX) (Hammond *et al.*, 1994; Hebert *et al.*, 1996; Ware *et al.,* 1995). In Lec 23, mutant CHO cells and $Pha^R$ 2.7, mutant BW 5147 mouse lymphoma cells which are defective in glucosidase I and II respectively CNX did not associate with substrate molecules confirming the importance of glucose trimming (Ora and Helenius, 1995). Also it has been seen that integral membrane glycoproteins like influenza hemagglutinin and vesicular stomatitis virus G associated with CNX and the presence of glucosidase inhibitors blocked the binding of CNX to HA or G proteins (Hammond *et al.*, 1994).

However, the presence of monoglucosylated oligosaccharide is not a prerequisite for CNX interaction as CNX can interact in more than one way (Ware *et al.,* 1995; Arunachalam and Cresswell 1995; Ihara *et al.,* 1999; Danilczyk and Williams 2001; Leach *et al.,* 2004). Recently it was show from this lab that CNX associates with MHC class II molecules in channel catfish (Fuller *et al.,* 2004) but it was not known whether this association is through the α chain alone or the β chain. Upon treatment of cells with glycosidase inhibitor, the surface expression of MHC class II molecules was reduced substantially. Thus, it would appear that the N-linked sugar on the β chain might facilitate MHC class II assembly in catfish. The data suggests that inhibition of N-linked glycan processing affects either the assembly or the transport properties of MHC class II molecules in the teleost. The most likely explanation for this would be the CNX might be involved in the biogenesis and subunit assembly of MHC class II molecules by interacting with the N-linked glycans. In human cells, assembly of class II histocompatibility molecules appears to be facilitated by association with CNX (Anderson and Cresswell 1994). This would imply that lectin type of interaction also operates in CNX-MHC class II assembly in channel catfish. But other kinds of interactions between CNX and substrate molecules are possible as seen with CD3 ε of TCR complex which are not glycoproteins (Rajagopalan *et al.,* 1994); MHC class II DR α and DR β (Arunachalam and Cresswell, 1995); human MHC class I (Zhang *et al.,* 1995). Even without CNX mediated folding in glucosidase treated cells, glycoproteins can fold and reach mature conformational form by associating with other chaperons and

folding enzymes (Ora and Helenius, 1995). This may explain for the low levels of MHC class II expression seen in CAS treated cells.

In conclusion, these results suggest that the N-linked glycan on class II β chain is important for the efficient surface expression MHC class II molecules in catfish. However, it remains unclear whether the MHC class II molecules are processed differently in T cells and other antigen presenting cells. Additional experiments are necessary to understand the role of glycans in the assembly of MHC class II molecules in the ER.

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