University of Alberta

Defining a Physical and Functional Interaction Between CD45 and Glucosidase II

by

Troy Allen Baldwin

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Abstract

Expression of the protein tyrosine phosphatase CD45 on cells of hematopoeitic origin is necessary for the transduction of signals derived from antigen receptors. CD45 is comprised of a relatively well characterized cytoplasmic phosphatase domain, and a poorly understood extracellular domain. Recently, our laboratory described the association between the external domain of CD45 and glucosidase II (GII). The goal of this study was to elucidate the biochemical mechanism of the binding of GII to CD45 and determine any functional outcomes from this interaction.

The association between CD45 and GII is specific and based on a lectin interaction. The α-subunit of GII provides the lectin activity through the active site, while removal of the N-linked carbohydrate from CD45 results in the loss of GII binding. Specificity for this interaction is dictated by both CD45 and GII. Only GIIα containing an alternatively spliced sequence call Box A1 are able to associate with CD45, and mannose residues found on CD45 carbohydrate are required for initiating the interaction.

Association of GII with CD45 is developmentally regulated and appears to modify the carbohydrate expressed by surface CD45. T cells of an immature phenotype contain GII associated CD45, while their mature counterparts show a significant reduction in the association. As well, surface glycoproteins, including CD45, from immature cells are recognized by the mannose binding lectin (MBL),

while no recognition is seen with mature cells. Inhibition of GII activity in mature cells results in the expression of surface ligands for MBL, including CD45, demonstrating the ability of GII activity to regulate carbohydrate expressed on surface glycoproteins.

In examining the trafficking of the CD45-GII complex, we found CD45 and GII interact in the ER and remain associated throughout the secretory pathway. Interestingly, a pool of newly synthesized CD45 is expressed on the cell surface five minutes after synthesis and contains entirely immature N-linked glycans. This population of CD45 appears to utilize a transport pathway which by-passes the Golgi complex and employs a Brefeldin A (BFA) resistant mechanism.

Overall, these data provide mechanisms for the expression of immature carbohydrate on surface CD45 which may impact functional characteristics of CD45.

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List of Abbreviations

2-ME	2-mercaptoethanol
APC	antigen presenting cell
ARF	ADP-ribosylation factor
BAP31	B cell antigen receptor associated protein 31 kDa
BFA	brefeldin A
$CaCl_2$	calcium chloride
CFTR	Cystic fibrosis transmembrane conductance regulator
CTL	cytotoxic T-lymphocyte
DC-SIGN	Dendritic cell-specific ICAM-3 grabbing nonintegrin
DN	double negative thymoctyes
DNM	deoxynorjirmycin
DOC	deoxycholate
DP	double positive thymocytes
Endo F	endoglycosidase F
Endo H	endoglycosidase H
ER	Endoplasmic reticulum
ERGIC	ER-Golgi intermediate compartment
FACS	fluorescence activated cell sorter
FITC	fluoroscein isothiocyanate
GEF	Guanine nucleotide exchange factor
GII	glucosidase II

GIIα	α-subunit of glucosidase II
GIIβ	β-subunit of glucosidase II
glc	glucose
h	hour
hrp	horseradish peroxidase
ip	immunoprecipitation
mAb	monoclonal antibody
man	mannose
MBL	mannose binding lectin
MBP	mannose binding protein
MHC	major histocompatability complex
min	minute
MRH	Mannose 6-phosphate receptor homology
NMdNM	N-methyl deoxynorjirmycin
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PE	R-phycoerythrin
PNS	post-nuclear supernatant
RPTPα	Receptor protein tyrosine phosphatase α
SA	streptavidin
SDS	sodium dodecyl sulfate
SMAC	supramolecular activation complex
SP	single positive thymocytes

TBS	tris buffered saline
TCR	T cell receptor
TGN	Trans-Golgi network
UGGT	UDP:glucose glycoprotein transferase
WB	western blot
WCL	whole cell lysate

Chapter 1: General Introduction

A. Basics of the Immune System

The immune system provides an essential defense mechanism against viuses, bacteria and other parasites, and may play a role in controlling cancer. There are two separate arms of the immune system, the innate and adaptive responses, each with their own specific roles in immunity, ultimately functioning in concert to confer protection. Components of the innate system include physical barriers such as the skin epidermis and internal mucosa, cellular mediators including natural killer cells (NK cells) and macrophages, and soluble factors such as complement system. The innate response is thought to serve as a first line defense mechanism which can act rapidly, but non-specifically against forgein invaders. The adaptive response is mediated mainly by T- and B- lymphocytes. This arm endows the immune system with the ability to respond specifically and acutely to pathogens. Because of the ability to respond specifically to forgein pathogens, it is necessary for the adaptive response to be able to discriminate between foreign and self antigens. Therefore, multiple mechanisms exist to prevent the adaptive system from responding to self. One of the most important mechanisms for this education is called central tolerance where thymocytes with a high reactivity towards self are deleted before entering the periphery thereby reducing the chance of autoimmunity.

B. Thymic Education

The process of educating the T cell arm of the adaptive immune response to ignore self antigens called central tolerance, occurs in the thymus. Multi-potent progenitor cells emigrate from the bone marrow and traffic to the thymus where they begin their journey to a mature T-cell. The thymus is an organ densely packed with numerous cell types, including fibroblasts, dendritic cells, epithelial cells, and thymocytes (Shortman and Wu, 1996). Interactions between thymocytes and other resident thymic cells, collectively termed the stroma, are thought to decide the fate of thymocytes. Some of the most important interactions are facilitated by the recognition of major histocompatability complex (MHC) proteins loaded with peptide on the stromal cells, by the T cell receptor (TCR) (Sebzda et al., 1999). One model developed to predict the result of contact between a thymocyte and stromal cells is the avidity model. The avidity model states that the outcome of the interaction between thymocytes and stromal cells is dictated by the strength/duration of the binding between the two cells. Specifically, high avidity interactions between the thymocyte and stroma results in death of the thymocyte or negative selection, because those thymocytes are likely to be autoreactive. However, weaker binding of the thymocyte to stromal cells results in survival signals for the thymocyte leading to positive selection (Sebzda et al., 1999). Ultimately, those thymocytes that are positively selected exit the thymus and enter the periphery as mature T cells. Recognition of self-MHC during positive selection ensures that once in the periphery, T cells will be able to recognize forgein antigens displayed by self-MHC.

The development of thymocytes proceeds in an ordered fashion with one sub-population of thymocytes giving rise to the next. Phenotypically, the sub-populations of thymocytes can be identified by the expression of the T cell co-receptors, CD4 and CD8. The most immature population of thymocytes do not express either CD4 or CD8 and are therefore termed double negative (DN) thymocytes (Figure 1-1). Progression to the next stage in thymocyte development results in the expression of both the CD4 and CD8 co-receptors. This population of thymocytes is termed double positive (DP). At the double positive stage, thymocytes test the TCR they express, and if the TCR expressed is of the correct affinity, they can be positively selected to either the CD4 or CD8 lineage. After positive selection, thymocytes are considered mature and express either CD4 or CD8 and are therefore termed single positive (SP) thymocytes.

For the most part, the contribution of other molecular interactions within the thymus is not well understood. These interactions however, may be important for regulating signaling thresholds, initiating interactions between thymocytes and stromal cells, or directing thymocytes to various areas of the thymus.

C. CD45 in the Thymus

CD45 is a type 1 transmembrane protein with tandem phosphatase domains in its cytoplasmic region and a large, complex external domain (Figure 1-2). CD45 was first identified as a positive regulator of immune cell function by studying the response of a T cell clone that was deficient in the expression of CD45. Proliferation of the CD45-deficient clone in response to its cognate

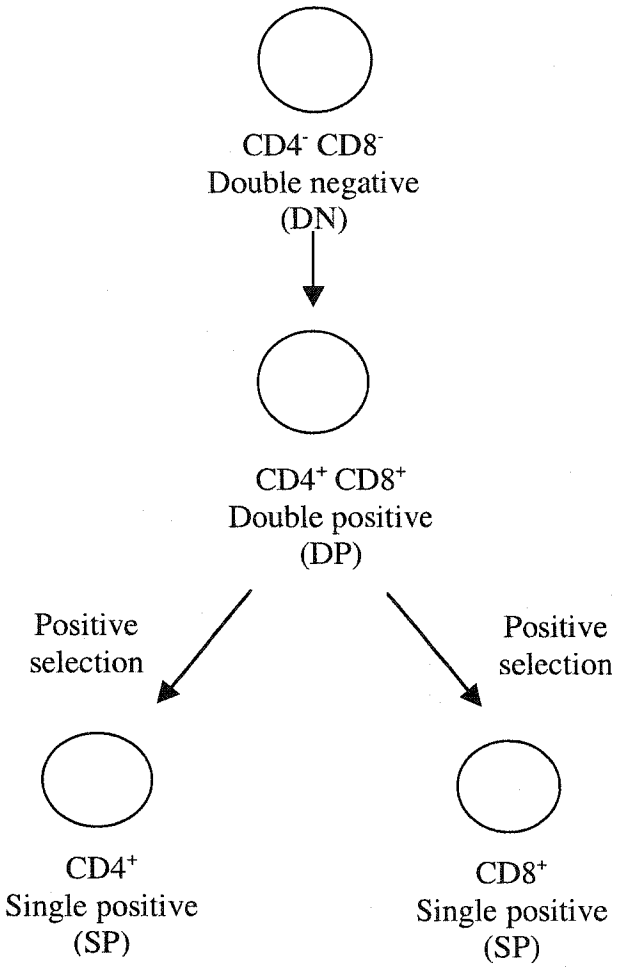


Figure 1-1: Overview of positive selection.

Thymocytes can be sub-divided into three basic populations based on the expression of the CD4 and CD8 co-receptors. The most immature population expresses neither CD4 nor CD8 and are termed double negative (DN). At the next stage thymocytes express both CD4 and CD8 and are double positive (DP). Finally, after positive selection, thymocytes only express either CD4 or CD8 and are mature single positive (SP) thymocytes.

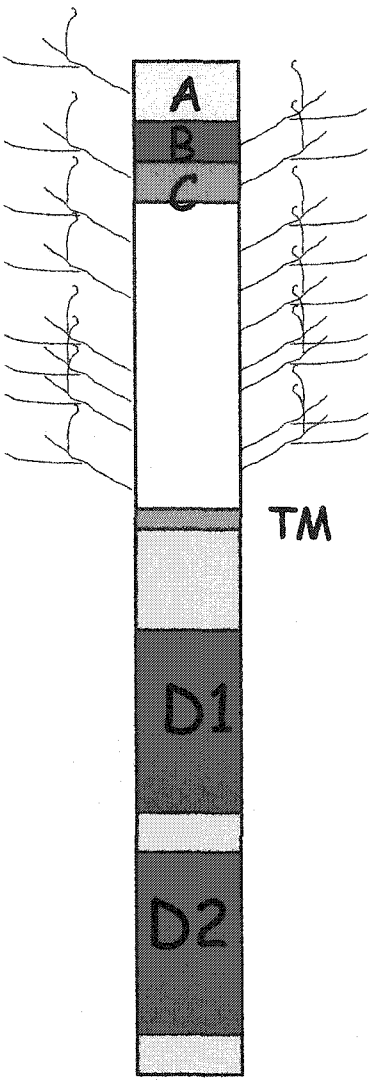


Figure 1-2: A schematic representation of CD45.
Illustrated are the tandem intracellular phosphatase domains (D1 and D2), the transmembrane region (TM) and the alternatively spliced exons A-C. The O-linked carbohydrate additions found within the alternatively spliced sequences are not depicted for simplicity. Shown decorating the entire ectodomain are the numerous N-linked carbohydrate additions.

antigen was severely diminshed (Pingel and Thomas, 1989). A number of subsequent studies have supported a positive role for CD45 in T cell responses (Koretzky et al., 1991; Koretzky et al., 1990; Weaver et al., 1991). To further elucidate the role of CD45 in T cell biology, mice were constructed where the gene encoding the CD45 protein was disrupted resulting in the lack of CD45 expression (Byth et al., 1996; Kishihara et al., 1993; Mee et al., 1999). Not only was there a drastic decrease in the population of mature peripheral T cells, but the T cells present showed an impairment in their responsiveness to antigen. In agreement with the immune deficiency seen in the CD45 knockout mice, some humans with a severe combined immunodeficient phenotype were shown to lack expression of CD45 due to mutations within the CD45 gene (Cale et al., 1997; Kung et al., 2000; Tchilian et al., 2001). Interestingly, examination of the thymocyte population from the CD45 knockout mice revealed a significant reduction in both total thymocyte numbers, and a block at the level of positive selection. The loss of mature peripheral T cells can be accounted for by a lack of positive selection, while the decrease in thymocyte numbers can be attributed to both a block in positive selection and a decrease in the ability of DN thymocytes to reach the DP stage. This major defect in positive selection was later confirmed by examining a CD45-deficient mouse with a transgenic TCR (Mee et al., 1999). In TCR transgenic mice heterozygous for CD45 expression, there is both enhanced positive and negative selection (Wallace et al., 1997). These data suggest that CD45 can alter signaling thresholds during both positive and negative selection. Intriguingly, in the absence of CD45, there does appear to be an

alteration in the amount of signal received by thymocytes during positive selection as the few thymocytes which are positively selected appear to have self-reactivity (Trop et al., 2000). Collectively, these data suggest that the expression of CD45 during thymocyte development plays a key role in determining the fate of those thymocytes, possibly through regulating signaling thresholds at the various stages of development.

The role of CD45 in lymphocyte activation appears to involve regulation of the phosphorylation status of the src-family non-receptor protein tyrosine kinases. In T cells, the major members of this family are p56lck and p59fynT. There are two key regulatory tyrosine residues found within src-family kinases. One tyrosine residue located near the active site, Y394 in Lck, has been shown to be a positive regulatory residue, while Y505 found in the carboxy-terminus, has been determined to be a negative regulatory site (Cooper and Howell, 1993). A model to support these data was obtained when the crystal structures of Hck and c-Src, src-family kinase members, were solved. The structures depicted an intramolecular interaction between tyrosine phosphorylated Y505 and an internal src-homology 2 (SH2) domain resulting in a "closed" conformation for Lck. This "closed" conformation sterically occluded the active site rendering the kinase inactive (Sicheri et al., 1997; Xu et al., 1997). To elucidate the mechanism of Lck regulation by CD45, Lck from cells lacking CD45 was examined. Interestingly, Lck from these CD45-deficient T cells was found to be hyperphosphorylated on residue Y505 (Ostergaard et al., 1989). Therefore, it was concluded that CD45 serves as a positive regulator of the src-family kinases by preferentially

dephosphorylating the negative regulatory site. Further examination of Lck in both wildtype and CD45-deficient cells revealed that CD45 was also able to dephosphorylate the positive regulatory site in addition to the negative regulatory site (Ostergaard and Trowbridge, 1990). Analysis of Lck from CD45-deficient thymocytes has demonstrated hyperphosphorylation at both Y505 and Y394, and an increase in kinase activity compared to wildtype thymocytes (Ashwell and D'Oro, 1999; D'Oro and Ashwell, 1999; Thomas and Brown, 1999). These data indicated that CD45 may not only be a positive regulator of Lck, but a negative regulator as well. Therefore, the role CD45 plays in regulating Lck activity is quite complex and still not entirely understood, nevertheless CD45 expression is required for T cell activation.

Given the regulatory effect of CD45 on Lck, it has been suggested that the defect in thymocyte development seen in CD45-deficient mice was due to a decrease in Lck activity. However, Lck from CD45-deficient animals contains high intrinsic enzymatic activity (D'Oro and Ashwell, 1999). Expression of a genetically modified form of Lck where Y505 is mutated to F505 is derepressed and can restore most, but not all, of the developmental defects seen in CD45-deficient animals (Pingel et al., 1999; Seavitt et al., 1999). The expression of F505 Lck did not appear to fully restore the percentage of mature CD4 SP thymocytes when introduced with a transgenic TCR (Seavitt et al., 1999). These data indicate that Lck kinase activity is not the major defect in CD45-deficient mice. Two separate hypotheses regarding the effect of CD45 on thymic development can be generated from the above data. One hypothesis is that Y505

dephosphorylation of Lck is important for rescue by allowing Lck to adopt an "open" conformation and facilitate the recruitment of and interaction with other proteins. Alternatively, CD45 may perform other functions in thymic development separate from regulating the phosphorylation status of Lck at Y505, possibly related to the external domain. These two hypotheses are not necessarily mutually exclusive, and both may be important.

CD45 possesses in addition to the phosphatase domains, an extracellular region that is extremely large and complex in nature. This ectodomain contains three alternatively spliced exons, designated A-C respectively, and within those exons are numerous sites for potential O-linked carbohydrate additions (Figure 1-2) (Thomas, 1989). Therefore, the inclusion of any combination of the three exons adds tremendous heterogeneity to CD45. In fact, addition of exons 4-6 (ABC) increases the size of CD45 by approximately 60 kDa. The usage of these alternatively spliced exons appears to be tightly controlled and developmentally regulated with immature thymocytes expressing predominately lower molecular weight isoforms while their mature counterparts express higher molecular weight forms (Wallace et al., 1992). To date there is no definitive role for any of the isoforms of CD45, or the entire external domain of CD45. However, addition of a pan-extracellular domain specific antibody to fetal thymic organ culture can inhibit the differentiation of double positive thymocytes to the mature single positive stage (Benveniste et al., 1994). This data suggests that either blockade or ligation of the CD45 external domain has functional effects on thymocyte development. Studies initiated to determine a specific role for the CD45RABC

and CD45R0 splice forms of CD45 in thymic development illustrated that expression of either isoform was sufficient to allow the progression of thymocytes from the DP to SP stage, but in mice expressing a single specific TCR, the CD45ABC form displayed a defect in the ability to promote positive selection of that TCR compared to the CD45RO form (Kozieradzki et al., 1997). One caveate to this study is the CD45 knockout mouse used had an insertional deletion at exon 6, which is alternatively spliced, and a small population of peripheral T cells from these mice were subsequently shown to express CD45 isoforms that lacked exon 6 (Kishihara et al., 1993). The expression of other CD45 isoforms by the positively selected T cells prevents firm conclusions from being drawn regarding a role for the individual isoforms of CD45 in thymic development. Therefore, it is possible that different isoforms of CD45 contribute specific functions within the thymus impacting T cell development, however further experiments need to be performed to answer this question fully.

In addition to the complexity of the extracellular domain of CD45 generated by the alternatively spliced sequences, there are 18 potential sites for N-linked carbohydrate additions with only 4 occurring in exons A-C (Figure 1-2). It has been estimated that approximately 25% of the total weight of CD45 is contributed by the N-linked carbohydrate and has therefore been proposed that the majority of the possible addition sites are used (Uemura et al., 1996). As with the various isoforms of CD45, and the entire extracellular domain in general, no definitive role for the N-linked carbohydrate on CD45 has been ascribed. It is clear however that the N-linked carbohydrate moieties are important for CD45

trafficking and surface expression as treatment of cells with tunicamycin, an inhibitor of N-linked carbohydrate addition, resulted in reduced cell surface expression, and a reduction in the stability of the protein (Pulido and Sanchez-Madrid, 1992).

Although much work has been performed in an attempt to elucidate specific functions for CD45 and its splice variants within the thymus, a number of questions still remain. Specifically, it is of interest to clearly define a role for the external domain of CD45 and the contribution each of the different isoforms, as well as determine a role for the N-linked carbohydrate in CD45 biology.

D. Regulation of CD45

Although much is known regarding the requirement for CD45 phosphatase activity in both T cell development and activation, a major mystery remaining in the field is how the activity of CD45 is regulated causing changes in the phosphorylation of Lck. A number of models regarding the regulation of CD45 activity have been proposed including phosphorylation, membrane localization, and dimerization. Recently dimerization and membrane localization models have received considerable attention as potential modes of regulation for CD45. Evidence for the potential contribution of receptor dimerization as a mode of regulation for CD45 began accumulating a number or years ago. Desai and colleagues constructed a chimeric CD45 molecule consisting of the extracellular and transmembrane domains of the epidermal growth factor receptor fused to the cytosolic portion of CD45 (Desai et al., 1993). The chimeric protein was

expressed in CD45-deficient HPB-ALL T cells and was found to rescue the CD45 dependent signaling defect seen in these cells. However, addition of EGF, which could dimerize the CD45 construct, resulted in a reduction in calcium flux and potentiation of signaling compared to untreated cells. Therefore, it was concluded that dimerization of CD45 resulted in an inhibition of phosphatase activity and a decrease in TCR signaling. Crystallographic studies on a related phosphatase, receptor protein tyrosine phosphatase (RPTP) α suggested that dimers of RPTPα had a reduced enzymatic activity due to the interaction of a "wedge" from one RPTPα molecule with the active site of the adjacent molecule (Bilwes et al., 1996). Due to the conservation of sequence between CD45 and RPTPα in the wedge region, it was proposed that CD45 dimerization caused a reduction in enzymatic by this "wedge" model. Mutation of a putative residue in the EGFR-CD45 chimera involved in the wedge formation resulted in EGF having no effect on downstream signaling, presumably due to the lack of formation of the inhibitory wedge (Majeti et al., 1998). Interestingly, a "knock-in" mouse was created where this residue involved in wedge formation was mutated. This mouse displayed a lymphoproliferative disorder, suggesting that the mutated CD45 could not be appropriately regulated by dimerization resulting in a constitutively active phosphatase (Majeti et al., 2000). However, using recombinant proteins consisting of the full length cytoplasmic domain and first phosphatase domain of CD45 demonstrated that an intramolecular interaction occurred more readily than did an intermolecular interaction as would be suggested by the inhibitory

dimerizaton model. As well, the intramolecular interaction did not inhibit the phosphatase activity of the proteins (Felberg and Johnson, 1998).

Another model for the regulation of CD45 is based on the segregation of CD45 from its putative substrates. It has been suggested that due to its size, structure and potential negative effect on Lck, CD45 may be physically sequestered from its substrates during target cell recognition by the T cell, thereby preventing the dephosphorylation of active Lck (Shaw and Dustin, 1997). The idea of compartmentalized signaling and formation of higher ordered structures at the interface between T cells and target cells has perpetuated this idea. These higher ordered structures at the T cell-APC interface have been termed immunological synapses (Bromley et al., 2001) or supramolecular activation clusters (SMACs) (Monks et al., 1998). In this model, active Lck is recruited to the SMAC while CD45 is excluded from the SMAC. However, it was demonstrated that a portion of CD45 does localize to the immunological synapse (Johnson et al., 2000). This evidence does not discount a role for sequestration or compartmentalization of CD45, but it does suggest that there may be a complex mechanism in place to control the access of CD45 to its substrates.

E. CD45 and lectins

Because of the structure of the external domain of CD45 and the fact that CD45 is a cell surface protein, there has been, and continues to be, a search for a potential ligand(s) for the extracellular region. To date this search has been relatively fruitless, however a few proteins have been shown to bind the

ectodomain of CD45. Interestingly, the majority of the proteins shown to recognize the external domain of CD45 were interacting with carbohydrate expressed on CD45. For example, the B cell protein CD22 was shown to bind CD45 and this interaction was dependent on the presence of sialic acid on CD45 (Sgroi et al., 1995; Stamenkovic et al., 1991). As well, galectin-1 has been demonstrated to recognize lactosamine units expressed on core 2-O glycan structures present on CD45 as well as other surface glycoproteins such as CD2, CD3, CD4, CD7 and CD43 (Galvan et al., 2000; Pace et al., 1999; Walzel et al., 2000). Recognition by galectin-1 triggered apoptosis in immature thymocytes as well as in mature T cells and induction apoptosis was regulated by expression of CD45. (Nguyen et al., 2001; Perillo et al., 1995). In addition, a component of the innate immune system, mannose binding lectin (MBL), has also been shown to bind CD45. This work demonstrated that MBL bound CD45 from thymocytes, but not mature T cells and was dependent on N-linked carbohydrate (Uemura et al., 1996). Finally, work from our laboratory demonstrated the interaction between the heterodimeric enzyme glucosidase II (GII) and CD45 (Arendt and Ostergaard, 1995; Arendt and Ostergaard, 1997). The mode of interaction was undetermined, however it was clear that Endo H sensitive carbohydrate was required for the interaction (Arendt and Ostergaard, 1997). With the exception of galectin-1, no functional significance for the interaction between CD45 and the lectins, including GII, has been demonstrated.

F. Glucosidase II

Glucosidase II is a heterodimeric enzyme consisting of an α-chain (GIIα) with an apparent molecular mass of 116 kDa, and a β-chain (GIIβ) of 80 kDa (Trombetta et al., 1996). The catalytic site of GII contains high sequence similarity with the family 31 gluco-hydrolases and resides within the α-chain (Arendt and Ostergaard, 1997; Flura et al., 1997). There is also one potential N-linked carbohydrate addition site in the N-terminus of the protein as well as two alternatively spliced sequences giving the potential to generate four possible isoforms (Figure 1-3) (Arendt et al., 1999). GIIβ also contains one alternatively spliced sequence and a number of different domain structures (Arendt et al., 1999). Differential biochemical or functional properties of the various isoforms of GIIα or GIIβ is currently unknown. There are two domains within GIIβ that are particularly interesting, the carboxy-terminal tetra-peptide HDEL sequence (Trombetta et al., 1996) and a mannose 6-phosphate receptor homology (MRH) domain (Figure 1-3) (Munro, 2001). The HDEL sequence along with its relatives, KDEL, RDEL and other variants, have been implicated in a retrieval role for proteins bearing this sequence via binding to the KDEL receptor. The KDEL receptor is a membrane protein that constitutively recycles between post-endoplasmic reticulum (ER) compartments and the ER (Lewis and Pelham, 1992). Therefore, if proteins bearing a tetrapeptide KDEL sequence escape the ER, they can be returned to the ER by the KDEL receptor. No direct role for the MRH domain in GIIβ has been ascribed, but it may be involved in GII function within

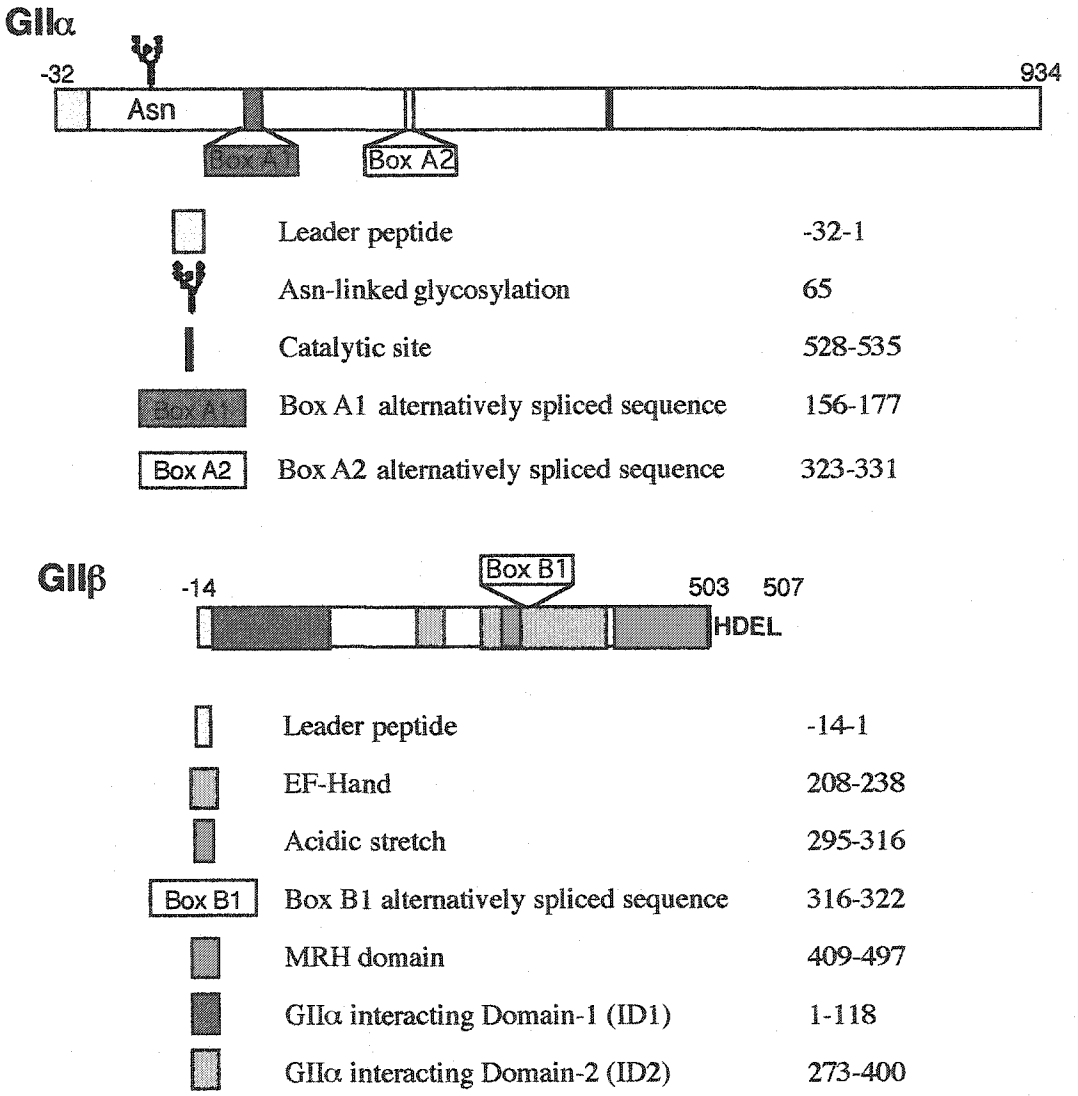


Figure 1-3: Structural representation of the heterodimeric enzyme GII. GII is composed of two polypeptides, GIIα and GIIβ. Within GIIα are the catalytic domain, one putative N-linked carbohydrate additon site, and two alternatively spliced sequences. GIIβ contains a number of interesting domains including a carboxy-terminal HDEL, and EF hand, an acidic stretch, a mannose 6-phosphate receptor homology region (MRH) and a single alternatively spliced exon.

other sub-cellular compartments. MRH domains are found in proteins with functions related to lysozomal and endosomal compartments. Interestingly, GII has been found to reside in a post-Golgi endocytic compartment (Brada et al., 1990). Overall, there is limited biochemical information available regarding how GII might interact with CD45 except for the fact that GII is a carbohydrate processing enzyme and CD45 contains carbohydrate capable of being processed by GII.

G. GII function within the ER

The mechanism of glycoprotein folding within the ER is a tightly controlled process involving the co-ordinated action of numerous enzymes and chaperones. The addition of N-linked carbohydrate to proteins imposes quality control steps important for the maturation of these glycoproteins (for review see (Helenius and Aebi, 2001)). A large oligosaccharide is added on asparagine residues found within the nascent polypeptide during the co-translational translocation of the protein into the lumen of the ER (Kornfeld and Kornfeld, 1985). This oligosaccharide contains a tri-glucosyl extension on one antennae of the sugar. The stepwise cleavage of this glucose extension first by glucosidase I, and subsequently glucosidase II leaves a mono-glucosylated carbohydrate structure capable of being bound by the lectin class of ER chaperones, calnexin and calreticulin (Figure 1-4) (Hammond et al., 1994; Hebert et al., 1995; Wada et al., 1997). The interaction of calnexin/calreticulin with the polypeptide promotes protein folding resulting in the adoption of the appropriate tertiary structure by the

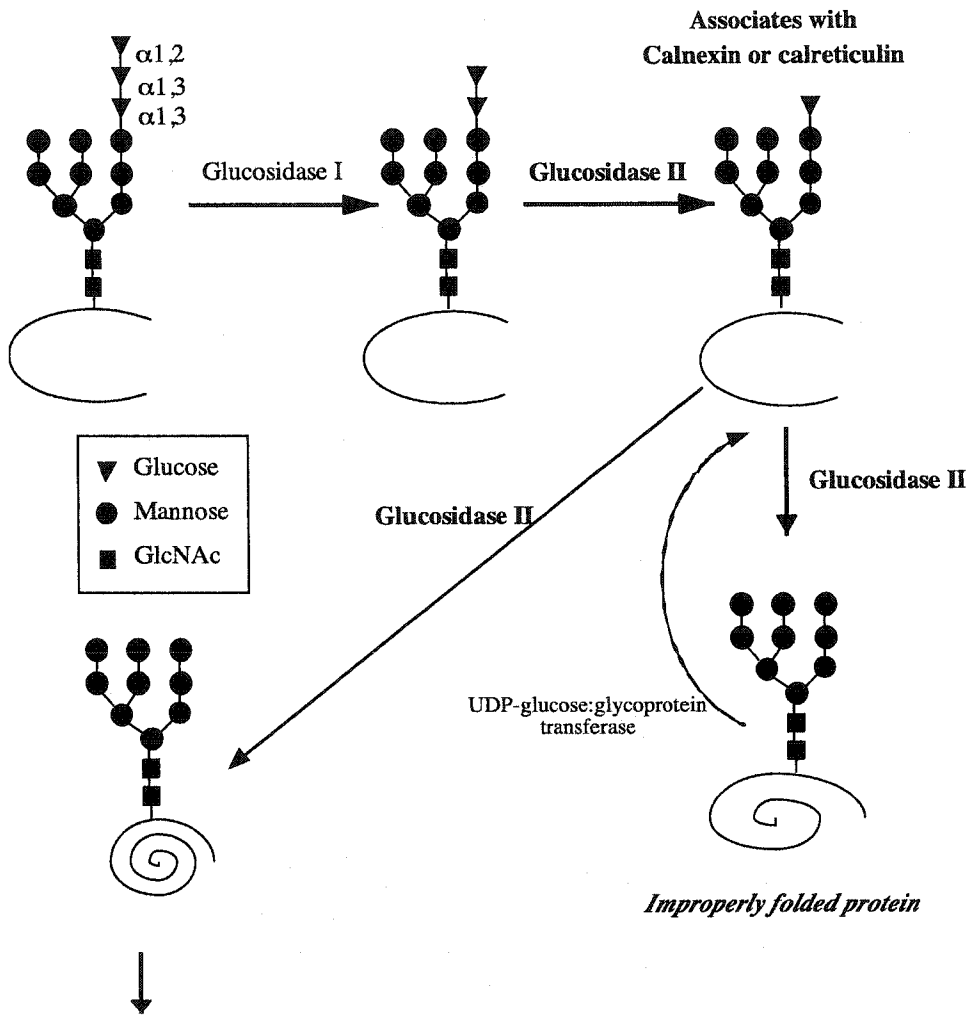


Figure 1-4: GII function within the ER.

Upon addition of the large oligosaccharide moiety to the nascent polypeptide, a tri-glucosyl extension is cleaved in a step-wise fashion by first glucosidase I, and second glucosidase II, leaving a mono-glucosylated structure. This structure is recognized by calnexin/calreticulin prompting a round of protein folding. After a folding reaction is complete, glucosidase II can then cleave the final glucose residue preventing the further association with calnexin/calreticulin. Proteins deemed properly folded can leave the ER, while improperly folded proteins can re-enter the folding cycle.

nascent polypeptide. After a round of protein folding occurs, GII can then cleave the last glucose residue thereby preventing further association with calnexin/calreticulin (Hebert et al., 1996; Helenius et al., 1997; Rodan et al., 1996). Egress of the glycoprotein from the ER can then proceed (Sousa and Parodi, 1995; Zapun et al., 1997). However, a single round of protein folding is generally insufficient for the adoption of the final tertiary structure, therefore mechanisms exist within the ER to discriminate between properly and improperly folded proteins. One such mechanism involves the glucosyl-transferase, UDP-glucose:glycoprotein transferase (UGGT), which is capable of adding a glucose residue to the oligosaccharide, reforming the binding site for calnexin/calreticulin, and promoting a subsequent round of protein folding (Figure 1-4) (Parodi, 2000b). The exact mechanism for the recognition of improperly folded proteins is not entirely understood, but likely involves the binding of exposed hydrophobic sequences in the improperly folded protein by motifs within UGGT (Trombetta and Helenius, 1999; Trombetta and Helenius, 2000). If after a number of folding cycles, the protein is still incorrectly folded due to mutation or other factors, this protein can be retro-translocated from the ER lumen to the cytosol where it can be degraded by the proteasome and the various components of the protein can be recycled (Parodi, 2000a; Sitia et al., 1990). This complex process of protein folding ensures that proteins leaving the ER are properly folded and therefore have a good chance of being functional.

H. The Secretory Pathway

After proper folding and adoption of the correct tertiary and quaternary structure, the protein or protein complex is transported from the ER to its appropriate sub-cellular location. This trafficking is mediated by transport machinery collectively termed the secretory pathway (Figure 1-5). Transport out of the ER is initiated by recruitment of cargo to distinct locations found randomly distributed throughout the ER membrane termed "ER exit sites" (Barlowe et al., 1994; Lippincott-Schwartz et al., 2000). To this point it is unclear whether or not cargo recruitment to ER exit sites is an active process where cargo receptors within the ER direct cargo to the exit site, or if a more passive mechanism is involved. For some classes of cargo, it appears that interaction with cargo receptors does occur and is important for transport out of the ER, however this may or may not be necessary for all classes of cargo (Herrmann et al., 1999).

Two proteins implicated as cargo receptors are p58 or ERGIC53, and BAP31 (Hauri et al., 2000; Spiliotis et al., 2000). Interestingly, ERGIC53 contains a mannose specific lectin domain and has been hypothesized to interact with putative cargo by virtue of high mannose carbohydrate on that cargo (Hauri et al., 2000). Once at the ER exit site, vesicles containing the coatamer complex COPII bud from the ER membrane and travel through an intermediate compartment called the ER Golgi intermediate compartment (ERGIC) (Figure 1-5). After passing through the ERGIC, vesicles fuse at the cis face of the Golgi complex where the contents of the vesicle are delivered to the cis-Golgi. In the Golgi complex, proteins move through the different Golgi stacks or cisternae

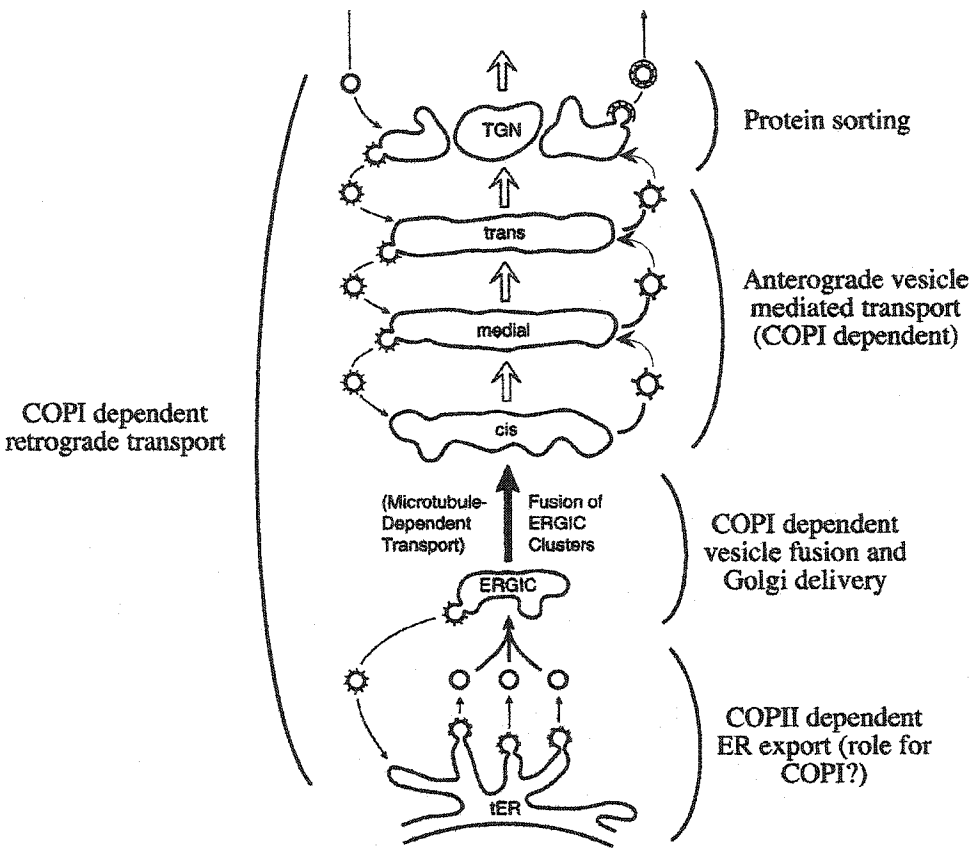


Figure 1-5: Transport through the exocytic pathway.

Transport from the ER is initiated by a COPII dependent mechanism. After leaving the ER, vesicles traffic through the ERGIC and ultimately are delivered to the cis-face of the Golgi. The delivery to the Golgi appears to be COPI dependent. Once in the Golgi, proteins traverse the entire stack where they are ultimately sorted for their final destination. Movement through the Golgi is thought to occur by bulk flow as illustrated by the open arrows, or by vesicular transport, closed arrows (figure adapted from (Glick and Malhotra, 1998)).

(Figure 1-5) (Lippincott-Schwartz et al., 2000). Within each of the different cisternae resides a subset of resident Golgi glycosylation enzymes that act sequentially on the glycoprotein ultimately shaping the final structure of the carbohydrate. Given the complexity of carbohydrate additions, and the diversity of carbohydrate structures found on glycoproteins, a tightly regulated system must be in place to ensure appropriate processing of carbohydrate (Colley, 1997; Roth, 2002). It is unclear what factors ultimately influence the final structure of the carbohydrate on a glycoprotein, but glycoprotein associated proteins, or developmentally regulated expression of Golgi glycosylation enzymes are possible factors (Roth, 2002). After passing through the entire Golgi stack, cargo is sorted at the trans-Golgi network (TGN) for delivery to its final destination within the cell including the plasma membrane and lysosomal compartments (Keller and Simons, 1997).

Movement of cargo containing vesicles from the ER to Golgi or between the Golgi stacks is a necessary process for maintaining the integrity and function of the secretory pathway. There are two important complexes of proteins involved in the directed movement of vesicles between the various members of the secretory pathway. Those two complexes are the COPI and COPII coatmer complex proteins. The COPII complex has been implicated in anterograde transport, or the trafficking of vesicles from the ER toward the Golgi (Barlowe et al., 1994), while the COPI complex mediates transport from distal Golgi stacks to more proximal stacks, or retrograde traffic (Figure 1-5) (Pelham, 1994). COPI also appears to play a role in anterograde transport past the ERGIC (Scales et al.,

1997). An important step in COPI mediated transport involves a G-protein dependent recruitment of the coatamer complex to the surface of the vesicle (Donaldson et al., 1991). The most well characterized G-proteins involved in COPI dependent transport is the ADP-ribosylation factor-1 (ARF-1) family of GTPases. When found in its GTP loaded form, ARF-1 can bind to the budding vesicle and subsequently provide a high affinity binding site for the COPI complex (Rothman and Orci, 1996). Exchange of GTP for GDP is catalysed by guanine nucleotide exchange factors, GEFs, so inhibition of the GEFs for ARF-1 prevents COPI dependent transport. A fungal metabolite, Brefeldin A (BFA) has been demonstrated to inhibit ARF-1 GEF activity and therefore block protein transport (Donaldson et al., 1992; Helms and Rothman, 1992). Upon treatment of cells with BFA, Golgi resident enzymes re-distribute to the ER while Golgi matrix proteins localize to Golgi remnant bodies (Klausner et al., 1992). These data suggest that upon BFA treatment, anterograde traffic is completely inhibited while retrograde transport may still proceed. Further work demonstrated the existence of a BFA insensitive GEF for ARF-1 which localized to the Golgi compartment and was therefore hypothesized to mediate the BFA insensitive retrograde transport of Golgi resident enzymes to the ER (Claude et al., 1999). Importantly, the fact that no anterograde trafficking has been observed after BFA treatment suggests that either COPI is involved in this trafficking, or COPII utilizes a similar ARF-1 dependant membrane recruitment. However, COPII has been shown to constitutively cycle between ER bound and free states irrespective of the presence of BFA (Ward et al., 2001), therefore COPI likely plays a role in

anterograde trafficking. Interestingly, there appears to be a sequential mode of action for COPI and COPII in anterograde tranport where COPII acts early in transport, while COPI acts slightly later, possibly after the ERGIC (Figure 1-5) (Scales et al., 1997). Consistent with this model, a cell line defective in ε-COP, a member of the COPI complex, showed no movement of vesicles towards the Golgi, but accumulation of transport intermediates at the ERGIC (Scales et al., 1997). Collectively, these data suggest that transport out of the ER is not dependent on COPI function, while trafficking beyond the ERGIC is COPI dependent.

I. Rationale and Objectives

Previous work from our laboratory suggested that the association between CD45 and glucosidase II was mediated through the external domain of CD45, specifically the Endo H sensitive carbohydrate expressed on CD45 (Arendt and Ostergaard, 1997). As GII is an enzyme capable of recognizing carbohydrate moieties we hypothesized that this association was based on a lectin interaction. Furthermore, the association appeared to be specific as GII was not found to associate with other glycoproteins. Because carbohydrate plays important roles in a number of cellular processes such as glycoprotein folding, trafficking, and ligand binding, we wished to dissect the biochemical basis of the association between CD45 and GII. Additionally, we hoped to determine a functional consequence for the stable binding of GII to CD45. By gaining further

knowledge of the interaction between CD45 and GII, we could then apply this information to the potential role of the external domain of CD45 in T cell biology.

Chapter 2: Specific isoforms of the resident ER protein glucosidase II associate with the CD45 protein tyrosine phosphatase via a lectin-like interaction

A version of this chapter has been published. Baldwin T.A., Gogela-Spehar M, and Ostergaard H.L., The Journal of Biological Chemistry, 2000 , 275: 32071-76. M. Gogela-Spehar generated the individual GIIα isoforming expressing clones used in this chapter.

A. INTRODUCTION

CD45 is a highly abundant, transmembrane, protein tyrosine phosphatase (PTPase) expressed on all cells of hematopoietic origin (Trowbridge and Thomas, 1994). The cytoplasmic phosphatase activity of CD45 has been shown to be essential for the early signal transduction events leading to both thymocyte maturation and T cell activation (Trowbridge and Thomas, 1994). There is substantial evidence to suggest that CD45 regulates the tyrosine phosphorylation of src-family kinases (Ashwell and D'Oro, 1999; Thomas and Brown, 1999). The external domain of CD45 is extremely heterogeneous with respect to size and carbohydrate content due primarily to three alternatively spliced exons, which encode for potential O-linked glycosylations (Thomas, 1989). The usage of these exons appears to be developmentally regulated (Thomas, 1989). As well, the extracellular domain encodes attachment sites for numerous N-linked glycans, and these glycosylations appear to be important for cell surface expression and protein stability of CD45 (Pulido and Sanchez-Madrid, 1992). Finally, although CD45 is a cell surface protein, no specific ligand for the extracellular domain has been definitively identified. Perhaps relevant to the present study, there have been studies suggesting that some lectins such as CD22 (Sgroi et al., 1995; Stamenkovic et al., 1991), galectin-1 (Pace et al., 1999; Perillo et al., 1995) and the mannan binding protein (MBP) (Uemura et al., 1996) are able to bind to CD45

carbohydrate although the biological significance of these interactions are largely not understood.

Recently, our laboratory has demonstrated that the carbohydrate processing enzyme α–glucosidase II (GII) physically interacts with CD45 (Arendt and Ostergaard, 1997). GII is found within the ER, and catalyses the hydrolysis of the inner two α 1,3 linked glucose residues present on all N-linked immature oligosaccharides (Hubbard and Ivatt, 1981; Lucocq et al., 1986). This processing of glucose in the ER has been shown to be intimately involved in protein folding by regulating the interaction between the nascent polypeptide and the lectin chaperones calnexin and calreticulin. More specifically, removal of the first α1,3 linked glucose by GII creates a substrate for calnexin/calreticulin binding (Hammond et al., 1994; Hebert et al., 1995; Ora and Helenius, 1995; Wada et al., 1997), while removal of the second glucose causes dissociation of calnexin/calreticulin from the polypeptide (Hebert et al., 1995; Hebert et al., 1996; Helenius et al., 1997; Rodan et al., 1996). The hydrolysis of the second α 1,3 linked glucose is necessary for the progression of properly folded glycoproteins from the ER to the Golgi (Sousa and Parodi, 1995; Zapun et al., 1997).

The GII enzyme is composed of a 116 kDa α-subunit, which contains a catalytic motif of the Family 31 glucosidases (Arendt and Ostergaard, 1997; Flura et al., 1997), and an 80 kDa β-chain of unknown function (Trombetta et al., 1996). We and others hypothesize that the β-chain is involved in enzyme localization (Arendt and Ostergaard, 1997; Trombetta et al., 1996). Both subunits

of GII have been shown to be alternatively spliced (Arendt et al., 1999). There is one alternatively spliced sequence (Box B1) within GIIβ which gives rise to two potential isoforms, while within GIIα, there are two alternatively spliced sequences (Box A1 and A2) which have the potential to generate four distinct isoforms (Arendt et al., 1999). These different splice forms may vary in their subcellular localization, enzymatic activity, or substrate specificity.

The association between CD45 and GII may be surprising given the subcellular distribution and function of the two proteins, nevertheless, this interaction may prove to be instrumental in elucidating aspects of CD45 biology. Therefore, we wished to dissect the biochemical basis for this stable interaction. We found that only isoforms of GIIα containing Box A1 are capable of interacting with CD45. It also appears that the active site of GIIα is required for the interaction with the N-linked carbohydrate on CD45. As well, the addition of mannose significantly decreases the association between CD45 and GII. Together, these data suggest that the association between GII and CD45 is a lectin-based interaction.

B. EXPERIMENTAL PROCEDURES

Cell Lines and Antibody Reagents

BW5147 (BW), and a CD45-negative variant (BW/T200$^-$), mouse T-lymphoma cells were maintained as described previously (Arendt and Ostergaard, 1995). The PHA^{r}2.7 cell line, generously provided by Dr. Ian Trowbridge (Salk Institute, La Jolla), is a BW-derived mutant deficient in GIIα subunit expression (Flura et al., 1997), which was maintained in an identical manner to the parental BW line. Monoclonal antibody I3/2.3, which was provided by Dr. Ian Trowbridge (Salk Institute, La Jolla), recognizes a pan-specific determinant within the CD45 extracellular region. I3/2.3 was purified and directly coupled to cyanogen-activated Sepharose 4B. Rabbit antiserum H2, specific for GIIβ, was previously described (Arendt and Ostergaard, 1997), while rabbit antiserum J37 was generated to the tandem intracellular phosphatase domains of CD45. The anti-GIIα antiserum was purchased from Stressgen (Vancouver, BC.).

Establishment of BW/PHA^{r}cell lines expressing individual GIIα isoforms

Complete sequences of clones 116FL.A (A1-/A2-), 116 FL.E (A1-/ A2-), 116FL.B (A1+/A2-) and 6R5-14 (A1+/A2+) were obtained by the dideoxy chain termination method and compared to a previously published GII alpha sequence (Arendt and Ostergaard, 1997). The 116FL.A sequence contained no mutations, while mutations/truncations in the 116FL.B and 6R5-14 sequences were repaired

by standard subcloning procedures. The 116FL.E and corrected 6R5-14 clones were then used to construct the A1-A2+ clone and all four final clones were re-sequenced as above. Upon sequence verification, the c-DNA fragments were cloned into the mammalian expression vector pcDNA3 (Invitrogen) and endotoxin-free preparations of the four constructs were prepared using the Qiagen EndoFree Plasmid Maxi Kit. 20 ug of endotoxin-free DNA was electroporated into BW/PHA^r cells using BioRad Gene Pulser at 300 mV. Transfected cells were allowed to recover for 24-36 h., after which G-418 (Life Technologies) was added to a final concentration of 2 mg/ml. Selected transfectants were grown up in presence of G-418 and screened for expression of GIIα by western blotting.

Cell lysis, immunoprecipitation, and reconstitution assays

Cells were lysed at a density of 5 x 10^7/ml in 0.5% Nonidet P-40 (Pierce, Rockford, IL.), 150 mM NaCl, 10 mM Tris, pH 7.6, and incubated on ice for 20 min. Post nuclear supernatants were incubated for 1-2 h with I3/2 coupled beads, or for 20-30 min. with polyclonal antisera followed by a 1-2 h incubation with Protein A-Sepharose beads (Boeringer Manheim, Laval, Que.). Immunoprecipitates were washed three times with ice cold lysis buffer, resuspended in reducing sample buffer and boiled. The reconstitution assay was performed as previously described (Arendt and Ostergaard, 1997). Briefly, CD45 immunoprecipitates were washed three times in 0.5% DOC, 20 mM Tris, pH 7.6, to remove bound GII, followed by one wash in lysis buffer. A BW/T200$^-$ lysate

was then added to the CD45 immunoprecipitate for 1-2 h, followed by three washes with lysis buffer. All antibody incubations took place at 4°C.

Polyacrylamide Gel Electrophoresis, and Immunoblotting

Proteins were resolved on 7.5% polyacrylamide gels and transferred to PVDF-Immobilon (Millipore) as previously described (Arendt and Ostergaard, 1995). Western blot analysis was carried out with the indicated antisera, followed by Protein A-HRP (Pierce), and visualized by enhanced chemiluminescence (DuPont NEN). The mobility of molecular weight standards are indicated on the left side of the gel panel with arrows.

Endoglycosidase treatment

CD45 or GIIβ immunoprecipitates were prepared as described above. For treatment under reducing and denaturing conditions, the immunoprecipitates were boiled in 1% NP-40, 0.1% SDS, 1% β-mercaptoethanol, 10mM phosphate, 150mM NaCl, pH 7.2, then allowed to cool before the addition of 0.3U Endo F (Sigma, St. Louis, MO). The beads were then incubated at 33°C for 16-18 h. For treatment under native conditions a 50 mM imidazole, pH 6.8 buffer was used. The beads were incubated for 16-18 h at 33°C in 50μl of imidazole buffer and 0.3U Endo F (Sigma, St. Louis, MO). The reactions were quenched by adding reducing sample buffer and boiling, or the beads were washed three times with lysis buffer prior to the addition of reducing sample buffer and boiling.

GII enzymatic assay

Determination of the GII enzymatic activity was performed as previously described (Arendt and Ostergaard, 2000). Briefly, samples were incubated with 5mM p-nitrophenyl α-D-gluocopyranoside (Sigma, St. Louis, MO) in phosphate-buffered saline, pH 7.2 for 16-18 h at room temperature. Color change was quantified by measuring the absorbance at 405 nm. Background absorbance, defined as the average value obtained when the colorimetric reagent was incubated with lysis buffer alone, was subtracted from all values obtained.

Inhibitor treatment of lysates

Deoxynorjirmycin (dNM) and australine (Oxford Glycosystems, Wakefield, MA) were reconstituted with water to a concentration of 100 mM, and aliquots were stored at –20°C. Lysates were treated with the inhibitors for one hour at room temperature with rotation. Samples of inhibited lysates were kept and analyzed for GII activity as described above.

C. RESULTS

The CD45-GII interaction requires the GIIα subunit

Recently, it was demonstrated that the BW5147 mutant cell line, BW/PHA^r is deficient in expression of the GIIα subunit (Flura et al., 1997). However, we find that BW/PHA^r expresses normal levels of CD45 and GIIβ (Figure 2-1). We were therefore able to utilize the BW/PHA^r cell line to determine which GII subunit mediates the interaction with CD45 that we have described previously (Arendt and Ostergaard, 1997). An association of both GII subunits with CD45 is observed in the BW cells, but neither subunit associates with CD45 in the BW/PHA^r cells (Figure 2-1). This result demonstrates two important features of the CD45-GII association. First, expression of the GIIα subunit is required for the association of the GII complex with CD45. Second, the β-subunit alone can not interact directly with CD45, rather, GIIβ associates with CD45 by virtue of its binding to GIIα. This observation does not preclude the possibility that GIIβ might enhance binding of GIIα to CD45 perhaps through a protein-protein interaction, or by a causing a conformational change in GIIα.

A minor, higher molecular weight isoform of GIIα associates with CD45

As stated previously, there are four potential protein isoforms of GIIα that can be generated by alternative splicing (Arendt et al., 1999). Variable isoform usage has not yet been conclusively demonstrated at the protein level, mainly due

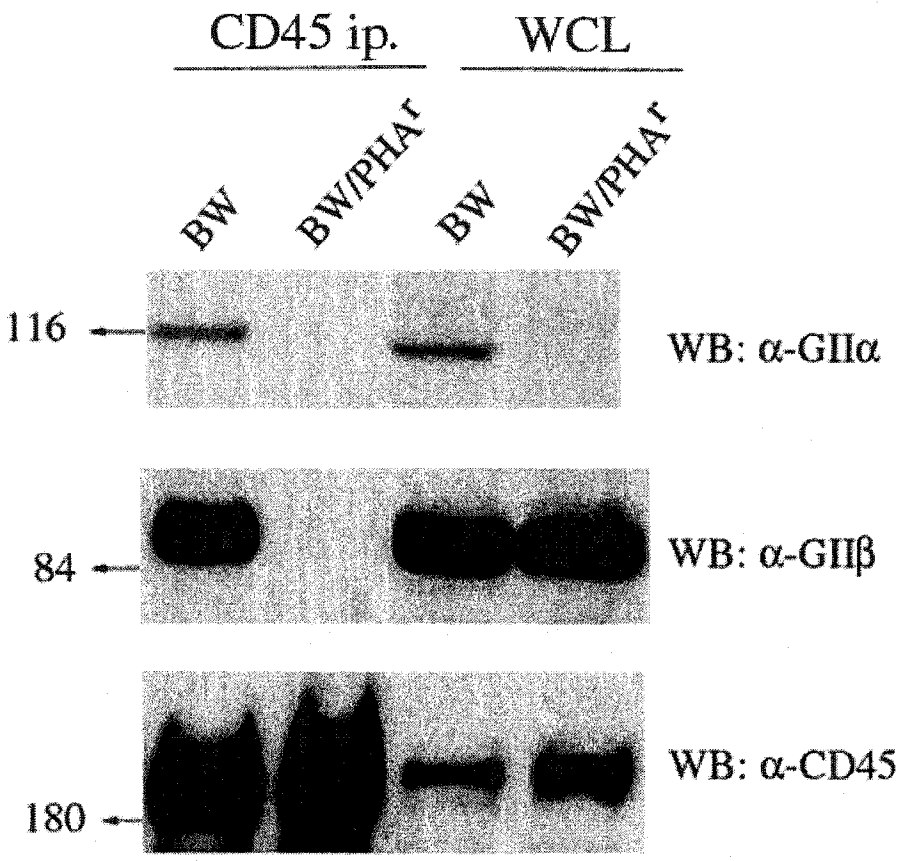


Figure 2-1: GIIα protein expression is required for the association with CD45.

I3/2 beads were incubated with 2.5 X 10^7 BW or BW/PHA^r post-nuclear extracts and the immunoprecipitates were resolved by SDS-PAGE. Western blotting for the presence of GIIα (top panel), GIIβ (middle panel), or CD45 (lower panel) was performed. Whole cell lysate (WCL) from the indicated cell type was probed for protein expression. Mobility of molecular weight standards are indicated on the left side of the gel panel by an arrow.

to a lack of specific antibody reagents. In comparing the molecular weight of GIIα that co-immunoprecipitates with CD45 and the total GIIα pool found in lysates, it appears that a higher molecular weight isoform of GIIα is found in association with CD45 (Figure 2-1). This higher molecular weight isoform is not readily detectable in a Western blot of total lysate, and is therefore thought to constitute a minor portion of total GIIα. When we compare CD45 and GIIβ immunoprecipitates from BW cells, a higher molecular weight isoform of GIIα is observed in CD45 immunoprecipitates (Figure 2-2). This is the same pattern that was observed when comparing CD45 immunoprecipitates and total cell lysate from BW cells (Figure 2-1). The difference in relative mobility of GIIα subsets can arise from at least two distinct mechanisms. First, the difference may be due the result of alternative splicing with a different size polypeptide backbone, or second, the difference may arise from post-translational modifications such as differential glycosylation. To address this issue, CD45 and GIIβ immunoprecipitates were digested with Endoglycosidase F (Endo F) under reducing and denaturing conditions, followed by Western blot analysis for GIIα. The samples were spiked with additional Endo F enzyme periodically throughout the incubation to achieve complete digestion. When the CD45 associated GIIα was treated with Endo F, the entire band shifted to a faster migrating form owing to the release of the N-linked carbohydrate (Figure 2-2). Therefore, all of the CD45 associated GIIα appears to be glycosylated. When the GIIβ associated GIIα is digested with Endo F, two distinct bands are revealed (Figure 2-2) likely

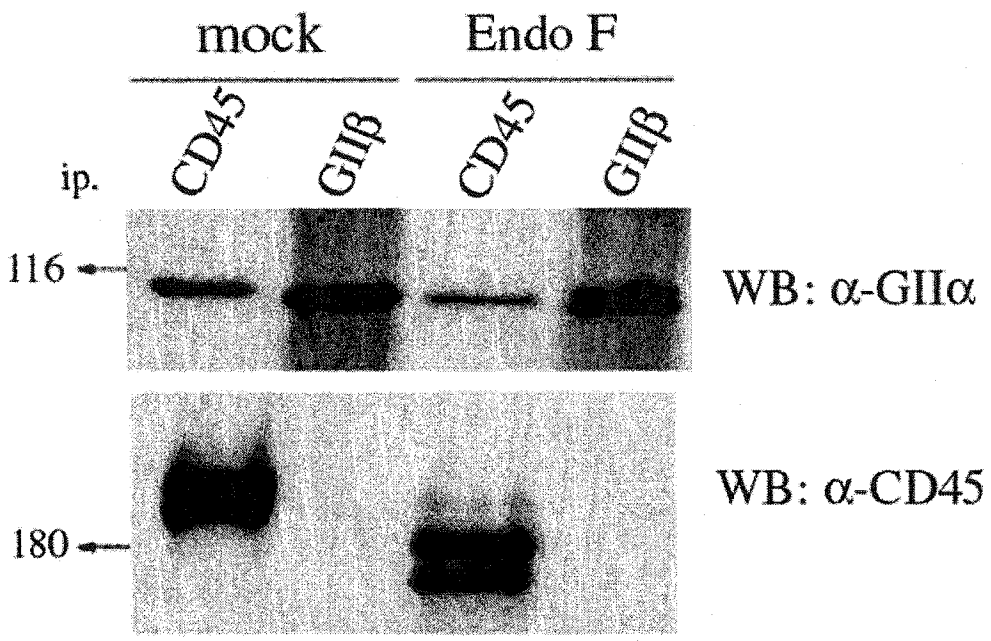


Figure 2-2: A higher molecular weight isoform of GIIα associates with CD45. CD45 or GIIβ was immunoprecipitated from 2.5 X10^7 BW cell lysates followed by mock treatment, or treatment with 0.3U Endo F under reducing and denaturing conditions. Proteins were resolved by SDS-PAGE and immunoblotted for either GIIα (upper panel), or CD45 (lower panel).

representing two distinct polypeptide backbones that contain, or lack, the larger differentially spliced sequence (Box A1).

Only a higher molecular weight isoform of GIIα is capable of associating with CD45

From the data presented in Figure 2-2, it appears that only a higher molecular weight form of GIIα associates with CD45. It is possible however, that other isoforms of GIIα are capable of associating with CD45. To address this issue, we employed a sequential reconstitution strategy. First, CD45 immunoprecipitates were prepared for reconstitution assays by washing with 0.5% DOC to remove endogenously bound GII. Next, a BW/T200$^-$ lysate was added to the first CD45 immunoprecipitate. After incubation, that same lysate was then added to the second CD45 immunoprecipitation. This was repeated for a total of six CD45 immunoprecipitates. A sample of the BW/T200$^-$ lysate before and after the sequential reconstitution assays was taken and examined for the presence of GIIα protein, and GII enzymatic activity. We found that only the higher molecular weight GIIα associated with CD45 and no lower molecular weight GIIα associated with CD45 even after depletion of the higher molecular weight form of GIIα (Figure 2-3). In examining the lysate after the sequential reconstitutions, there was little change in the amount of the lower molecular weight isoform of GIIα (Figure 2-3), demonstrating that there was still plenty of GIIα available. As well, there was still 75% of the initial GII enzymatic activity

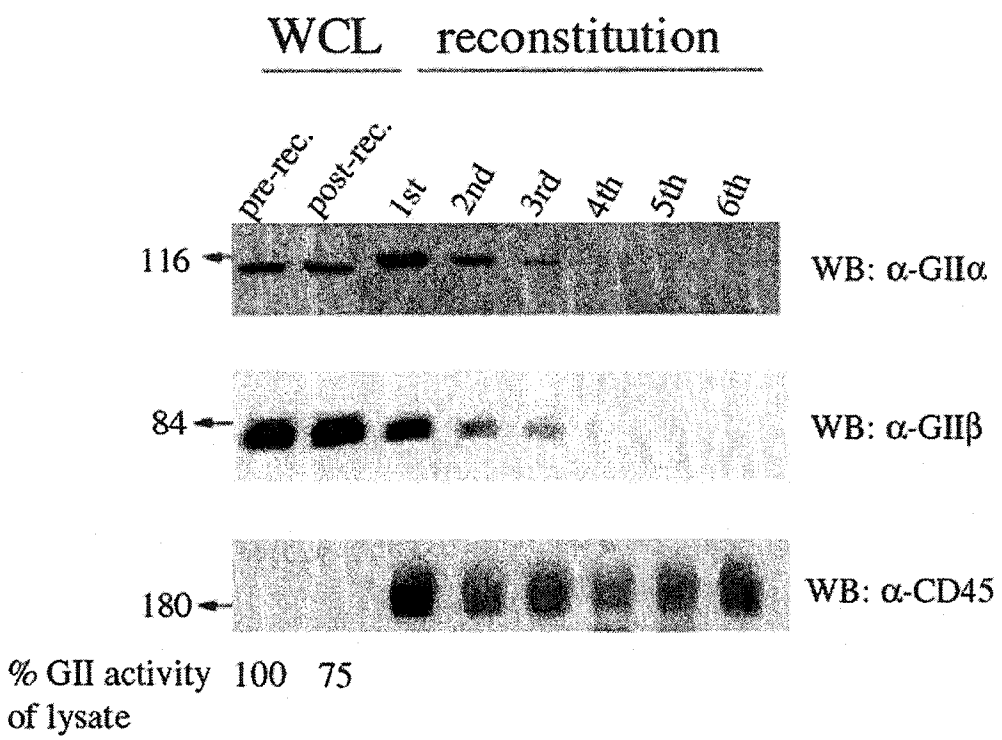


Figure 2-3: Only a higher molecular weight isoform of GIIα is capable of associating with CD45.

CD45 immunoprecipitates from 2.5 X 10^7 BW post nuclear extracts were washed with 0.5% DOC, 20 mM Tris, pH 7.6 to remove bound GII. A lysate corresponding to 1.5 X 10^7 cell equivalents of BW/T200$^-$ was added sequentially to the washed CD45 immunoprecipitates. Beads were washed three times in lysis buffer and subjected to SDS-PAGE analysis. Western blot analysis was performed for GIIα (top panel), GIIβ (middle panel) and CD45 (lower panel). Whole cell lysate (WCL) before (pre-rec.) and after (post-rec.) the sequential reconstitutions was also analyzed for the presence of the indicated proteins and GII enzymatic activity.

remaining, so loss of activity can not explain the lack of binding. Therefore, in a competitive situation where multiple GIIα isoforms are expressed, a higher molecular weight form of GIIα preferentially associates with CD45.

Only isoforms of GII containing alternatively spliced sequence A1 are capable of associating with CD45

The observation that only a higher molecular weight isoform of GIIα is capable of association with CD45 raises the obvious question regarding the molecular identity of this isoform. To answer this question, we performed transfection studies utilizing the BW/PHA^r cell line. Stable transfectants expressing each of the four isoforms of GIIα were generated in the BW/PHA^r cells, and used for immunoprecipitation experiments. In examining CD45 immunoprecipitates from lysates of the BW, BW/PHA^r parentals, and each of the transfectants, it is clear that the association between CD45 and GII only occurs in cells which express GIIα isoforms containing the alternatively spliced sequence A1 (Figure 2-4), while the second alternatively spliced region (Box A2) does not appear to influence the binding of GII to CD45 (Figure 2-4). Therefore, the binding of GII to CD45 is dictated by presence of Box A1. Of note, all isoforms appear to have similar catalytic activities, and therefore, alteration of GII activity can not account for the isoform-specific GII binding to CD45 (data not shown).

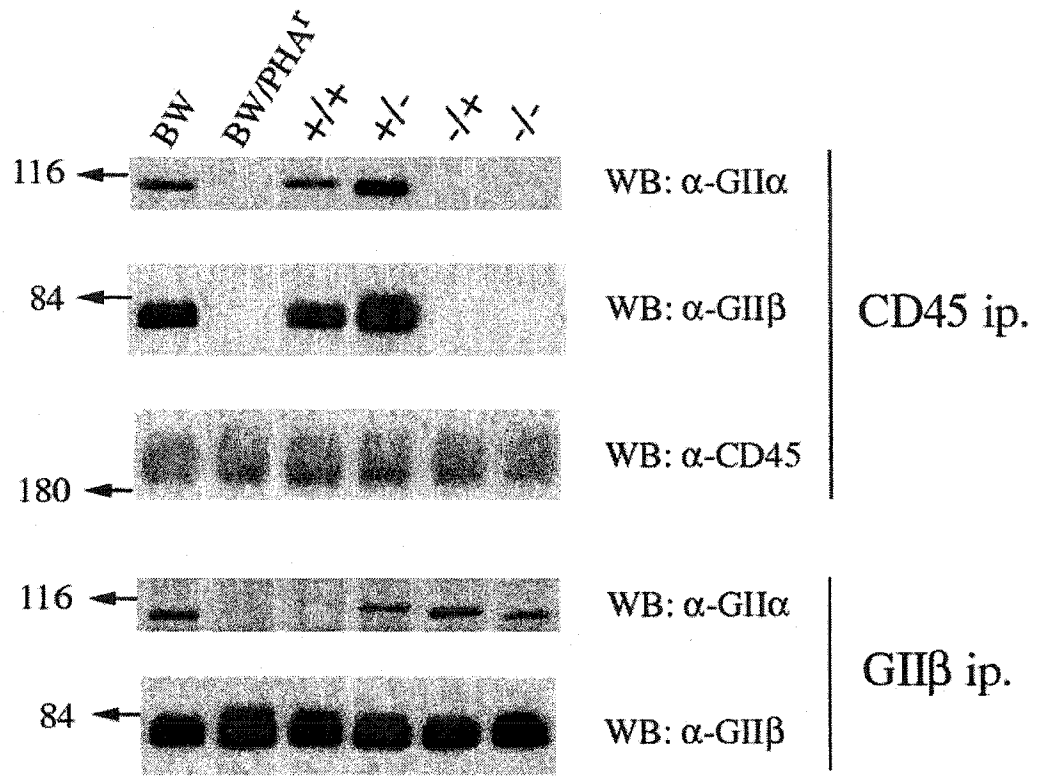


Figure 2-4: Only the GIIα isoforms containing Box A1 are capable of associating with CD45.

CD45 or GIIβ immunoprecipitates were prepared from the post nuclear extracts of 2.5 X 10^7 BW; BW/PHA^r; or the BW/PHA^r +/+ (BoxA1$^+$/ Box A2$^+$), +/- (Box A1$^+$/ Box A2$^-$), -/+ (Box A1$^-$/ Box A2$^+$), -/- (Box A1$^-$/ Box A2$^-$) transfectants. The immunoprecipitates were either resolved by SDS-PAGE, or a GII enzymatic activity assay was carried out. Western blot analysis for GIIα, GIIβ, and CD45 was performed.

The GIIα catalytic site is essential for the binding of GII to CD45

From the data presented in Figure 2-1, GIIα protein is required for binding of GII to CD45. We next wanted to determine whether or not GIIα activity is necessary for CD45 binding by making use of competitive, active site directed inhibitors. We performed a reconstitution assay where CD45 immunoprecipitates from BW cells were stripped of endogenously bound GII. To these immunoprecipitates, we added BW/T200$^-$ lysates pre-treated with either deoxynorjirmycin (dNM), a GII inhibitor (Kaushal et al., 1990), or australine, a glucosidase I (GI) specific inhibitor (Tropea et al., 1989). When dNM-treated lysates were added to CD45 immunoprecipitates devoid of GII, the ability of GII from that lysate to bind CD45 was inhibited (Figure 2-5). On the other hand, australine treated lysates showed no impairment of GII binding to CD45 (Figure 2-5). Further, treatment of GII bound CD45 immunoprecipitates with dNM, but not australine, resulted in a loss of GII binding (data not shown). These data indicate that in order to initiate the formation of the CD45-GII complex, an unoccupied GII active site is required. Another interesting point can be made regarding these data. In the 0.01mM dNM treated lysate, there was almost a complete inhibition of reconstitution, yet there was still 81% of the GII activity remaining in the lysate (Figure 2-5). Since all isoforms appear to have a similar enzymatic inhibition curve with dNM (data not shown), this data indicates that the binding of GIIα to CD45 is inhibited at a lower concentration of inhibitor than is

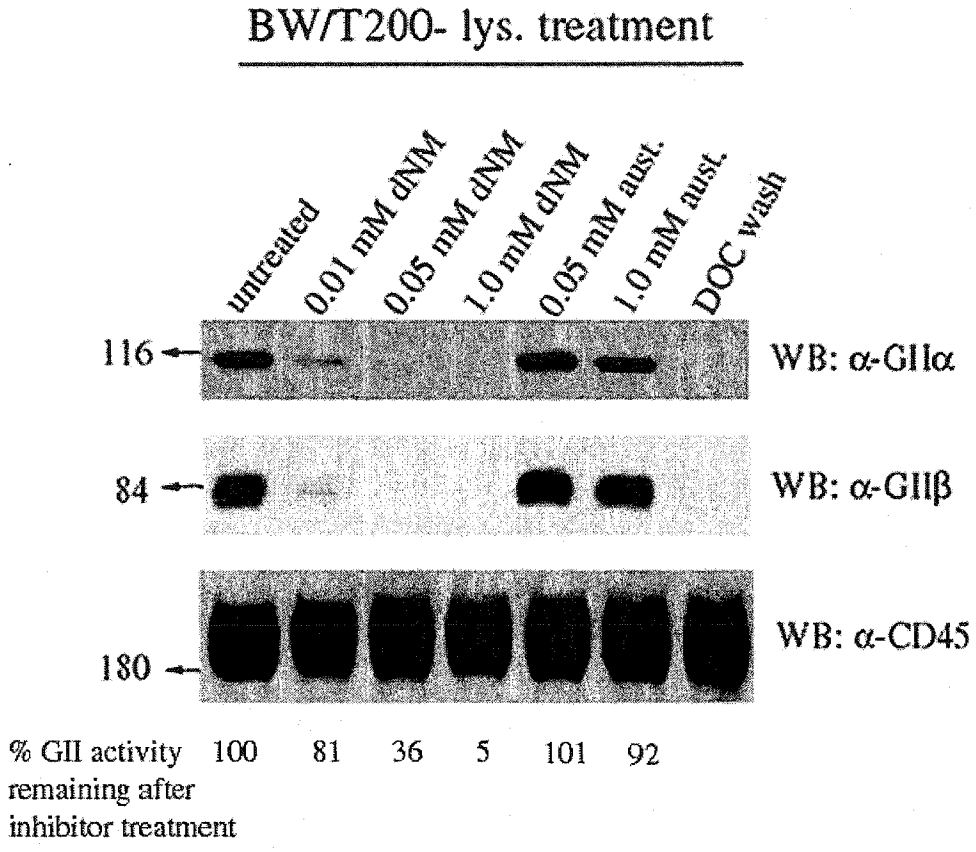


Figure 2-5: An unoccupied GIIα active site is required for the CD45-GII association.

I3/2 coupled beads were incubated with extracts from 2.5 $X10^7$ BW cells, and washed with 0.5% DOC, 20 mM Tris, pH 7.6 to remove bound GII. BW/T200 lysates were incubated with the indicated concentration of dNM, or australine (aust.) for 1 h. at room temperature. The inhibited lysates were mixed with the DOC washed CD45 immunoprecipitates for 2 h. at 4°C. The ability of GII from the inhibited lysate to re-associate with CD45 was assessed by Western blot analysis for GIIα (top panel) and GIIβ (middle panel). A sample of the inhibitor treated lysate was analyzed for GII activity by the colorimetric assay described in "Materials and Methods", and reported as a percentage of the untreated lysate.

the activity, which may reflect a differential affinity of GII for CD45 and substrate.

Removal of CD45 N-linked carbohydrate can disrupt the preformed CD45-GII association

In a previous report, we demonstrated that in order to reconstitute the association between CD45 and GII, Endo H sensitive carbohydrate on CD45 was required (Arendt and Ostergaard, 1997). In addition, in this report, we show that an unoccupied active site on GII is also required to reconstitute the association(Figure 2-5). We then asked if we could disrupt the pre-formed complex by removing the N-linked carbohydrate. To determine the contribution of N-linked carbohydrate to the association, we needed a buffering system that maintained the association in native form, yet allowed Endo F enzymatic activity. Therefore, we utilized a 50mM imidazole, pH 6.8 buffer and treated CD45 immunoprecipitates with Endo F. Treatment of CD45 immunoprecipitates with Endo F resulted in the loss of GII association, while mock treatment preserved the interaction (Figure 2-6). This data indicates that removal of the N-linked carbohydrate on both GII and CD45, while GII is bound, will disrupt the interaction between CD45 and GII. This experiment does not discriminate between the contribution of CD45 N-linked carbohydrate and the contribution of GIIα N-linked carbohydrate. Given the inability of GII to bind CD45 treated with Endo H, it is reasonable to suggest that immature N-linked carbohydrate on CD45 is important for the interaction (Arendt and Ostergaard, 1997). This does not

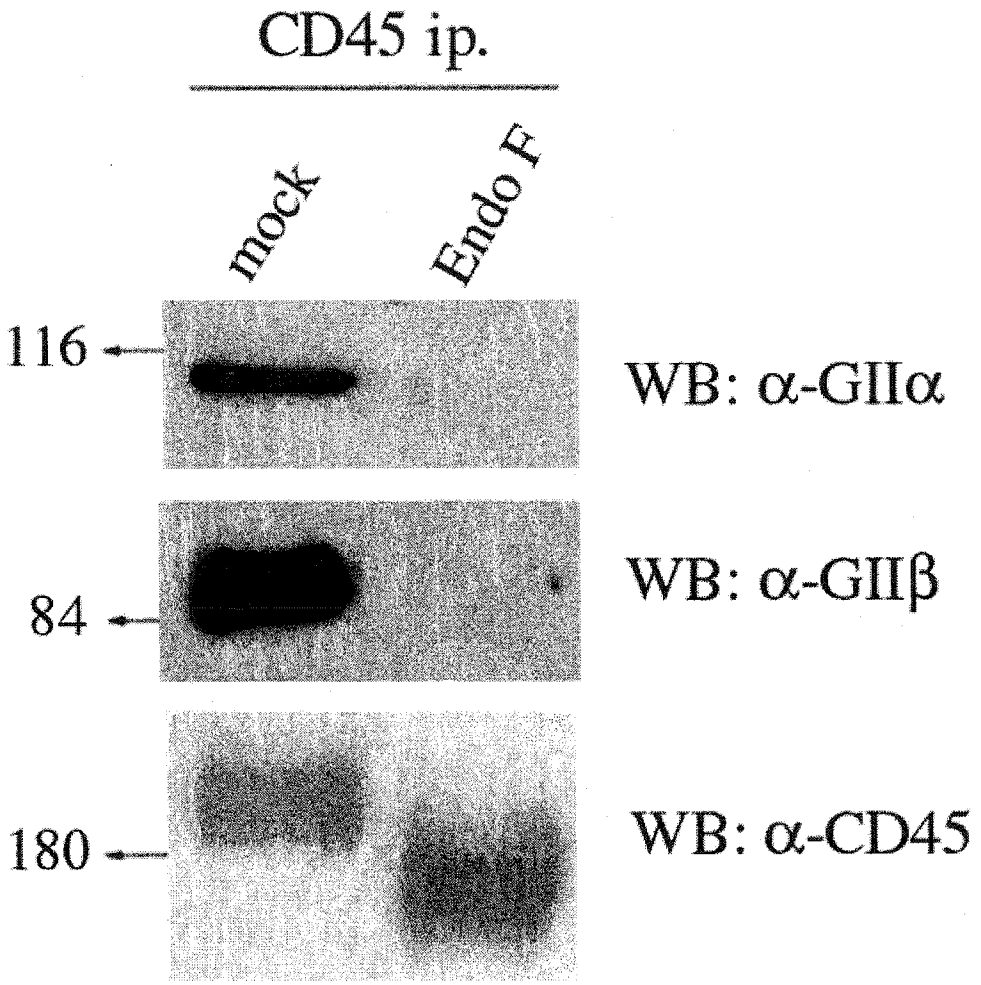


Figure 2-6: CD45 N-linked carbohydrate is required for the CD45-GII interaction.

CD45 immunoprecipitates from 2.5 X 10^7 BW post nuclear lysates were either untreated (mock), or treated with Endo F under native conditions for 16h. at 33°C. All immunoprecipitates were washed with lysis buffer prior to SDS-PAGE analysis. Immunoblotting was performed for GIIα (top panel), GIIβ (middle panel), and CD45 (lower panel).

preclude the possibility of GIIα N-linked carbohydrate contributing to the association.

The addition of mannose prevents reconstitution of the interaction between CD45 and GII

From the data presented in Figure 2-5 and Figure 2-6, the association between CD45 and GII requires both the active site of GII and the CD45 N-linked carbohydrate. These data suggest that the association between CD45 and GII is based on a lectin interaction. A report from Grinna et al. demonstrated that in order to obtain optimal GII enzymatic activity, branched mannose residues are required (Grinna and Robbins, 1980). It is therefore possible that GII also possess a mannose binding activity. To address the issue of whether or not GII does indeed possess a mannose binding function, we performed a reconstitution assay where either glucose or mannose monosaccharides were included in the reconstitution assay. While the addition of 10mM glucose caused little change in the ability of GII to bind CD45, the addition of 10mM mannose significantly inhibited the binding of GII to CD45 (Figure 2-7). As well, the addition of either glucose or mannose had no effect on the enzymatic activity of GII (data not shown). Therefore, these data indicate that the presence of mannose can inhibit the association of CD45 and GII, and suggest that GII does in fact possess mannose binding activity.

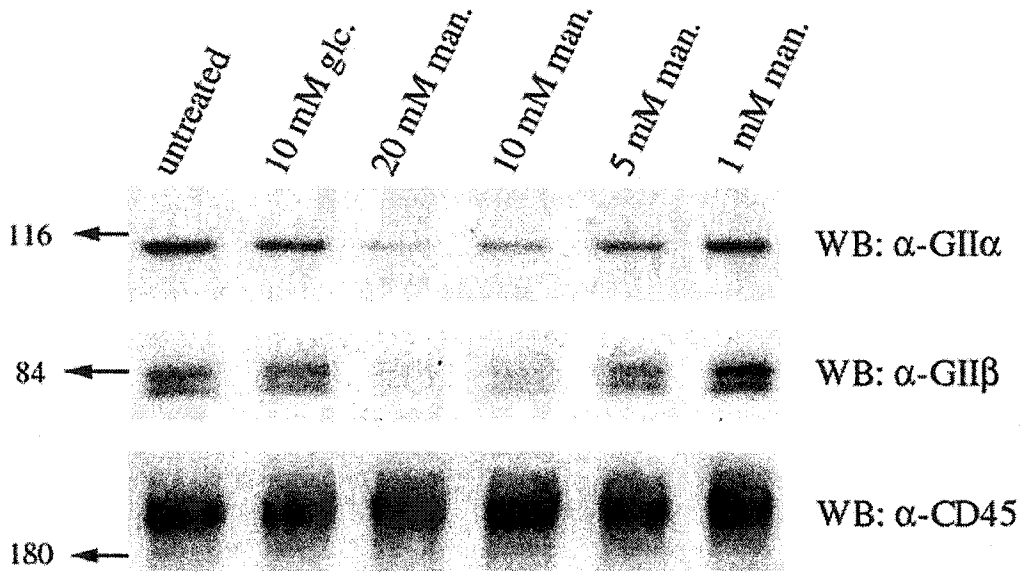


Figure 2-7: The addition of mannose inhibits the re-association of GII with CD45.

I3/2 coupled beads were incubated with extracts from 2.5 $X10^7$ BW cells, and washed with 0.5% DOC, 20 mM Tris, pH 7.6 to remove bound GII. BW/T200$^-$ lysates containing the indicated concentration of monosaccharide was incubated with the washed CD45 immunoprecipitates for 2 h. at 4°C. The beads were then washed three times with lysis buffer prior to analysis by SDS-PAGE. Western blotting was performed for GIIα (top panel), GIIβ (middle panel), and CD45 (lower panel). No inhibition of GII enzymatic activity by the addition of the monosaccharides was observed.

D. DISCUSSION

In a previous report, we described the physical association between the protein tyrosine phosphatase CD45, and the resident ER protein glucosidase II (Arendt and Ostergaard, 1997). This association appears to be extremely stable, and has the ability to be reconstituted *in vitro* under the appropriate conditions (Arendt and Ostergaard, 1997). As well, in order to reconstitute the association between CD45 and GII, there is a dependence on Endo H sensitive carbohydrate on CD45 which suggests that GII is binding via a lectin-like interaction (Arendt and Ostergaard, 1997). Our present studies sought to further elucidate the biochemical basis for the association between CD45 and GII in an attempt to understand the potential implications of this association on CD45 biology.

In this report, we show that CD45 appears to strongly interact with a minor, higher molecular weight form of GIIα. Through our transfection studies, we found that only the Box A1+/A2+ and the A1+/A2- isoforms are capable of binding CD45. Therefore, the alternatively spliced sequence A1 is necessary for the interaction of GIIα with CD45, while Box A2 is dispensable for binding. This finding is consistent with our amino acid sequencing data obtained from the α-subunit purified on the basis of its association with CD45, which demonstrated that the first alternatively spliced sequence was present (Arendt and Ostergaard, 1997). Data presented in Figure 2-3 suggests that the higher molecular weight CD45-interacting GIIα is only a relatively minor subset of total GIIα found in the BW cell line. Consistent with this, RT- PCR analysis of a number of different

cell lines indicated that transcripts containing the first alternatively spliced exon were significantly less abundant than the transcripts which lacked this sequence (Arendt et al., 1999). The fact that CD45 only associates with a minor population of GIIα indicates that there is a high degree of specificity to the interaction. This association is not taking place simply because CD45 is an abundant, carbohydrate containing protein and GII is an enzyme which is capable of modifying this carbohydrate. In further support of the specificity of this interaction, we have never been able to detect an association of GII with other abundant N-linked glycan containing proteins such as Class I MHC (Arendt and Ostergaard, 1997), LFA-1, or CD44 (unpublished results). Taken together, these results suggest that CD45 specifically associates with a minor, Box A1 containing, subset of GII.

Because of the requirement for the first alternatively spliced sequence in GIIα, we posit that Box A1 functions to stabilize the association between CD45 and GII. Based on the primary sequence, Box A1 is not adjacent to the active site (Arendt and Ostergaard, 1997; Trombetta et al., 1996), however the tertiary structure of the protein may place this sequence in close proximity to the active site, thereby modifying the enzymatic properties of GII resulting in binding but not glucose cleavage. We find this possibility unlikely since all isoforms contain equal activity (data not shown). Furthermore, CD45-associated GII is fully active (data not shown). We find it more likely that Box A1 could provide GIIα with a novel binding activity or stabilize an existing binding activity. Interestingly, we show that the addition of mannose can inhibit the ability of GII to associate with CD45. Therefore, Box A1-containing GIIα not only possess a glucose specific

catalytic activity, but also displays a mannose specific lectin activity. This lectin activity is not of high enough affinity to allow GIIα to bind to branched mannose oligosaccharides alone as incubation of lysates from any one of the GIIα transfectants with mannan-agarose beads does not result in the binding GIIα to the beads (data not shown). From these data, we believe that both the active site and the lectin activity of Box A1 containing GIIα are required for the stable binding of GII to the N-linked carbohydrate on CD45.

The finding of lectin activity in addition to the enzymatic activity within a carbohydrate processing enzyme, though unusual, is not unprecedented. Both structural and biochemical evidence exists in the literature for this type of enzyme organization. The interplay between enzymatic activity and the lectin binding domain can be quite complex and unpredictable. For example, mutations in the ricin-like lectin motif of UDP-*N*-acetyl-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase do not affect the activity of the enzyme (Hagen et al., 1999). However, in the case of cell surface β 1,4 galactosyltransferase, the galactosyl transferase activity depends on the ability of its lectin domain to bind a substrate, laminin (Begovac et al., 1994). Further, the physical state of the enzyme can alter its substrate binding and affect enzymatic activity yet, maintain its lectin binding capabilities. This is the case for β-galactoside α2,6-sialyltransferase where dimerization reduces donor substrate binding, but does not affect galactose binding (Ma and Colley, 1996). Therefore, it is conceivable that by utilizing both the GIIα active site and the lectin binding domain, there may be modulation of either one or both of the activities causing the stable association of

GII with CD45. For example, simultaneous engagement of the active site and the lectin domain may result in a stable association.

It is reasonable to deduce that the association between CD45 and GII may have significant biological implications for CD45. GII may act as a molecular chaperone that could help to retain CD45 within the ER longer than other proteins to allow for further post-translational modifications, the interaction with additional proteins, or regulated cell surface expression of CD45. The association with GII may also affect the enzymatic activity of CD45. While in the ER, reduced phosphatase activity could prevent the inappropriate dephosphorylation of proteins. In other compartments reduced activity may alter the signalling thresholds for cellular functions. As well, the stable interaction of GII with CD45 could perhaps lead to additional modifications on the carbohydrate of CD45, which could regulate CD45 function. Determining where the association between CD45 and GII occurs will be of interest. Our preliminary data suggests that the association does not take place on the cell surface, and since GII is an ER protein, we believe the interaction is at least initiated within the ER, thus supporting a function related to ER localization.

To date, most of the potential ligands and binding proteins of CD45 have been shown to interact with the carbohydrate on CD45, the biological significance of which is elusive. Our data suggests that GII is another example of a lectin that binds CD45. It is possible that CD45 does not have just one single ligand, rather there are multiple ligands that bind to CD45 carbohydrate and regulate the function of CD45.

We have begun to understand the biochemical basis for the GII-CD45 association, and this information has provided us with insight into the possible biological functions related to the interaction. These biological functions may help to identify the role that the carbohydrate and extracellular region CD45 plays in lymphocyte biology. As well, by understanding how GII interacts with its substrates, we hope to gain information into the role GII plays in the quality control system within the ER.

Chapter 3: Developmentally Regulated Changes in Glucosidase II Association with, and Carbohydrate Content of, the Protein Tyrosine Phosphatase CD45

A version of this chapter has been published. Baldwin T.A and Ostergaard H.L, The Journal of Immunology, volume 167, p. 3829-3835, 2001.

A. INTRODUCTION

The protein tyrosine phosphatase CD45 is an abundant, heavily glycosylated, type I integral membrane protein. CD45 is expressed on all nucleated cells of hematopoietic origin, and constitutes approximately 10% of all cell surface protein (Thomas, 1989). The tandem intracellular phosphatase domains of CD45 are responsible for the regulation of the src-family kinase members Lck and Fyn through dephosphorylation of the negative regulatory tyrosine residue found in the carboxy-terminus of the kinase. This regulation of Lck and Fyn has been demonstrated to be important for both T cell activation and thymocyte development using both CD45-deficient cell lines as well as gene targeted mice (Ashwell and D'Oro, 1999; Thomas and Brown, 1999). The external domain of CD45 is large, and heterogeneous with respect to both size and carbohydrate content. There are three alternative splice exons, 4-6, in the extracellular region whose usage is variable depending on the developmental stage of the cell (Trowbridge and Thomas, 1994). Within the alternatively spliced exons are numerous sites for O-linked carbohydrate attachment. As well, there is an abundance of N-linked carbohydrate sites mostly found outside of the alternatively spliced exons (Okumura et al., 1996). A biological role for the external domain remains elusive, however, addition of monoclonal antibodies specific for the extracellular domain of CD45 in fetal thymic organ culture disrupts normal thymic selection events (Benveniste et al., 1994). Therefore, it is

possible that the external domain of CD45 is involved in the process of thymocyte development.

Carbohydrate additions to proteins are evolutionarily conserved and extremely important for a number of different processes such as protein folding, transport, and ligand binding. The processing of N-linked carbohydrate is quite complex, tightly controlled and ultimately decides the fate of that particular glycoprotein, whether it is appropriate transport and function or degradation (Kopito, 1997; Wiertz et al., 1996). The machinery involved in carbohydrate processing lies within the ER and Golgi apparatus. The ER machinery is primarily responsible for ensuring proper folding through the actions of enzymes such as glucosidase I and II (Kearse et al., 1994; Keller et al., 1998; Moore and Spiro, 1993); calnexin and calreticulin (Helenius, 1994; Ou et al., 1993); and UDP-glucose glucosyl transferase (Parodi, 2000b). The carbohydrate modifying enzymes within the Golgi stacks ultimately shape the final structure of the carbohydrate through various cleavages and additions by enzymes such as the mannosidases (Kornfeld and Kornfeld, 1985), and various glycosyl-transferases. Numerous examples exist where carbohydrate is extremely important for biological function of a protein, as in the case of the selectins (Bevilacqua, 1993), the DC-SIGN and ICAM-2 (Geijtenbeek et al., 2000b), or ICAM-3 (Geijtenbeek et al., 2000a) interaction, and the recognition of bacterial antigens by the complement system (Drickamer and Taylor, 1993). For the most part, these examples feature a lectin binding to its ligand via the carbohydrate displayed by

the ligand. Therefore, in order to achieve proper biological outcomes, the carbohydrate processing by the cell expressing the ligand is extremely important.

We have previously demonstrated that CD45 and GII associate by way of a mannose-dependent lectin interaction (Baldwin et al., 2000). Here we show that the carbohydrate structure found on CD45 changes as immature thymocytes mature into T cells. This change in carbohydrate structure probably involves an intrinsic change in the carbohydrate processing machinery of the cell. An example of such a change in CD45 carbohydrate during development is the induction of the CT1 epitope after stimulation through the pre-TCR (Reed et al., 1998). The presence of CD45 which can be bound by MBP or related lectins on immature cells could be involved in the process of T cell maturation.

B. MATERIALS AND METHODS

Cell Lines and Antibody Reagents

The mouse T-lymphoma cells SAKRTLS.12.1 (SAKR), BW5147 (BW) and their CD45-deficient derivatives (SAKR/T200- and BW/T200-) were maintained as described previously (Arendt and Ostergaard, 1995). Thymocytes and splenocytes were isolated by gentle teasing of the thymus or spleen from C57B/6 mice and used immediately after isolation. Cloned CTL AB.1 was maintained as previously described (Blakely et al., 1987). Monoclonal antibody I3/2.3 that recognizes a pan-specific determinant within the CD45 extracellular domain was kindly provided by Dr. Ian Trowbridge (Salk Institute, La Jolla, CA). Fluorochrome-coupled anti-CD4 and anti-CD8 monoclonal antibodies RM4-4 and 53-6.7 respectively were purchased from PharMingen (Mississauga, ON). The hybridoma secreting the Class I MHC specific monoclonal antibody M1/42.3.9.8 was purchased from ATCC (Rockville, MD). Rabbit antisera H2 and J37, specific to GIIβ and the intracellular region of CD45 respectively, were previously described (Arendt and Ostergaard, 1997; Baldwin et al., 2000). Rabbit antiserum L177 was generated to a peptide fragment corresponding to the alternatively spliced Box A1 region of GIIα coupled to KLH (Arendt et al., 1999).

Cell Surface Biotinylation, Cell lysis, Immunoprecipitation, MBP pull-down Assays, Reconstitution Assay and Endoglycosidase Treatment.

Cell surface biotinylation was performed with 50 μL of 10 mM sulfo-NHS biotin (Pierce, Rockford, IL) per 5 X 10^7 cells/ml in PBS for 10 min. at room temperature. The reaction was quenched by washing cells two times in PBS containing 5 mM glycine. All cells were lysed at a density of 5 X 10^7/mL in 0.5% Nonidet P-40 (Pierce, Rockford, IL), TBS buffer, and incubated on ice for 20 min. Post-nuclear supernatants were incubated for 1-2 h. with I3/2 coupled Sepharose 4B at 4°C with rotation. Immunoprecipitates were washed three times with lysis buffer prior to the addition of reducing sample buffer and boiling. For MBP pull-down assays, cell surface biotinylated lysates were made to 1.25 M NaCl, 20 mM $CaCl_2$, +/- 5 mM glucose or mannose prior to addition of immobilized MBP (Pierce, Rockford, IL) and then incubated for 4 h. at 4°C with rotation. Pull-downs were washed three times with lysis buffer containing 1.25 M NaCl, and 20 mM $CaCl_2$. For elution of MBP bound proteins, immobilized MBP was incubated with lysis buffer containing 1.25 M NaCl, and 5 mM EDTA for 3 X 10 min. Eluted proteins were then either subjected to I3/2 immunoprecipitation or streptavidin pull-down for examining either bound CD45 or total bound cell surface proteins respectively. Reconstitution assays were performed as previously described (Baldwin et al., 2000). Briefly, CD45 devoid of GII was incubated with a CD45-deficient, GII containing lysate for 1 hour at 4°C, followed by washing three times with lysis buffer. Immunoprecipitates were treated with

Endoglycosidase H and F (Calbiochem, La Jolla, CA) in PBS containing 0.1% SDS, 1% 2-ME for 16 h. at 33°C.

Polyacrylamide Gel Electrophoresis and Immunoblotting

Proteins were resolved on 7.5% polyacrylamide gels and transferred to polyvinylidene difluoride-Immobilon (Millipore, Bedford, MA) as described previously (Arendt et al., 1995). Western blot analysis was carried out with the indicated antiserum, followed by protein A^{hrp} or with $streptavidin^{hrp}$ (Pierce, Rockford, IL) and visualized by enhanced chemiluminescence (PerkinElmer Life Sciences)

Serum MBP purification

Rabbit serum MBP was purified as previously described (Kawasaki et al., 1983). Briefly, whole rabbit serum proteins (Sigma, St. Louis, MO) were precipitated with PEG 6000 and resuspended in TBS containing 1.25 M NaCl, 50 mM $CaCl_2$ (MBP binding buffer) and applied to an equilibrated 10 mL mannan-agarose (Sigma, St. Louis, MO) column at 4°C. The column was washed with twenty volumes of MBP binding buffer before eluting bound proteins with TBS containing 1.25 M NaCl, 5 mM EDTA (MBP elution buffer). Fractions were collected and analyzed by SDS-PAGE and silver staining for presence of MBP. Fractions containing MBP were pooled, made to 50 mM $CaCl_2$ and re-applied to a 2 mL mannan-agarose column. The column was washed with twenty volumes of MBP binding buffer before elution. Fractions were analyzed for MBP by silver

staining and concentrated by centrifugal filtration. Concentrated MBP was biotinylated as per Pierce biotinylation kit (Pierce, Rockford, IL). Biotinylated MBP was detected by Western blotting using streptavidin^{hrp} as described above.

GII inhibitor treatment

AB.1 cells were treated with 2mM N-methyl deoxynorjirmycin (NMdNM) (Oxford Glycosystems, Wakefield, MO) for 20 hours. Cells were harvested, cell surface biotinylated and lysed as described above. Cell lysates were examined for GII enzymatic activity as previously described using the colorimetric p-nitrophenyl α-D glucopyranoside (Sigma, St. Louis, MO).

FACS analysis

1 X 10^6 cells were incubated with either 10 μg/mL biotinylated MBP, or 5 ug/mL of fluorochrome-conjugated anti-CD4 or anti-CD8 for 20 min. on ice in PBS containing 1 mM $CaCl_2$. For MBP detection, the cells were incubated with streptavidin^{FITC} for an additional 20 min. on ice in PBS containing 1 mM $CaCl_2$. All cells were then fixed in 1% paraformaldehyde prior to analysis. Statements regarding change in expression levels are quantified by the relative mean fluorescent intensities derived from the Cell Quest software.

Generation of Con A thymocyte blasts

Freshly isolated C57B/6 thymocytes were incubated with 2 μg/mL Concanavalin A (Amersham Pharmacia, Baie d'Urfé, Que.) and IL-2 for three days. The cultures were then split and allowed to proliferate for four days in the presence of IL-2. Thymocyte blasts were then harvested and either lysed or used for FACS analysis as above.

Purification of Splenic T cells

Freshly isolated C57B/6 splenocytes were passed over a mouse T cell immunocolumn (Cedarlane, Hornsby, ON) which purifies T cells based on negative selection. Splenic T cell preparations were greater than 80% T cells as determined by FACS analysis for TCRβ.

C. RESULTS

Association between CD45 and GII only occurs in immature cells

To gain additional insight into the possible biological role the association between CD45 and GII plays, we examined whether the association occurred in all cell types, or in a subset of cells. CD45 immunoprecipitates from lysates of cells of different developmental stages was performed and analyzed by western blot for the presence of GII. GII was only co-immunoprecipitated with CD45 in lysates from BW5147 and thymocytes, while virtually no GII was detected in CD45 immunoprecipitates from a CTL clone, AB.1, or splenic T cells (Figure 3-1). A small amount of GII was seen in CD45 immunoprecipitates from the purified splenic T cells, but only after over-exposure of the western blot (data not shown). This data suggests that the association between CD45 and GII is developmentally regulated and only occurs in immature cells.

MBP binds cell surface CD45 from immature cells only

Because the association between CD45 and GII is based on a lectin interaction, requires the active site of GIIα and can be inhibited by mannose (Baldwin et al., 2000), we wished to examine the mannose content of the carbohydrate on CD45 from various cell types using another lectin. We chose to use MBP as it is specific for mannose, and has been previously shown to bind CD45 (Uemura et al., 1996). Lysates from cell surface biotinylated SAKR, a T-

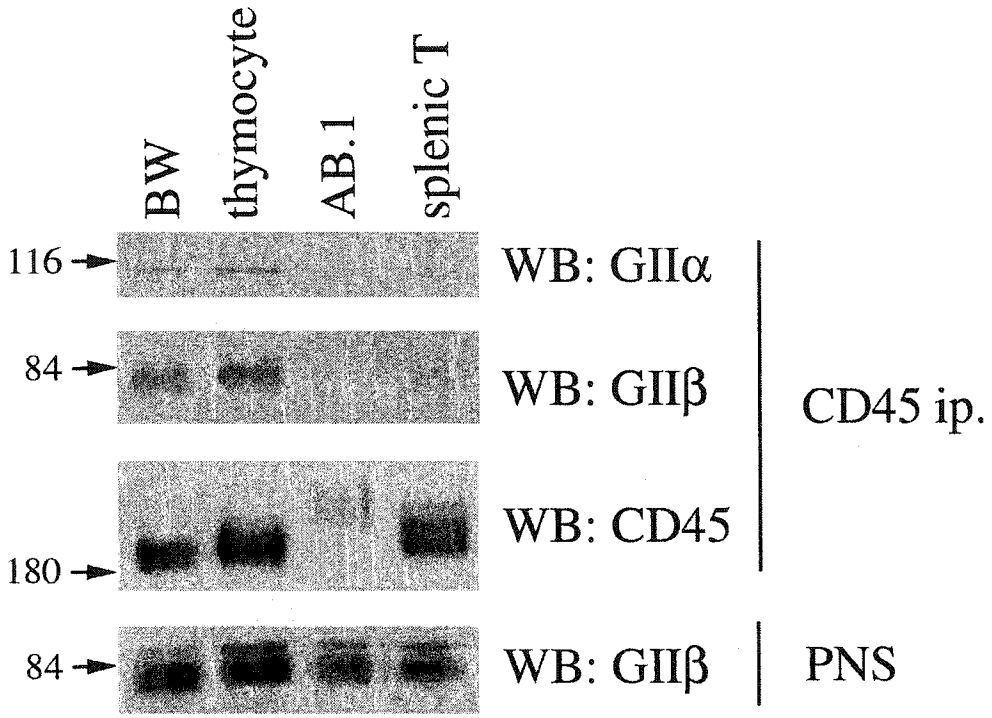


Figure 3-1: CD45 associates with GII only in immature cells.

CD45 immunoprecipitates from the indicated lysates were resolved by SDS-PAGE analysis and western blotted for GIIα using L177 antisera (1st panel), GIIβ (2nd panel) and CD45 (3rd panel). Post-nuclear supernatant (PNS) from each of the cell lysates was immunoblotted for GIIβ as a control (4th panel).

lymphoma, thymocytes, and AB.1 were incubated with immobilized MBP in the presence of Ca^{2+} to recover all proteins capable of binding MBP. The MBP beads were washed, bound proteins eluted with 5mM EDTA, and then captured with anti-CD45 coated beads. The captured CD45 was resolved by SDS-PAGE and detected by western blotting. CD45 immunoprecipitates from each of the cell types was also performed. Using streptavidin to specifically detect cell surface CD45 illustrated that MBP can recognize cell surface CD45 in immature cells such as SAKR and thymocytes, but not from mature cells such as AB.1 (Figure 3-2a). Examining total MBP bound CD45 reveals that there are two different forms of CD45 recognized by MBP, only one of which is readily cell surface biotinylated (Figure 3-2a). These two different forms are likely to be different glycoforms of CD45RO as western blotting for CD45RB showed only minor amounts of CD45RB brought down by MBP, and the CD45RB has a higher relative molecular weight than did the upper most band in the MBP bound CD45 immunoblot (data not shown).

Using FACS analysis as another method to determine cell surface binding by MBP, we see that MBP recognizes a cell surface protein(s) on SAKR, BW (data not shown) and thymocytes, but not AB.1 (Figure 3-2b). This MBP binding is Ca^{2+} dependent and sugar specific as the binding is inhibited by the inclusion of EDTA or the addition mannose (data not shown). Interestingly, CD45-deficient variants of SAKR (Figure 3-2c) and BW (data not shown) both bind MBP, but at approximately 50% the level seen in wildtype cells. Consistent with these data is the observation that in MBP pull-downs of cell surface biotinylated lysates, we

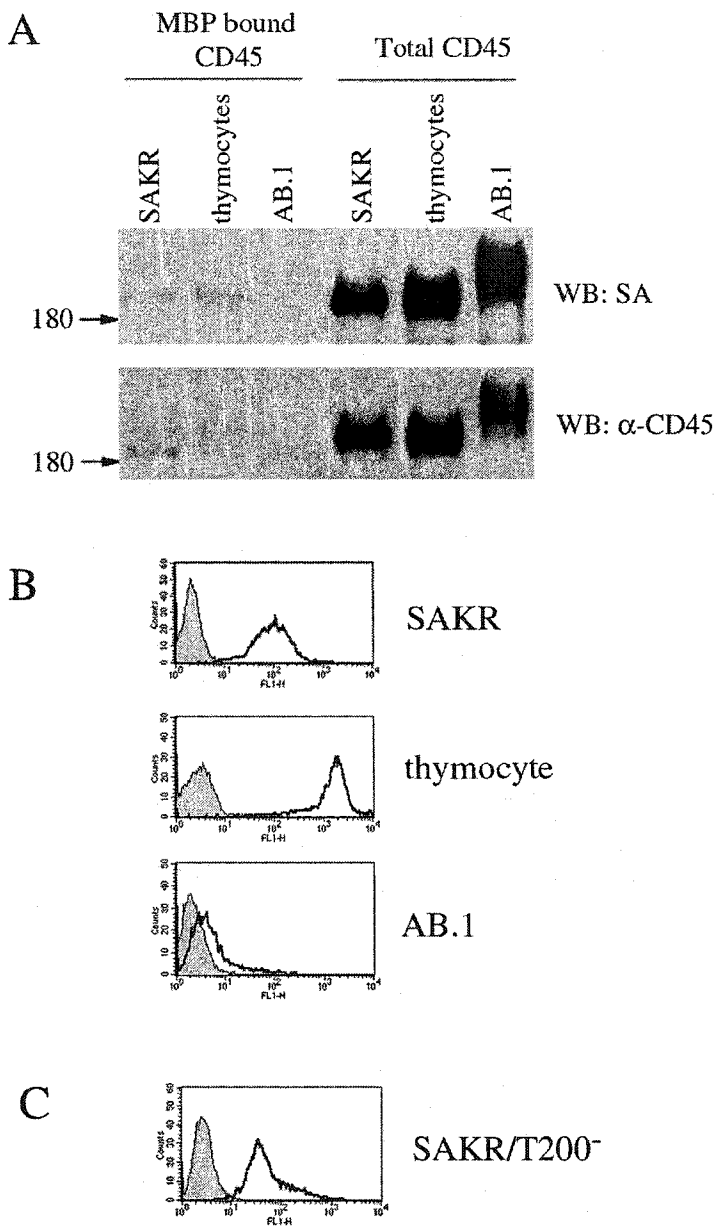


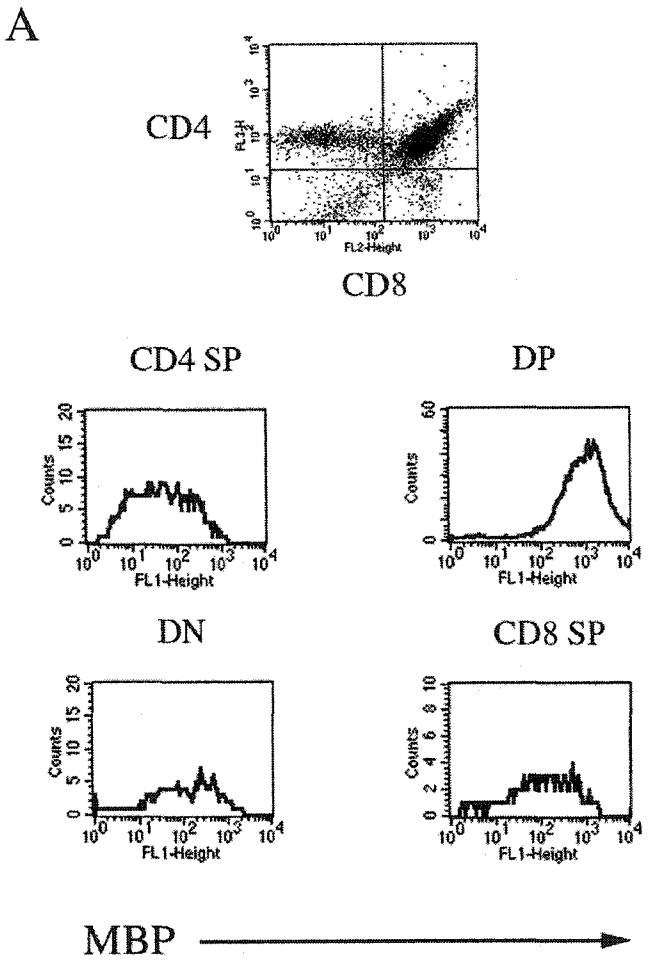
Figure 3-2: Cell surface CD45 is recognized by MBP in immature cells.

A. MBP bound CD45 and total CD45 from cell surface biotinylated lysates were resolved by SDS-PAGE and western blotted with streptavidin (top panel) and anti-CD45 antibodies (bottom panel). B and C. 1 X 10^6 cells were stained with 10 ug/mL biotinylated MBP for 20 min. on ice followed by streptavidin^{FITC}. Shaded area represents staining with streptavidin^{FITC} alone.

see that CD45 is the predominant MBP-bound protein, however it is certainly not the only protein (data not shown) as previously reported (Uemura et al., 1996). These data suggest that cell surface CD45 from immature cells is the major glycoprotein recognized by MBP, but there appears to be other minor cell surface glycoproteins that can be recognized by MBP.

Developmental differences in the amount of MBP binding and CD45-GII association

We have demonstrated that the ability of MBP to bind CD45 and the amount of GII associated with CD45 correlates with the maturation state of the cell. Next, we wanted to examine these changes more closely using *ex vivo* thymocytes. *Ex vivo* thymocyte populations contain a mixture of immature double negative and double positive cells, as well as mature single positive cells. By using three-color flow cytometric analysis one can separate the four different developmental populations of bulk thymocytes and compare the level of MBP binding to each population. Three-color analysis of bulk thymocytes demonstrated the there is a two to three fold higher level of MBP binding on immature double positive thymocytes compared with either immature double negative or mature single positive thymocytes (Figure 3-3a). As an alternative approach to compare developmental differences with respect to MBP binding and CD45-GII association, Con A stimulation of thymocytes was performed. Stimulation of thymocytes with Con A leads to an increase in the percentage of single positive thymocytes and a corresponding decrease in the percentage of



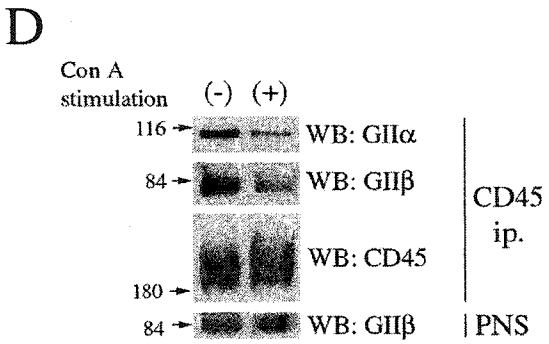


Figure 3-3: An increase in MBP recognition and CD45-GII association is seen in double positive thymocytes compared to single positives.

A. 1 X 10^6 freshly isolated thymocytes were triple stained with biotinylated MBP, $CD4^{CyChrome}$ and $CD8^{PE}$ followed by $streptavidin^{FITC}$. Thymocyte sub-populations were gated and analyzed for MBP binding. B. 1 X 10^6 bulk or Con A-stimulated thymocytes were double stained with 1 μg anti-$CD4^{FITC}$ and anti-$CD8^{PE}$ followed by FACS analysis. C. 1 X 10^6 bulk or Con A stimulated thymocytes were stained with biotinylated MBP followed by $streptavidin^{FITC}$. Shaded area represents staining with $streptavidin^{FITC}$ alone. D. CD45 immunoprecipitates from unstimulated bulk thymocytes (-) or Con A stimulated (+) thymocytes were prepared and analyzed for the presence of associated GII by immunoblotting for GIIα (1^{st} panel), GIIβ (2^{nd} panel) and CD45 (3^{rd} panel). Post-nuclear supernatant (PNS) was assessed for relative amounts of protein used in the initial immunoprecipitates by western blotting for GIIβ (4^{th} panel).

double positive cells (Figure 3-3b). Comparing the bulk thymocytes and Con A thymocyte blasts for MBP binding in FACS analysis, there is approximately a two-fold decrease in MBP binding by the Con A thymocyte blasts (Figure 3-3c). Co-ordinate with the decrease in MBP binding, we see an approximate two-fold decrease in the amount of GII bound to CD45 in the Con A thymocyte blasts (Figure 3-3d). These data suggest that there is an overall change in the mannose content of CD45 as double positive thymocytes mature into single positive thymocytes.

The pool of CD45 recognized by MBP is also recognized by GII

To this point a correlation existed between the cell types where the CD45-GII association occurred and the cell types where MBP could recognize CD45. We then sought to determine if perhaps both MBP and GII could recognize the same pool of CD45. To this end we performed a reconstitution assay where MBP-purified CD45 from BW5147 was incubated with a lysate of BW/T200$^-$ cells, the CD45-deficient variant of BW5147. The bound proteins were separated by SDS-PAGE and visualized by western blotting with GII specific antibodies. GII from the BW/T200$^-$ was able to bind MBP purified CD45, which suggests that the same pool of CD45 is capable of associating with both MBP and GII (Figure 3-4).

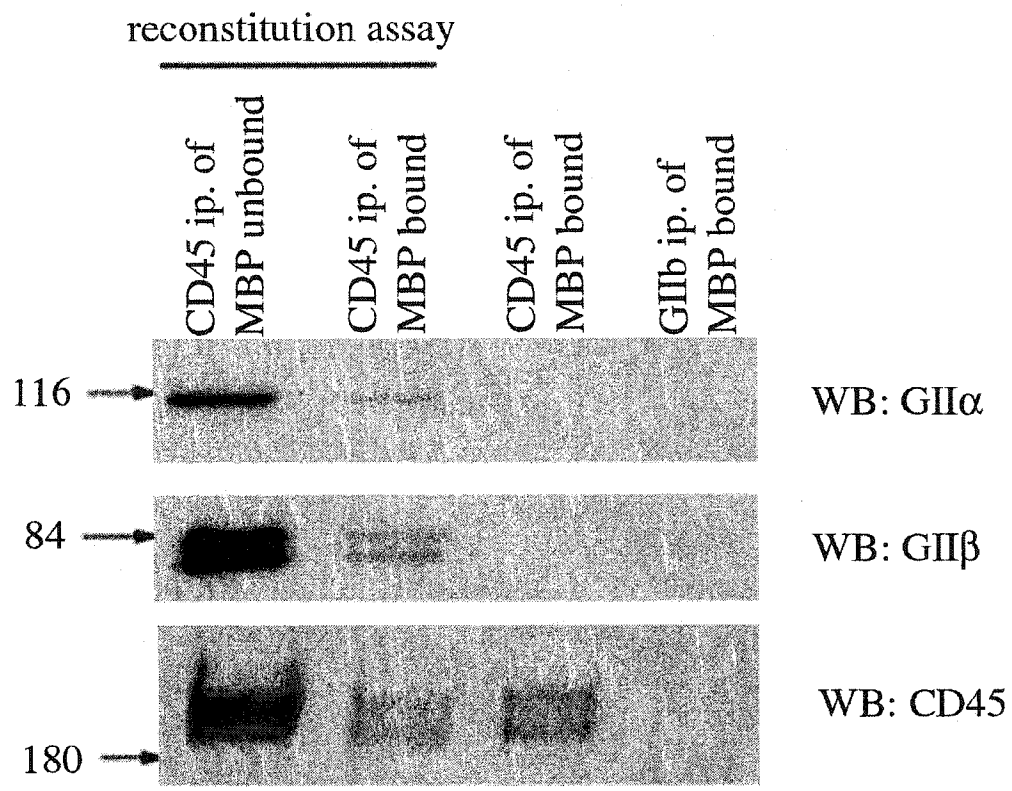


Figure 3-4: The same pool of CD45 is recognized by both MBP and GII. Immunoprecipitated CD45 purified by immobilized MBP (MBP-bound), or the CD45 remaining in the PNS after MBP purification (MBP-unbound) was subjected to a GII reconstitution assay. A CD45-deficient lysate was incubated with the two pools of CD45 isolated above to determine whether or not the CD45 bound by MBP could also bind GII. Western blot analysis was performed for GIIα (upper panel), GIIβ (middle panel) and CD45 (lower panel) to detected the indicated protein.

Cell surface CD45 from both immature and mature cells contain Endo H sensitive carbohydrate

Because of the ability of both GII and MBP to recognize CD45 on immature cells,and our previous data demonstrating that GII only associates with Endo H sensitive carbohydrate, we postulated that immature cells express higher levels of Endo H sensitive carbohydrate on the cell surface than mature cells. To test this hypothesis, we performed a CD45 immunoprecipitate from cell surface biotinylated lysates from various cells. The bound proteins were treated with either Endo H or Endo F under reducing and denaturing conditions, resolved by SDS-PAGE analyzed by blotting with streptavidin. Upon treatment of cell surface CD45 from either BW, thymocytes, AB.1, or splenic T cells with Endo H, there is a shift in the mobility of CD45 owing to the presence of immature high mannose or hybrid type carbohydrate (Figure 3-5). This shift is not as dramatic as treatment with Endo F which suggests there is a mixture of both Endo H sensitive and resistant carbohydrate. In examining control glycoproteins such as Class I MHC, CD44 and LFA-1, we see virtually no Endo H sensitive carbohydrate (Figure 3-5 and data not shown). Therefore, cell surface CD45 from all cells examined contains a mixture of both immature and mature carbohydrate while other glycoproteins examined contain only mature carbohydrate. While the high level of Endo H sensitive carbohydrate on CD45 expressed on the cell surface is surprising, it does not appear to be the basis for the preferential GII and MBP binding to CD45 from the immature cells.

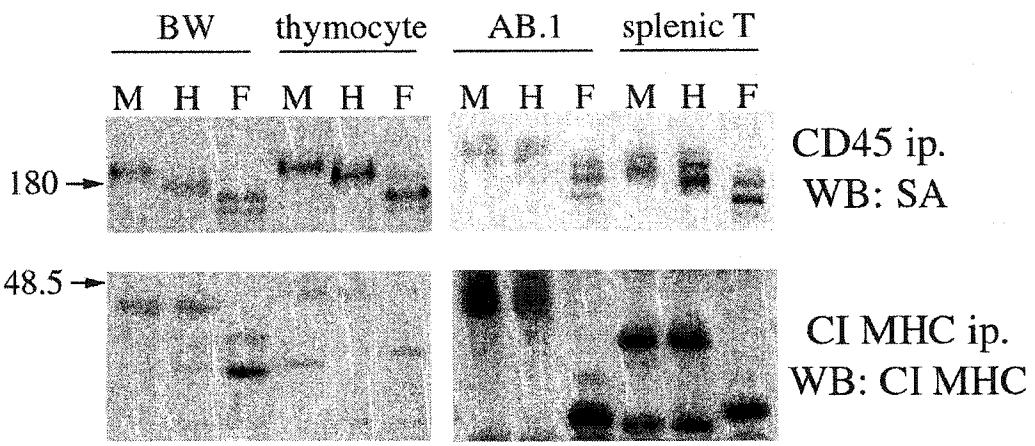


Figure 3-5: Cell surface CD45 from both immature and mature T cells contains Endo H sensitive carbohydrate.

CD45 immunoprecipitates were prepared using cell surface biotinylated lysates from the indicated cell types. Mock (M), Endoglycosidase H (H) or Endoglycosidase F (F) treatment of the immunoprecipitates was completed and the cell surface CD45 was detected by western blotting with streptavidin (upper panel). Class I immunoprecipitates were also digested with Endoglycosidase H or F and immunoblotted with a polyclonal anti-Class I MHC antiserum (lower panel).

MBP recognizes two distinct glycoforms of CD45

In a recent report by Hansen et al. (Hansen et al., 2000) it was shown that MBP from a number of different species comes in two different forms, denoted MBP-A and MBP-C. The difference between the two forms lies in the amino-terminus where MBP-A has three cysteines while MBP-C has only two. This difference leads to MBP-A being capable of forming hexamers while MBP-C is found predominantly in dimers or trimers (Hansen et al., 2000). As well, it was demonstrated through sugar competition, that MBP-A can be inhibited from binding mannan with a significantly lower concentration of glucose that can MBP-C, while the concentration of mannose required to compete off either form of MBP from mannan is comparable (Hansen et al., 2000). In examining the binding of CD45 by MBP with either glucose or mannose as competitors, the presence of two different glycoforms of CD45 bound by MBP was revealed (Figure 3-6). Cell surface biotinylated lysates from SAKR, BW5147, and thymocytes were subjected to an MBP pull-down assay without any competitor, or in the presence of either glucose or mannose. We then compared the cell-surface versus the total CD45 bound to MBP in each case. In the SAKR and thymocyte lysates, only the higher molecular weight glycoform of CD45 is readily surface biotinylated, and that form disappears with the inclusion of glucose or mannose (Figure 3-6). The lower molecular weight glycoform is only slightly sensitive to glucose, but is entirely competed from binding by mannose (Figure 3-6). A similar pattern is seen with the BW5147 cells, except the upper

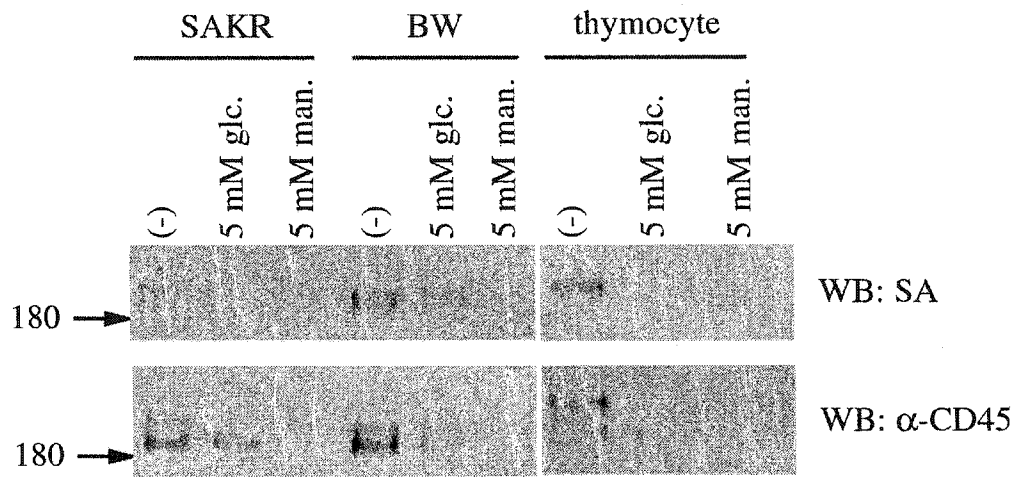


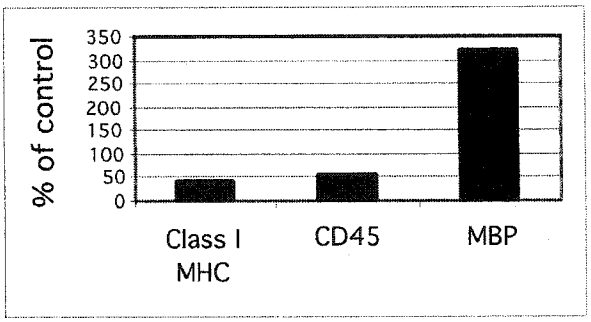
Figure 3-6: Two distinct glycoforms of CD45 are recognized by MBP.
Cell surface biotinylated lysates were incubated with MBP-coupled beads with or without 5 mM glucose (glc.) or mannose (man.). Bound proteins were eluted with EDTA and CD45 immunoprecipitates were prepared from the eluted material. Streptavidin (top panel) or anti-CD45 (lower panel) western blotting was used to detect cell surface or total CD45 respectively.

form is more resistant to competition with glucose (Figure 3-6). These data suggest that there are two glycoforms of CD45 bound by MBP, each with differential sensitivity to glucose competition.

In vivo glucosidase II inhibition results in increased surface expression of MBP ligands, specifically CD45

A strong correlation exists between cell types where CD45 and GII associate and where cell surface CD45 is bound by MBP. To determine whether or not GII activity directly influences the amount of cell surface CD45 bound by MBP, we treated AB.1 cells, which do not normally have endogenous MBP ligands on their cell surface, with the GII inhibitor 2mM N-methyl deoxynorjirmycin (NMdNM) overnight and examined them for the presence of MBP ligands. The overnight treatment of AB.1 with 2mM NMdNM resulted in a 50% inhibition of glucosidase II activity as measured by a colorimetric substrate (data not shown). This inhibition of GII activity resulted in a 56% decrease in cell surface Class I MHC expression, a 44% decrease in surface CD45 expression, and a 321% increase in MBP binding as measured by FACS analysis (Figure 3-7a). Cell surface expression of Class I MHC has been previously shown to be dependent on GII activity (Balow et al., 1995), however, it appears that CD45 cell surface expression is also somewhat dependent on GII activity. The increase in MBP binding after GII inhibition directly links GII activity with the production of MBP ligands. Further, in examining whether or not CD45 specifically acquires the appropriate carbohydrate for MBP recognition, an MBP pull-down from cell

A.



B.

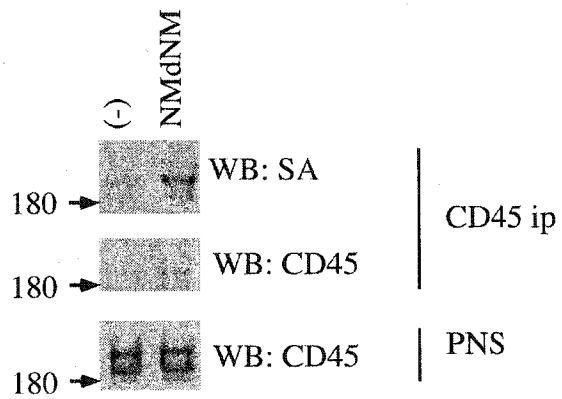


Figure 3-7: GII inhibition results in increased MBP ligands, specifically CD45, on the cell surface of mature T cells.

A. FACS analysis for Class I MHC, CD45 and MBP was performed on either untreated or 2mM NMdNM treated AB.1 cells. Values are expressed as a percent of mean fluorescence intensity compared to untreated cells. B. Cell surface biotinylated lysates from either untreated or 2mM NMdNM treated AB.1 cells were used in an MBP pull-down experiment. The MBP bound proteins were eluted and further subjected to anti-CD45 immunoprecipitation. Proteins recovered from the CD45 immunoprecipitate were resolved by SDS-PAGE and visualized by western blot with streptavidin (upper panel) and anti-CD45 (middle panel). Post-nuclear supernatants (PNS) from either untreated or treated cells were analyzed for CD45 content by anti-CD45 western blot (lower panel).

surface biotinylated AB.1 lysates either untreated or NMdNM treated showed that there is a significant increase in the amount of cell surface CD45 recognized by MBP in the GII inhibited AB.1 cells (Figure 3-7b). Taken together, these results demonstrate that GII has the ability to regulate the presence of cell surface MBP ligands.

D. DISCUSSION

We have demonstrated that the carbohydrate structure on CD45 changes as T-cells mature using three distinct biochemical systems and a number of different cell types. This change in the carbohydrate structure principally revolves around the ability of both MBP and GII to bind CD45. Changes in the carbohydrate on cell surface CD45 have biological implications for a number of processes such as adhesion, development or plasma membrane localization.

The association between CD45 and GII is seen in immature cells, but not mature cells (Figure 3-1). This change in the ability of GII to bind CD45 may be due to a number of different factors including changes in GII or changes in CD45. In examining the isoform expression of GIIα in immature versus mature cells, we find comparable amounts of GIIα that contain the first alternatively spliced sequence (Box A1), which are the only isoforms capable of associating with CD45 (Baldwin et al., 2000), as well as other GIIα isoforms (data not shown). Therefore, GIIα isoform expression does not appear to be significantly different between mature and immature cells. Previous data using a transfection system indicated that all isoforms of CD45 are capable of associating with GII (data not shown), and examination of two different isoforms of CD45 in BW cells, CD45RO and CD45RB, revealed that both isoforms are capable of associating with GII (data not shown). These data suggest that all isoforms of CD45 are capable of associating with GII within the same cell type. These data support the

notion that the ability of CD45 to associate with GII is intrinsic to the cell, and not dependent on the isoform of CD45 found within that cell.

We and others have also shown that there is a higher amount of MBP binding to cell surface CD45 (Figure 3-2a) in immature cells (Uemura et al., 1996). Because the CD45-deficient variants of the BW and SAKR cells also bind MBP (Fig. 2c), and MBP appears to pull down a limited set of cell surface proteins other than CD45 (data not shown), we suspect that there is an overall change in the carbohydrate processing machinery within immature cells giving rise to carbohydrate structures capable of being bound by MBP. The regulation of which glycoproteins acquire carbohydrate capable of being bound by MBP may involve GII, or other ER proteins; therefore we are interested in determining if there are other proteins which associate with GII, specifically Box A1 containing GIIα. In support of differential carbohydrate processing in thymocytes, detection of calnexin-associated CD3γ on the cell surface with immature carbohydrate has been reported (Wiest et al., 1997). In this case, it appears that calnexin may regulate the carbohydrate on CD3γ, whereas GII may regulate the CD45 carbohydrate.

The most striking data in support of developmentally regulated changes in the carbohydrate structure on CD45 comes from the analysis of *ex vivo* thymocytes. Double positive thymocytes are recognized by MBP to a significantly higher degree than either the double negative or single positive subsets (Figure 3-3a). As well, there is more GII bound CD45 in bulk thymocytes versus single positive Con A thymocyte blasts (Figure 3-3d). Interestingly,

during thymocyte development, the activity of CD45 is most crucial at the double positive stage during positive selection as illustrated by CD45-deficient mice (Byth et al., 1996). Therefore, lectins expressed on thymic stromal cells with similar specificity to MBP could bind to the abundant CD45 glycoprotein where their interaction could affect adhesion, signaling, or plasma membrane localization leading to changes in thymocyte selection. The binding of CD45 to lectins on the surface of thymic stromal cells may be of a low affinity and transient in nature, but may allow for some high avidity interactions to take place. This appears to be the case for the interaction between DC-SIGN and ICAM-3 where the interaction between DC-SIGN on the dendritic cell and ICAM-3 on the T cell seems to be the initial adhesion event allowing for the interaction between LFA-1 and ICAM-3 (Geijtenbeek et al., 2000a). The adhesion mediated by LFA-1 and ICAM-3 keeps the dendritic cell and T cell in contact long enough for TCR engagement by MHC-peptide (Geijtenbeek et al., 2000a).

Since a soluble lectin, MBP, has been demonstrated to bind CD45, it is also possible that a soluble, mannose specific lectin found within the thymus could bind CD45. The binding of CD45 by a soluble lectin may constrain CD45 in a particular spatial organization that may impact signaling thresholds. This was recently demonstrated to occur for the TCR where binding of the TCR by galectin-1 decreased TCR clustering (Demetriou et al., 2001). In light of the work published by Johnson et al. (Johnson et al., 2000) describing the movement of a small fraction of CD45 into the immunological synapse, it is possible that a soluble mannose specific lectin binding CD45 will perform a similar function to

galectin-1 and spatially restrict the movement of CD45. By restricting the movement of CD45 into and out of the immunological synapse, signaling thresholds may be altered. The altering of signaling thresholds may be particularly important for thymic selection events.

The use of monosaccharides to inhibit the MBP binding of CD45 demonstrated the existence of two glycoforms of CD45. Both glycoforms of CD45 could be inhibited from binding MBP by mannose, while only the higher molecular weight glycoform was competed by glucose (Figure 3-6). These data suggest that MBP may be binding CD45 via terminal mannose or terminal glucose. In analyzing the total carbohydrate on CD45 from immature SAKR cells for the presence of terminal glucose using Endo H digestion followed by mannosidase treatment, which is less active on carbohydrate containing terminal glucose, we found that there were two distinct pools of CD45 carbohydrate. One pool was susceptible to mannosidase treatment, while the other was not, which suggests the presence of terminal glucose on a portion of CD45 carbohydrate (data not shown). It is not clear which pool of carbohydrate was from cell surface CD45, however since the ratios of mannosidase sensitive to insensitive carbohydrate were approximately 1:1 we believe at least some of the mannosidase insensitive carbohydrate was cell surface derived. In fact, the calnexin associated, cell surface CD3γ was found to contain terminal glucose on its Endo H sensitive carbohydrate by a similar method (Wiest et al., 1997). In order for the cell surface CD45 carbohydrate to contain terminal glucose or mannose, there must be a mechanism in place to protect those sugars. Those residues may be protected

from processing by a number of different mechanisms including physical protection by interacting proteins, such as GII, or sequestration within the ER and Golgi, thereby preventing access to the carbohydrate.

In support of a model whereby GII directly regulates MBP binding, treatment of mature cells with a GII specific inhibitor results in an increase in MBP binding to cell surface CD45 (Figure 3-7a,b). Since GII is required for the removal of the terminal glucose residues from the immature carbohydrate, inhibition of its activity likely results in the maintenance of a fraction of those glucose moieties. Therefore, MBP may be binding to cell surface CD45 from GII inhibited AB.1 cells by way of terminal glucose residues. Since GII stably associates with CD45 in immature cells, it is possible that the enzymatic activity of GII is inhibited while bound to CD45 thus preserving the glucose residue on the immature carbohydrate expressed on cell-surface CD45. This protection hypothesis could explain our detection of cell surface CD45 which is capable of being bound by MBP, and is sensitive to glucose competition (Figure 3-6). MBP or other lectins with similar specificity could bind to this pool of CD45.

In summary, it appears that the regulation of CD45 glycosylation changes during T cell development, however future studies must be done to determine if these modifications are important for T cell maturation. Given the recent interesting example of how carbohydrate modification and lectin interaction regulates T cell responsiveness (Demetriou et al., 2001), it is tempting to speculate that carbohydrate interactions with CD45 and other proteins could be important for regulating signaling thresholds during thymocyte development.

Chapter 4: The Protein Tyrosine Phosphatase CD45 Traffics to the Cell Surface via Golgi Dependent and Independent Pathways

A version of this chapter has been submitted to The Journal of Biological Chemistry.

A. INTRODUCTION

The transmembrane protein tyrosine phosphatase CD45 is required for both thymocyte development and T cell activation (Byth et al., 1996; Trowbridge and Thomas, 1994). CD45 exerts its effects at least in part by regulating the phosphorylation state of src-family kinases through the dephosphorylation of a negative regulatory carboxy-terminal tyrosine residue (Ashwell and D'Oro, 1999; Thomas and Brown, 1999). In addition to the cytoplasmic phosphatase domains, CD45 contains a large extracellular region. Three alternatively spliced exons reside within the external domain of CD45, which contain numerous sites for potential O-linked carbohydrate additions. These exons are developmentally regulated with respect to usage in T cells and therefore cells of different developmental stages have the potential to express vastly different forms of CD45. In addition to the O-linked carbohydrate found in the alternatively spliced sequences, there are numerous potential N-linked carbohydrate attachment sites (Thomas, 1989). These N-linked carbohydrate additions have been demonstrated to be important for both cell surface expression and stability of CD45 (Pulido and Sanchez-Madrid, 1992). As well, we have recently shown that the composition of the CD45 N-linked carbohydrate is developmentally regulated, possibly through the action of the ER enzyme glucosidase II (GII) (Baldwin and Ostergaard, 2001). Intriguingly, no typical cell-surface ligand for the extracellular domain of CD45 has been identified, however, several lectins have been demonstrated to bind

CD45 carbohydrate (Baldwin and Ostergaard, 2001; Pace et al., 1999; Perillo et al., 1995; Sgroi et al., 1995; Stamenkovic et al., 1991; Uemura et al., 1996).

The intracellular transport of proteins from the ER to the cell surface is a tightly controlled process involving the co-ordinated action of many enzymes and proteins (Lippincott-Schwartz et al., 2000). For the most part, as a protein moves through the secretory pathway, a level of control is exerted at each stage of the transport process, from protein folding and vesicle budding at the ER to movement through the Golgi stacks and finally sorting at the TGN en route to its final destination (Roche, 1999). In many cases this regulation is necessary for proper function of the protein. For example, transport of major histocompatability complex (MHC) class I and II antigen presenting proteins, as well as CD1 each follow different routes to the cell surface, and those specific routes are necessary to ensure the appropriate antigens are loaded into their peptide or glycolipid binding grooves (Briken et al., 2002; Pamer and Cresswell, 1998; Pieters, 1997). For CD45, it is clear that cell surface expression is required for proper function and the N-linked carbohydrate on CD45 plays a role in this process (Pulido and Sanchez-Madrid, 1992). Given our recent demonstration of the association of GII with CD45 altering the carbohydrate structure on cell surface CD45 (Baldwin and Ostergaard, 2001), it is possible that this interaction somehow alters the trafficking of CD45 from the ER to the cell surface. In fact, mannose binding lectin (MBL) has been shown to bind cell surface CD45, which indicates that CD45 is able to escape complete carbohydrate processing leaving immature, high mannose carbohydrate (Baldwin and Ostergaard, 2001; Uemura et

al., 1996). This escape from processing may in fact be due to alternative trafficking routes. With the recent examples of carbohydrate influencing biological function such as DC-SIGN interactions (Geijtenbeek et al., 2000a; Geijtenbeek et al., 2000b), CD8-MHC interactions (Daniels et al., 2001; Moody et al., 2001), and TCR clustering (Demetriou et al., 2001), as well as the limited information on CD45 trafficking in general, we set out to determine the overall trafficking patterns of CD45 and the impact of GII on CD45 trafficking.

Our results indicate that CD45 and GII associate early in the secretory pathway, and remain associated during transit to the cell surface. As well, we find that two different pathways exist for the transport of CD45 to the cell surface; one involving the Golgi complex resulting in Endo H resistant carbohydrate modification, and the other by-passing the Golgi resulting in the maintenance of Endo H sensitive carbohydrate on the cell surface. The pathway leading to cell surface expression of CD45 which by-passes the Golgi complex is BFA resistant and traffics CD45 with extremely rapid kinetics. These data support the existence of a transport pathway where cargo can reach the cell surface extremely rapidly, without the requirement of the Golgi complex.

B. MATERIALS AND METHODS

Cell lines and antibody reagents

The T lymphoma cell line BW5147 (BW) and its CD45 and GIIα-deficient variants, BW/T200$^-$ and BW/PHA^r respectively, were maintained as previously described (Arendt and Ostergaard, 1995). The pan-extracellular domain specific CD45 monoclonal antibody I3/2.3 was described previously (Arendt and Ostergaard, 1995). Class I MHC D^b specific antiserum H137 (Maksymowych et al., 1998) was kindly provided by Dr. Kevin Kane (University of Alberta, Edmonton, AB, Canada). Rabbit antisera H2 and J37, specific for GIIβ and the intracellular region of CD45 respectively, were previously described (Baldwin et al., 2000).

Cell surface biotinylation, cell lysis, immunoprecipitation, streptavidin pulldowns and endoglycosidase treatment

Cell surface biotinylation was performed as previously described (Baldwin and Ostergaard, 2001). Briefly, cells were biotinylated with 50 μl of 10 mM sulfo-NHS biotin (Pierce, Rockford, IL) per 5 X 10^7 cells/ml in PBS for 10 min at room temperature, or for 20 min on ice. Reactions were quenched by washing cells twice in PBS containing 5 mM glycine. All cells were lysed at a density of 5 X 10^7/ml in 0.5% Nonidet P-40 (Pierce)/TBS buffer, and incubated for 20 min on

ice. 20 mM mannose was included in the lysis buffer when the prevention of newly formed CD45-GII complexes was desired. Post-nuclear supernatants were incubated with I3/2 coupled Sepharose 4B for 1-2 h, or with 10 µl/ml of rabbit antisera followed by capture of the immune complexes with Protein A coupled Sepharose 4B. Biotinylated proteins were isolated by incubation with streptavidin coupled agarose for 1-2 h. at 4°C. Immunoprecipitates and streptavidin pulldowns were washed three times with RIPA buffer, or three times with lysis buffer when maintenance of the CD45-GII complex was required. After washing, the immunoprecipitates were boiled in reducing sample buffer. Immunoprecipitates were treated with Endo H (Calbiochem, La Jolla, CA) in PBS containing 1% NP-40, 0.1% SDS, 1% 2-mercaptoethanol for 16 h at 33°C.

Pulse-chase analysis, isolation of cell surface proteins and inhbitor treatments

Cells were washed twice in PBS prior to depletion of intracellular methionine by incubation for 30 min at 37°C with methionine-free RPMI (Invitrogen, Carlsbad, CA). The cells were then pulsed for 10 min at 15°C with 0.5 mCi/ml of TRAN^{35}S-LABEL (ICN Biomedicals) at 5 X 10^7/ml. The pulse was performed for 5' at 37°C in Figure 1 and 10 only. Cells were then washed twice in ice-cold unlabeled media prior to initiation of the chase. Cells were chased at 37°C, or as indicated, followed by washing with PBS prior to lysis. For specific isolation of cell surface proteins, either an antibody or biotinylation method was employed. For isolation of cell surface protein by the antibody method, cells

were incubated for 20 min with 20 μg/ml I3/2 or 10 μl/ml rabbit antiserum at 4°C followed by washing three times with PBS. 5 X 10^6 cells were then lysed with 500 μl of a 2.5 X 10^7/ml unlabeled lysate. Immune complexes were recovered with secondary antibody coated Protein A Sepharose 4B. For isolation of cell surface CD45 by biotinylation, cells were surface biotinylated as described above, lysed at 2.5 X 10^7/ml in lysis buffer, followed by total CD45 immunoprecipitation. Captured CD45 was released by boiling for 2 X 5' in 50 μl of 2% SDS/TBS. Eluent was diluted to 1ml with lysis buffer. Biotinylated CD45 was isolated by a streptavidin pulldown described above. Cell surface proteins were selectively isolated by the antibody method unless otherwise stated. All immunoprecipitates and pulldowns were washed three times with RIPA buffer prior to boiling with reducing sample buffer unless the CD45-GII interaction was examined in which case immunoprecipitates were washed with lysis buffer. In the cases where BFA was used to block protein trafficking, cells were pre-incubated with 2 μg/ml of BFA for 30 min. at 37°C. BFA was also present during the pulse and chase at 2 μg/ml. N-ethyl maleimide (NEM) was used at a final concentration of 500μM. NEM was added post-pulse for 10 min. on ice, and included in the chase at 500μM.

Polyacrylamide gel electrophoresis, autoradiography and immunoblotting

Proteins were resolved on polyacrylamide gels and transferred to polyvinylidene difluoride-Immobilon (Millipore, Bedford, MA) as described

previously (Arendt and Ostergaard, 1995). For separation under non-reducing conditions, 2-mercaptoethanol was omitted from the sample buffer. Autoradiography was performed with the Kodak BioMax TranScreen Intensifying system (Eastman Kodak, Rochester, NY). Western blot analysis was conducted with the indicated antiserum followed by protein A^{hrp} (Pierce) and visualized by ECL (PerkinElmer Life Sciences, Norwalk, CT).

FACS analysis

1 X 10^6 cells were incubated with 10 µl/ml H2 antiserum for 20 min on ice, followed by two washes with PBS containing 0.1% serum. For detection of bound antibody, cells were further incubated with donkey anti-rabbit^{FITC} for an additional 20 min on ice. Cells were then fixed in 1% paraformaldehyde before analysis.

C. RESULTS

Newly synthesized CD45 and newly synthesized GII interact rapidly, remain associated for an extended period of time, and traffic together to the cell surface

Since CD45 and GII associate stably and specifically (Baldwin et al., 2000), we wished to determine where the association was initiated and examine the duration of the interaction. Given the role of GII within the ER, one might predict that CD45 and GII interact initially in the ER, but dissociate before CD45 exits the ER. To examine the association kinetics, BW5147 T-lymphoma cells were pulsed for 5' at 37°C with ^{35}S-methionine followed by a chase with unlabelled media for various periods of time. The cells were lysed in lysis buffer containing 20mM mannose, which prevents the formation of new CD45-GII complexes upon lysis (data not shown), thereby allowing the isolation of pre-formed CD45-GII complexes only. CD45 was then immunoprecipitated and examined for GII association. Newly synthesized GIIα was found to rapidly associate with newly synthesized CD45 and the interaction was maintained for at least 120' (Figure 4-1a). Newly synthesized GIIβ was also found to rapidly associate with CD45 (data not shown). As a control for examining interaction kinetics within the ER, GIIα association with its β-chain was studied. Newly synthesized GIIα and GIIβ interact rapidly after synthesis, displaying similar kinetics to those seen when examining CD45 and GIIα association (Figure 4-1a). Therefore, the interaction between CD45 and GII is initiated rapidly after

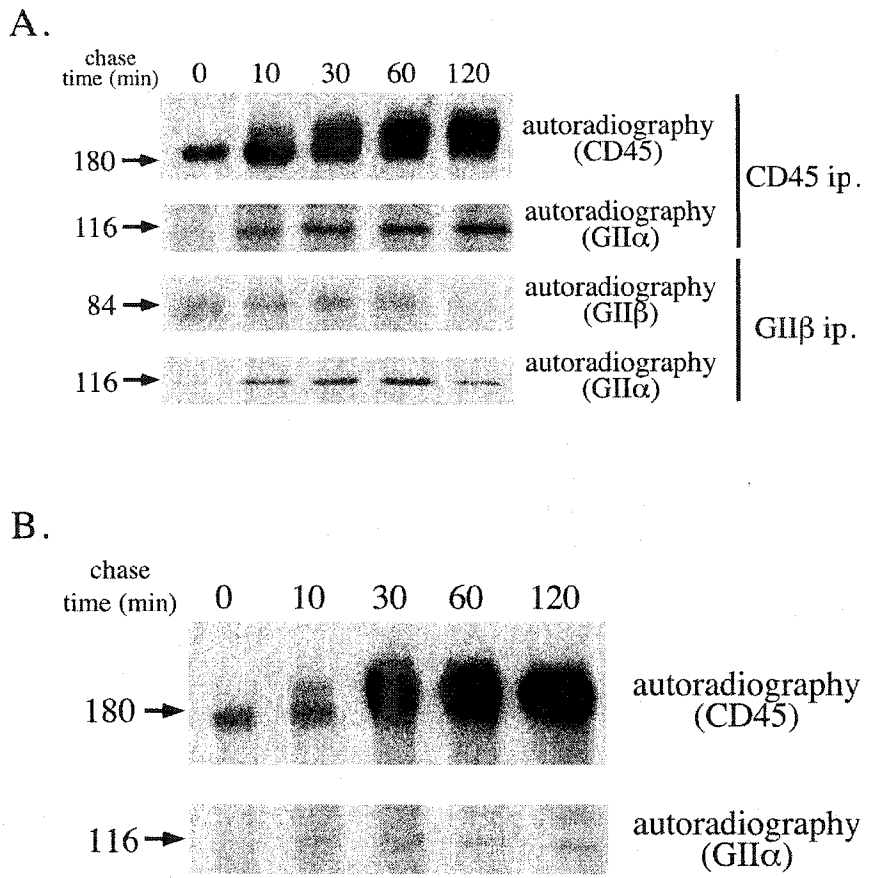


Figure 4-1: CD45 and GII associate early in the secretory pathway and remain associated during CD45 trafficking to the cell surface.

A. Cells were pulsed at 37°C for 5' with media containing ^{35}S-methionine and chased in unlabeled media over the indicated time course after which the cells were lysed. CD45 (top two panels) or GIIβ (bottom two panels) were immunoprecipitated and the immune complexes were resolved by SDS-PAGE followed by autoradiography. The identity of the proteins visualized by autoradiography was confirmed by western blot analysis (data not shown). B. Cells were pulsed and chased as in A followed by specific isolation of cell surface CD45 by the antibody mediated method. Resultant immune complexes were resolved by SDS-PAGE and autoradiography was performed. Identity of indicated band was again confirmed by western blot analysis.

synthesis, presumably in the ER, and appears to be maintained for an extended period of time.

As expected, the association between CD45 and GII is initiated rapidly within the ER after protein synthesis, however somewhat unexpectedly the association is maintained for at least 120'. Given the extended duration of the interaction between CD45 and GII, it is plausible that GII remains associated with CD45 throughout the transport of CD45 to the cell surface. Another possibility for the observed prolonged association is the interaction of radio-labeled GII remaining in the ER with newly synthesized, unlabeled CD45 translated during the chase. To distinguish between these two possibilities, cell surface CD45 from a pulse-chase experiment was specifically isolated. To isolate cell surface CD45, cells were incubated with an anti-CD45 antibody after the chase period followed by lysis with a non-radio-labeled BW lysate to saturate unoccupied sites of the CD45 antibody, followed by capture of the CD45 antibody. This protocol has been used in the past to isolate newly synthesized proteins on the cell surface (Williams et al., 1985). Radio-labeled GIIα was found to be associated with newly synthesized cell surface CD45 (Figure 4-1b). These data suggest that after formation of the CD45-GII complex in the ER, at least a fraction of these complexes traffic together to the cell surface.

GII, a putative resident ER protein, is found on the cell surface

Given the previous data demonstrating GII association with cell surface CD45, it is predicted that GII should be detected on the cell surface. Using FACS analysis with an antiserum directed against the β-chain of GII, it is clear that GIIβ is expressed on the cell surface of BW5147 as well as its derivatives; BW/T200$^-$, a CD45 deficient cell line, and BW/PHA^r, a cell line deficient in GIIα (Figure 4-2a). There is reduced cell surface expression of GIIβ in BW/PHA^r compared to either BW wildtype or BW/T200$^-$ which suggests that GIIα may be involved in the trafficking of the GII complex to the cell surface. As well, no substantial difference in the amount of GIIβ found on the cell surface was seen in comparing BW to the CD45 deficient cell line, which suggests that transport of GIIβ to the cell surface is not dependent on the presence of CD45. Due to the lack of GIIα specific reagents for FACS analysis, an alternative and complementary approach was taken to determine if GIIα is present on the cell surface. Cells were surface biotinylated and those biotinylated proteins were captured by incubation of the lysates with streptavidin coupled beads. Examination of the biotinylated proteins revealed that both GIIα and GIIβ were found on the cell surface (Figure 4-2b). Again, less GIIβ is seen on the cell surface from BW/PHA^r cells compared to BW wildtype, which supports a role for GIIα in the trafficking of GIIβ to the cell surface. Additionally, it appeared that all detectable isoforms of GIIα were found on the cell surface (data not shown). GII was also found on the cell surface of

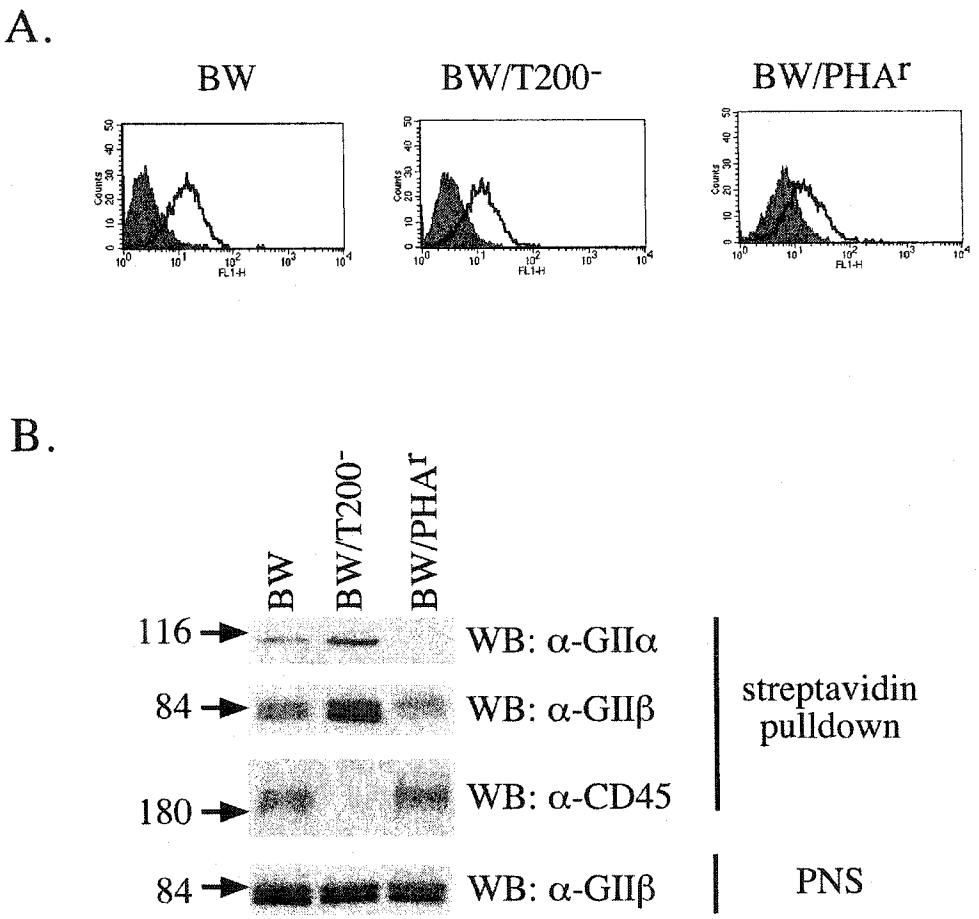


Figure 4-2: GII is found on the cell surface.

A. 1 X 10^6 cells were incubated with 2μl of antiserum specific for GIIβ on ice for 20 min, followed by protein A^{FITC}. Shaded area represents control staining with an antiserum specific for the intracellular domains of CD45. B. Cell surface biotinylated post-nuclear supernatants from the indicated cell line were incubated with streptavidin coupled agarose for 2 h. at 4°C. The bound proteins were resolved by SDS-PAGE and western blotting was performed for GIIα (1^{st} panel), GIIβ (2^{nd} panel) and CD45 (3^{rd} panel). Input post-nuclear supernatant was analysed for equal protein levels by western blotting for GIIβ (4^{th} panel).

other T cell lines as well as *ex vivo* thymocytes by both methods (data not shown) suggesting that escape of GII to the cell surface is a universal property of T cells.

CD45, but not Class I MHC appears on the cell surface with rapid kinetics

During the isolation of newly synthesized cell surface CD45 in Figure 4-1b, it was observed that newly synthesized CD45 appeared on the cell surface at time zero of the chase. These data suggest that the pulse time of 5' at 37°C used in Figure 1 was sufficient to allow newly synthesized CD45 to traffic to the cell surface. This time to cell surface expression seems to be extremely rapid compared to the kinetics reported for the majority of other cell surface proteins. However, Nori and Stallcup used CD45 as a control in a pulse chase experiment and reported similar results to those obtained in Figure 4-1b with respect to rapid CD45 cell surface expression (Nori and Stallcup, 1988). To more accurately determine the time required for newly synthesized CD45 to reach the cell surface, a modification to the previous pulse-chase protocol was necessary. A pulse condition was needed that prevented or substantially slowed bulk protein transport, but still allowed for sufficient incorporation of metabolic label. Incubation of cells at 15°C has previously been demonstrated to block protein transport at the ER-Golgi intermediate compartment (ERGIC) (Saraste and Kuismanen, 1984), therefore pulsing cells with ^{35}S-methionine at 15°C instead of 37°C should slow protein transport enough to reveal a true zero chase time. Using this new protocol, no radio-labeled CD45 was detected on the cell surface at time zero (Figure 4-3a), although bulk ^{35}S-methionine labeled CD45 is consistently

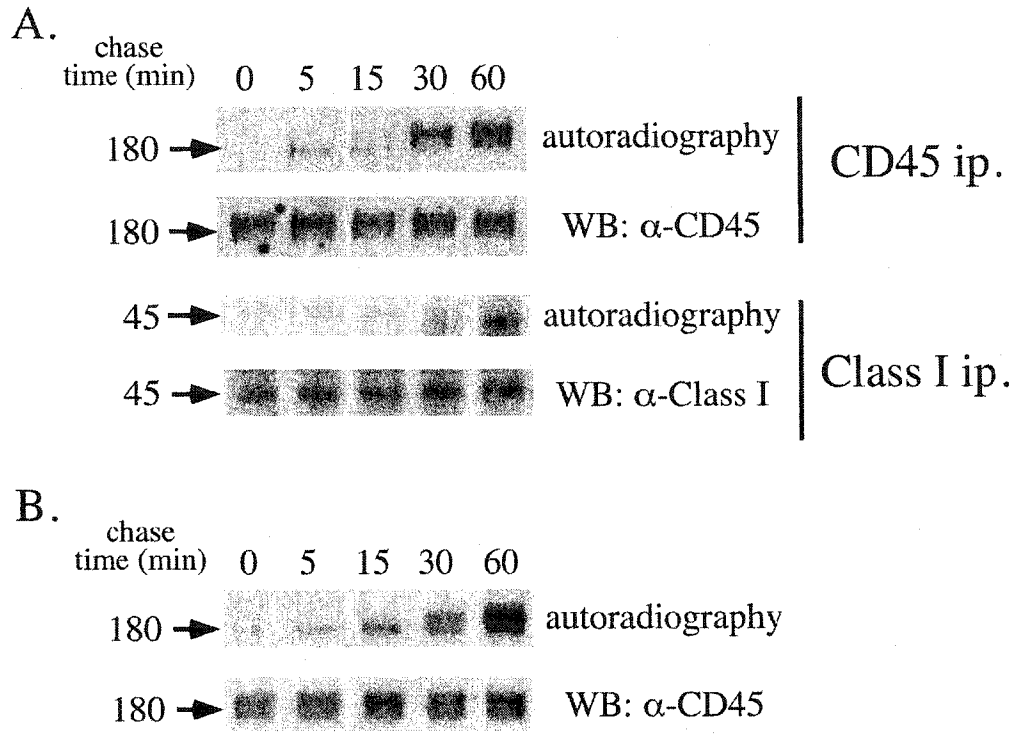


Figure 4-3: Two forms of newly synthesized CD45 can be isolated from the cell surface, each with distinct trafficking kinetics.

A. Cells were pulsed with ^{35}S-methionine at 15°C followed by chasing in unlabeled media at 37°C as described in Materials and Methods. Cell surface CD45 (top two panels), or Class I MHC (bottom two panels) were isolated by the antibody method followed by separation by SDS-PAGE. Autoradiography, followed by western blot analysis was performed. B. Cells were pulsed and chased as in A followed surface biotinylation and lysis. Total CD45 was immunoprecipitated, eluted from I3/2 beads by boiling in 2% SDS/PBS for 10 min., and a streptavidin pulldown was performed on the eluted material. Material bound to streptavidin beads was resolved by SDS-PAGE and subjected to autoradiography.

detected at time zero when analysing total CD45 (see Figure 4-5). By 5', newly synthesized cell surface CD45 was detected and the amount increased until a chase time of 60' (Fig. 3a). In addition, there appear to be two different forms of CD45 expressed over the entire chase time; a rapidly expressed form of lower molecular weight, and a higher molecular weight form which predominates by the end of the chase period (Figure 4-3a). Newly synthesized Class I MHC appeared on the cell surface at 30' and increased until 60' (Figure 4-3a). As well, there was only one form of Class I MHC to reach the cell surface (Figure 4-3a). These trafficking kinetics of Class I MHC are similar to those previously reported (Williams et al., 1988; Williams et al., 1985).

Another published method to isolate cell surface proteins involves surface biotinylation, isolation of the protein of interest, followed by streptavidin affinity enrichment (Briken et al., 2002; Hobman et al., 1993). Using the surface biotinylation approach to isolate cell surface CD45, we found identical results to the surface antibody method described above (Figure 4-3b). Newly synthesized, surface biotinylated CD45 began appearing on the cell surface at 5' and increased through to 60' chase time, with the existence of two different forms (Figure 4-3b). Interestingly, the lower molecular weight, rapidly expressed form of CD45 is readily detectable at steady state levels, indicating this is a stable population of CD45 on the surface (Figure 4-3a,b CD45 western blots). Using two different methods of isolating cell surface material, we find that CD45 appears on the cell surface rapidly after synthesis.

CD45 acquires Endo H resistance significantly more rapidly than Class I MHC

Given the rapidity with which CD45 reaches the cell surface, we wished to examine the trafficking kinetics of CD45 in more detail using the acquisition of Endo H resistance as a marker of protein location along the secretory pathway. Treatment of newly synthesized CD45 with Endo H after various chase times indicated that CD45 begins to acquire Endo H resistance approximately 15' after synthesis (Figure 4-4a). Class I MHC shows a lag period before acquiring Endo H resistance. This lag phase in glycoprotein trafficking seen with Class I would be predicted for most proteins as both protein folding and transport to the Golgi are required to achieve Endo H resistance. Figure 4b shows graphically the rapid acquisition of Endo H resistance for CD45 and the lag period seen with Class I MHC as quantified by densitometry.

CD45 achieves a transport competent form soon after synthesis

Due to the size, number of N-linked glycan additions and proposed complexity of the extracellular domain of CD45, one might predict that CD45 would require a significant amount of time in the ER to fold properly. The rapidity with which CD45 begins to acquire Endo H sensitive carbohydrate and the observation that a portion of CD45 traffics to the cell surface rapidly suggests that CD45 adopts a properly folded conformation quickly after synthesis. To

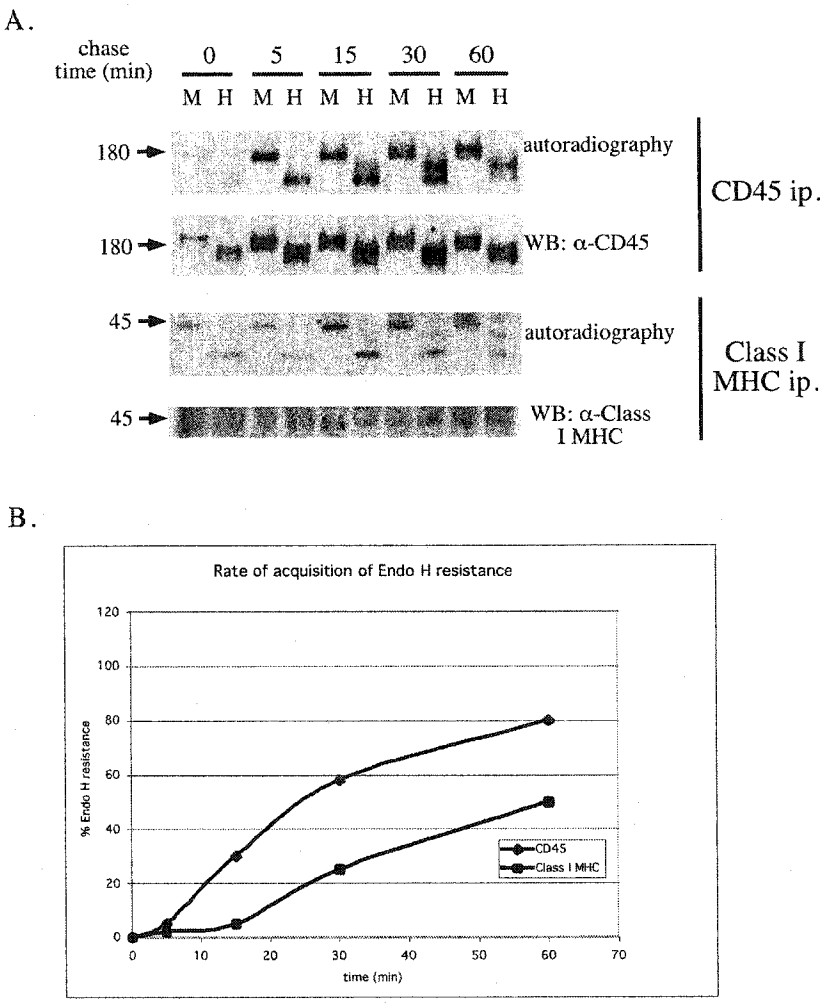


Figure 4-4: CD45 achieves Endo H resistance rapidly after synthesis.
A. Cells were pulsed and chased as indicated previously. Total CD45 (top two panels) or Class I MHC (bottom two panels) was immunoprecipitated from the lysates and either mock treated (M) or subjected to Endo H digestion (H) prior to resolving on SDS-PAGE. Autoradiography and western blot analysis was performed as indicated. B. The relative amount of Endo H sensitive and resistant material was obtained by densitometry. Percentage of Endo H resistant CD45 was calculated by dividing the amount of Endo H resistant material by the total Endo H sensitive and Endo H resistant fractions and multiplying by 100%. This value is plotted as a function of time.

directly determine the time necessary for CD45 to adopt its fully folded conformation, we performed pulse-chase analysis, and separated immunoprecipitated CD45 by either reducing and denaturing or non-reducing and denaturing SDS-PAGE. Separation of proteins under non-reducing conditions maintains the disulfide bonds in their original configuration. The mobility of proteins isolated from pulse-chase experiments under non-reducing conditions would be expected to increase over time as disulfides are formed within the protein resulting in a more compact structure. Interestingly, we find that there is no significant difference in the mobility of CD45 under reducing and denaturing or non-reducing and denaturing conditions indicating few disulfide bonds are formed or significantly altered after synthesis is completed (Figure 4-5). Separation of newly synthesized CD45 under non-reducing conditions results in a more diffuse banding pattern, but this observed with total CD45 as well. However, there are a number of limitations which can be placed on the interpretation of this result owing to both the pulse-chase protocol as well as the ability to detect changes in the mobility of CD45. First, due to the length of the pulse, 10 minutes, and lack of a sharp termination in label incorporation, at time zero the newly synthesized protein may have had sufficient time to undergo disulfide bond formation or isomerization and therefore, no mobility change would be seen. As well, if there are no substantial domain structures in CD45 created by disulfide bonds, it would be difficult to detect a mobility change. It does appear however, that the pulse condition used resulted in CD45 adopting a conformation that can be transported and support the idea that fully folded CD45

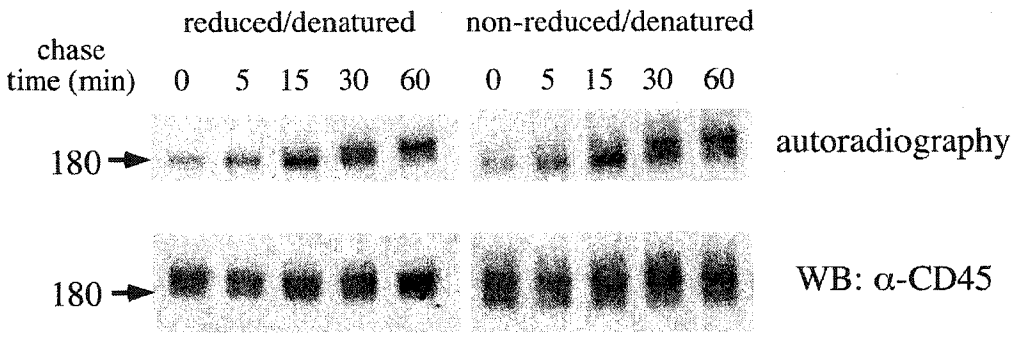


Figure 4-5: CD45 can adopt a transport competent conformation soon after synthesis.

Cells were pulsed for 10 minutes at 15°C and chased for the indicated time course at 37°C. Total CD45 was immunoprecipitated from the lysates and resolved by SDS-PAGE under either reducing and denaturing or non-reducing and denaturing conditions. Autoradiography and western blot analysis was performed as indicated.

can be detected at the plasma membrane within five minutes. Examination of Class I MHC at steady state levels reveals a population of Class I that forms aggregates, presumably due to an unfolded conformation, under non-reducing and denaturing conditions (data not shown), illustrating the need for resident time within the ER for Class I to achieve a fully folded state.

The lower molecular weight, rapidly expressed cell surface CD45 is Endo H sensitive, while the higher molecular weight form is Endo H resistant

Since bulk CD45 acquires Endo H resistance rapidly after synthesis, and there are two different forms of CD45 expressed on the cell surface with different kinetics, we wished to determine if both forms were in fact Endo H resistant, or if one was possibly Endo H sensitive. To determine the carbohydrate status of the two different forms of newly synthesized cell surface CD45, surface CD45 was isolated after pulse-chase and subjected to Endo H treatment. The rapidly expressed population of CD45 was entirely Endo H sensitive, while the higher molecular weight form achieved its full Endo H resistance (Figure 4-6). These data suggest that in fact the higher molecular weight form is a mature glycoform of CD45 with fully processed carbohydrate, while the lower molecular weight form is an immature glycoform with unprocessed carbohydrate. Note that the fully processed glycoform of CD45 still contains a significant fraction of Endo H sensitive carbohdyrate, but mature, cell surface CD45 always appears to contain this level of Endo H sensitive carbohydrate (Baldwin and Ostergaard, 2001).

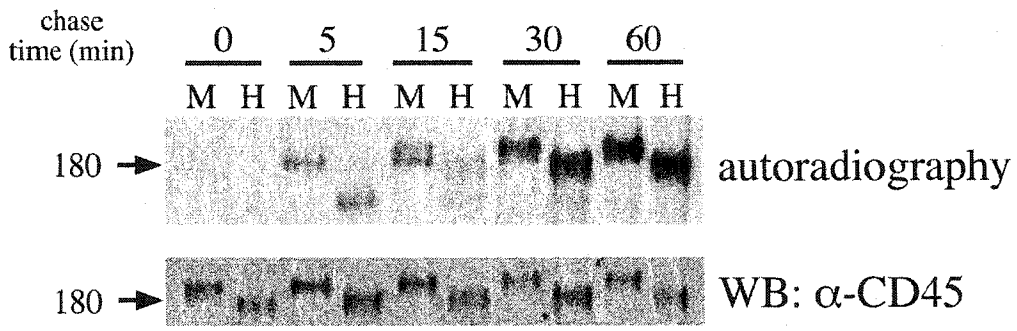


Figure 4-6: Rapidly expressed newly synthesized cell surface CD45 contains exclusively Endo H sensitive carbohydrate, while the majority of newly synthesized cell surface CD45 contains Endo H resistant carbohydrate.

Cells were pulsed and chased as described in Materials and Methods followed by isolation of cell surface CD45 by the antibody method. The immune complexes were split into two fractions and either mock treated (M) or treated with Endo H (H). The resultant proteins were separated by SDS-PAGE and autoradiography was performed (top panel) Western blot analysis of total protein is shown as a control.

The rapidly expressed pool of CD45 is partially sensitive to the 15°C blockade, but not a BFA induced blockade

The finding that CD45 is capable of reaching the cell surface without complete processing of its carbohydrate raises the question of how this material traffics from the ER to the cell surface. The prevailing model of glycoprotein transport states that once a glycoprotein leaves the ER it is transported through the ERGIC to the Golgi, where the carbohydrate is processed to a complex form, and finally to the cell surface. Given our finding that the rapidly expressed pool of CD45 reaches the cell surface with completely unprocessed N-linked carbohydrate suggests that this pool may by-pass the Golgi entirely on route to the cell surface. Protein transport is kinetically blocked at the ERGIC by incubation of cells at 15°C (Saraste and Kuismanen, 1984). Pulse-chase experiments performed with a chase temperature of 15°C resulted in a significant delay and decrease in the amount of the rapidly expressed pool of CD45 on the cell surface, but not complete blockage as seen with Class I MHC (Figure 4-7a). Another method commonly used to inhibit protein transport through the Golgi is treatment of cells with the fungal metabolite BFA. BFA interferes with the recruitment of the ARF-1 GTPase to COP I coated membranes effectively blocking protein transport through the prevention of ER export and subsequent Golgi re-distribution (Donaldson et al., 1992; Helms and Rothman, 1992). Treatment of cells with BFA effectively inhibited Class I MHC cell surface expression, and the trafficking of the higher molecular weight, mature glycoform of CD45, but it did

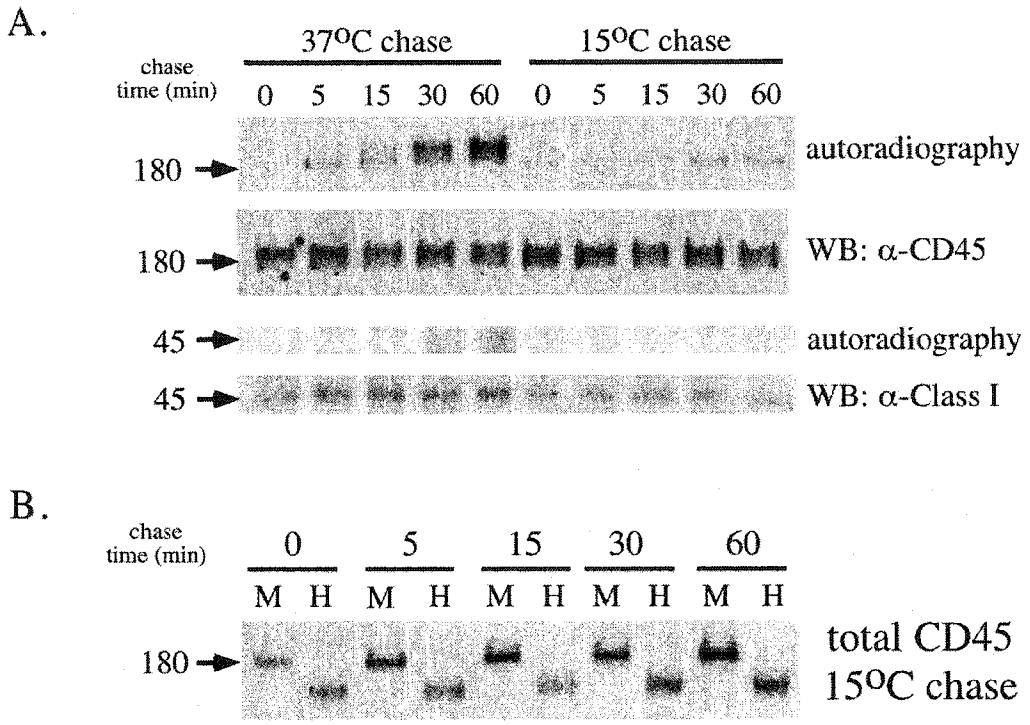


Figure 4-7: Trafficking of rapidly expressed cell surface CD45 is partially sensitive to the 15°C block.

A. Cells were pulsed at 15°C, followed by chasing at15°C. Cell surface CD45 (top two panels) or Class I MHC (bottom two panels) was isolated by the antibody method and separated by SDS-PAGE. Autoradiography was performed followed by western blot analysis for confirmation of protein identity. B. The remaining CD45 from the cell lysate was immunoprecipitated and treated with Endo H. Proteins were separated by SDS-PAGE and autoradiography was performed.

not inhibit the transport of the rapidly expressed, lower molecular weight glycoform of CD45 to the cell surface (Figure 4-8a). Examination of the carbohydrate on newly synthesized CD45 after either the 15°C chase or BFA block revealed that it contained entirely Endo H sensitive carbohydrate, as expected (Figure 4-7b, Figure 4-8b). These data suggest that the rapidly expressed population of CD45 completely by-passes the Golgi complex on its way to the cell surface, and does not rely on a BFA sensitive mechanism for transport.

The 20°C blockade results in delayed trafficking kinetics of the rapidly expressed pool of cell surface CD45

To further dissect the pathway used by the rapidly expressed pool of CD45, we took advantage of a block at the TGN imposed by incubation at 20°C (Griffiths et al., 1985; Griffiths and Simons, 1986). Chasing cells at 20°C after pulsing at 15°C and isolation of cell surface CD45 results in the appearance of the lower molecular weight form of CD45 beginning at fifteen minutes and increasing through the entire chase time, while no newly synthesized, higher molecular weight CD45 was recovered from the cell surface (Figure 4-9). As expected, no newly synthesized cell surface Class I MHC was recovered after the chase at 20°C (Figure 4-9). Examination of the remaining CD45 after the chase at 20°C revealed that it still contained predominantly Endo H sensitive carbohydrate (Figure 4-9). The observation of reduced kinetics of acquisition of Endo H

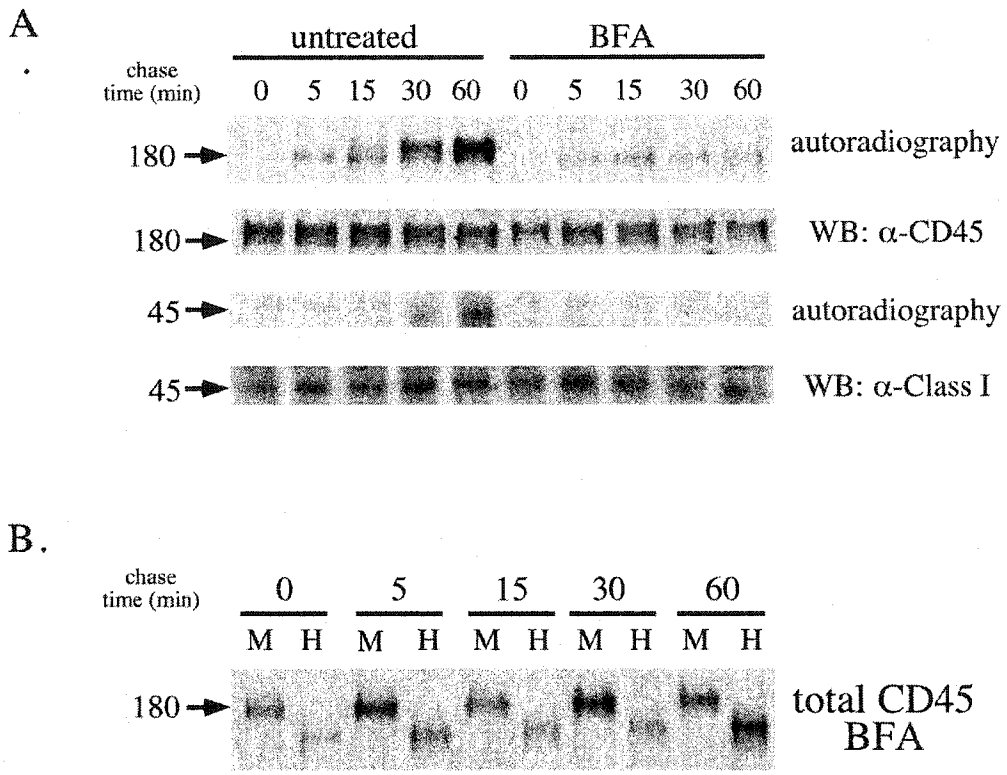


Figure 4-8: Trafficking of rapidly expressed cell surface CD45 is resistant to BFA treatment.

A. Cells were pre-treated with 2 μg/ml BFA for 30 min at 37°C followed by the pulse-chase protocol as described in Materials and Methods. BFA was present at the same level during the pulse and chase times. Cell surface CD45 (top two panels) or Class I MHC (bottom two panels) were isolated by the antibody method. The immune complexes were resolved by SDS-PAGE and subjected to autoradiography followed by western blot analysis with the indicated antisera. B. The remaining CD45 from the cell lysate was immunoprecipitated and treated with Endo H. Proteins were separated by SDS-PAGE and autoradiography was performed.

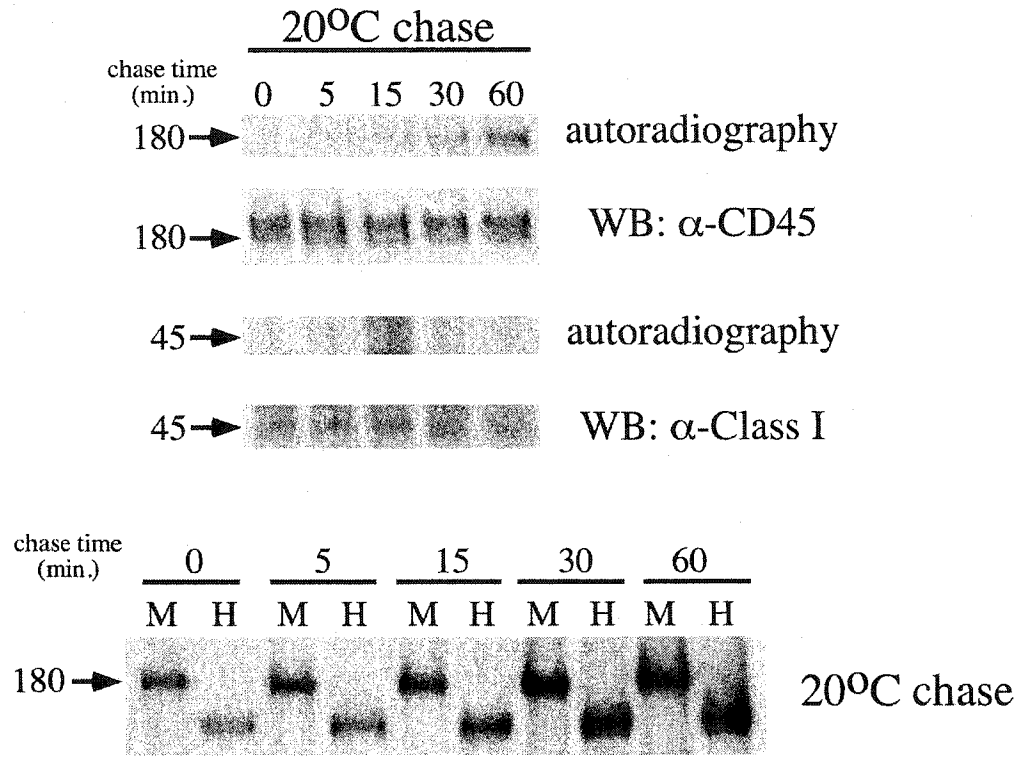


Figure 4-9: Trafficking of rapidly expressed Endo H sensitive CD45 to the cell surface occurs in a 20°C chase.

Cells were pulsed as described in Materials and Methods, and chased at 20°C. Cell surface CD45 (top two panels) or Class I MHC (bottom two panels) were isolated by the antibody method and resolved by SDS-PAGE. Autoradiography followed by western blot analysis was performed. Remaining CD45 was immunoprecipitated and subjected to Endo H digestion. Resultant proteins were separated by SDS-PAGE and autoradiography was performed.

resistance is in accordance with previously published data (Griffiths et al., 1985). Therefore, it appears that a block of glycoprotein traffic through the cis-Golgi or at the TGN does not prevent the expression of the lower molecular weight form of CD45, but it does appear to delay its trafficking kinetics.

N-ethyl maleimide can prevent surface expression and maturation of all forms of CD45 and Class I MHC

In an attempt to further dissect the mechanism of transport utilized by the rapidly expressed pool of CD45, we followed the trafficking of newly synthesized CD45 and Class I MHC to the plasma membrane after treatment with N-ethyl maleimide (NEM). NEM prevents vesicle fusion by inhibiting the activity of N-ethyl maleimide sensistive factor (NSF) through covalent modification (Malhotra et al., 1988). Treatment of cells prior to initiation of the chase with NEM prevented the appearance of both forms of CD45 as well as Class I MHC on the cell surface (Figure 4-10). Treatment of cells with NEM also prevented the processing of N-linked carbohydrate on both CD45 and Class I MHC as illustrated by the lack of change in mobility of CD45 and Class I (Figure 4-10). These data suggest that the process of vesicle fusion is necessary for the maturation and cell surface expression of both CD45 and Class I MHC.

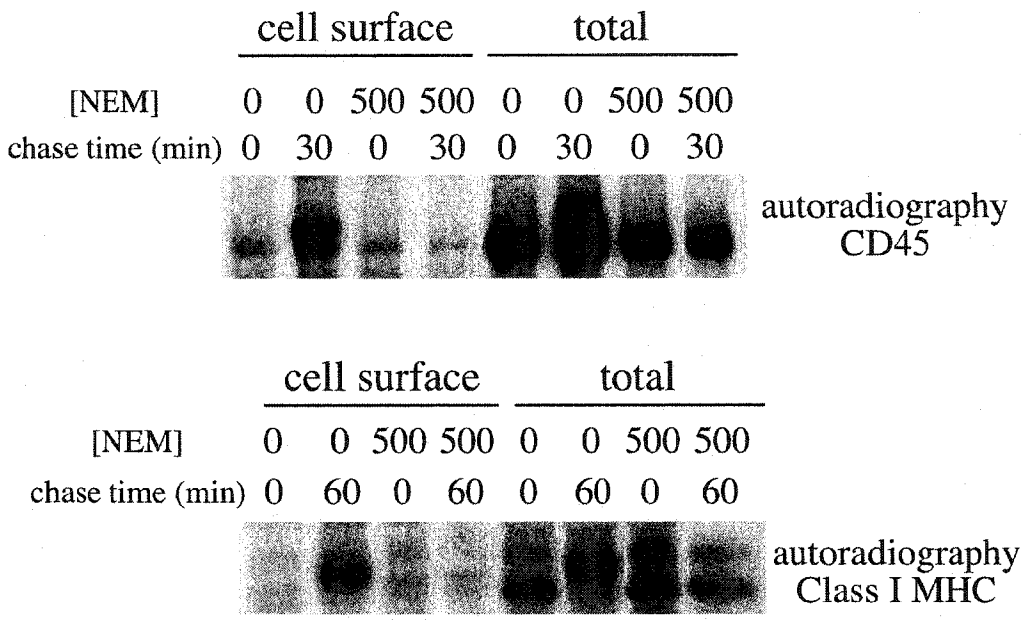


Figure 4-10: NEM treatment can inhibit the surface expression and maturation of CD45 and Class I MHC.

Cells were pulsed for 5 min. at 37°C followed by incubation with or without 500μM NEM for 10 min. on ice. After NEM treatment, cells were chased for indicated time at 37°C. Cell surface CD45 (top panel) or Class I MHC (bottom panel) was isolated by antibody method. The remaining CD45 or Class I MHC was also isolated. Proteins were resolved by SDS-PAGE and subjected to autoradiography for detection of proteins. Protein identity was confirmed by western blot.

Newly synthesized GIIα is expressed rapidly on the cell surface, and steady state levels of surface GIIα contain unprocessed carbohydrate

One question raised from the observation of GII on the cell surface is how a putative ER protein can traffic from the ER to the plasma membrane. To address this question, pulse chase analysis was performed where newly synthesized GII found on the cell surface was analyzed. Cells were again pulsed for 10' at 15°C, followed by chasing at 37°C for the indicated period of time. Newly synthesized GII was isolated by the antibody method and detected by autoradiography. Radio-labeled GIIα was detected on the cell surface beginning at 5' and increased until 30' after which levels remained constant (Figure 4-11). This form of newly synthesized GIIα was lacking Box A1 as confirmed by western blot analysis (data not shown). To determine if trafficking of GII to the cell surface resulted in processing of its N-linked carbohydrate, surface biotinylated GII was captured by immobilized streptavidin followed by Endo H or F treatment and visualized by western blot. Clearly, the carbohydrate found on GIIα was entirely Endo H sensitive in nature (Figure 4-11). Another glycosylated resident ER protein, grp94, has also been found on the cell surface (Wiest et al., 1997). The carbohydrate expressed by surface grp94 is also completely Endo H sensitive (Figure 4-11). These data indicate that during trafficking of resident ER proteins to the cell surface, the carbohydrate expressed by these proteins is not modified in the Golgi.

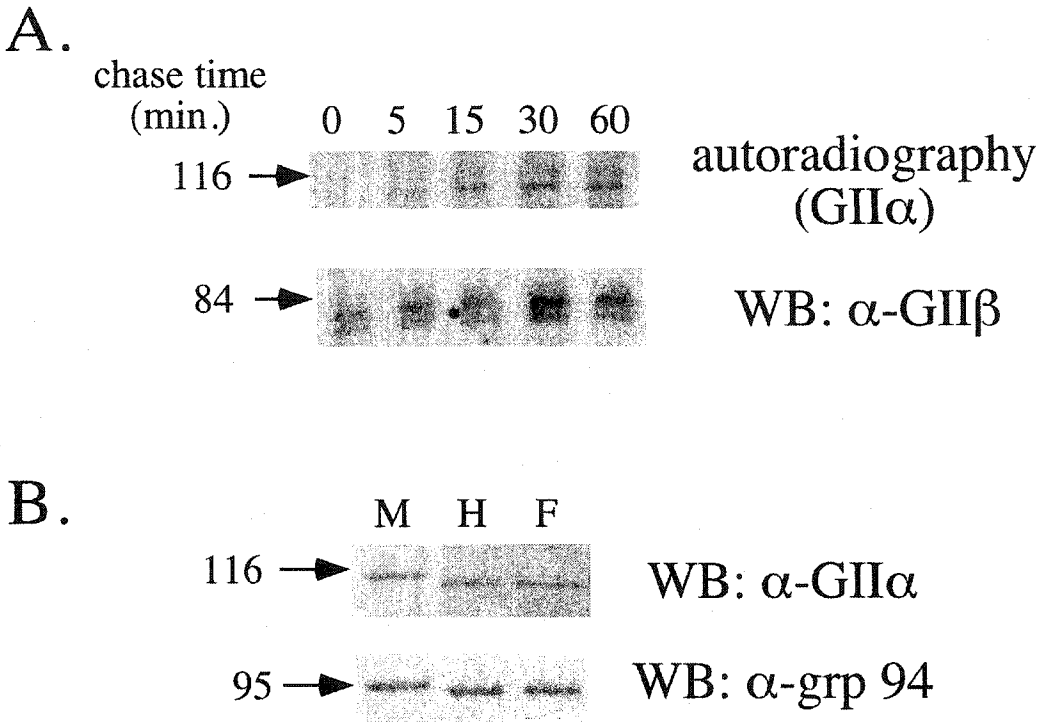


Figure 4-11: Newly synthesized GIIα traffics rapidly to the cell surface, and steady state surface GIIα possesses Endo H sensitive carbohydrate.

A. Cells were pulsed and chased as in Materials and Methods and surface GII was isolated by the antibody method. Isolated GII was resolved by SDS-PAGE and subjected to autoradiography for visualization of newly synthesized GIIα (top panel). Identity of GIIα was confirmed by western blot analysis (data not shown). Western blot analysis for isolated GIIβ was performed as a control (bottom panel). B. Surface biotinylated proteins were isolated with immobilized streptavidin. Bound proteins were treated with Endo H (H) or Endo F (F) under reducing and denaturing conditions followed by separation by SDS-PAGE. GIIα (top panel) and grp94 (bottom panel) were detected by western blot.

D. DISCUSSION

We have previously demonstrated the specific interaction between CD45 and GII, and that this association is capable of modifying the carbohydrate on CD45 (Baldwin and Ostergaard, 2001). Because of recent reports implicating carbohydrate in the regulation of cellular processes and the importance of CD45 in T-cell function, we have further investigated the association between CD45 and GII specifically examining the association in the early secretory pathway, as well as the trafficking characteristics of CD45.

The interaction between CD45 and GII is initiated rapidly after synthesis and is maintained stably through the early secretory pathway to the cell surface (Figure 4-1). The finding that CD45 and GII traffic as a complex offers a potential molecular explanation for the finding that GII can regulate the carbohydrate on CD45 by presumably protecting it from processing by resident Golgi enzymes. This protection may maintain the carbohydrate structure required for the binding of CD45 by MBL. A similar situation was observed with CD3 and calnexin where the association protected the carbohydrate on CD3 from processing and resulted in the maintenance of their association on the cell surface (Wiest et al., 1997).

Because the association between CD45 and GII is maintained during the trafficking to the cell surface, we should be able to detect GII itself on the cell surface. In fact, we do see cell surface expression of GII by two distinct methods, namely cell surface biotinylation and FACS analysis (Figure 4-2). This finding is

somewhat surprising since GII is a putative resident ER protein, however, many ER proteins such as calnexin (Okazaki et al., 2000), calreticulin (Arosa et al., 1999), gpr94, BiP (Wiest et al., 1997), and PDI (Mezghrani et al., 2000) have been detected on the cell surface. Interestingly, CD45 does not appear to be required for the cell surface expression of GII as GII is also detected on the cell surface in CD45-deficient cell lines. However, GIIα may play a role in the trafficking of the GII heterodimer to the cell surface as there is less GIIβ found on the cell surface of the GIIα-deficient BW/PHA^r cells compared to BW wildtype cells (Figure 4-2). The mechanism for the trafficking of these resident ER proteins to the cell surface is not obvious as there are multiple ways ER proteins can be retained in the ER or returned to the ER if they do escape (Teasdale and Jackson, 1996).

Unexpectedly, in examining the trafficking of the CD45-GII complex to the cell surface, we observed a striking pattern of CD45 cell surface expression. There appears to be two different glycoforms of CD45 that reach the cell surface, each with different kinetics. The lower molecular weight glycoform arrives within five minutes and achieves maximum expression at fifteen minutes, while the higher molecular weight form appears after fifteen minutes and increases through sixty minutes. The rapidity with which both forms reach the cell surface compared to other cell surface glycoproteins was quite surprising. The GII complex also reaches the cell surface five minutes after synthesis. This rapid trafficking of CD45 was confirmed by examining the rate of acquisition of Endo H resistance. As illustrated by Figure 4-4a, there is a shorter lag period in the

time required for CD45 to acquire Endo H resistance than there is with Class I MHC. This suggests CD45 does not remain in the ER long after synthesis, rather it traffics quickly to the Golgi where its carbohydrate can be processed. Given the size and number of carbohydrate additions on CD45, it is quite surprising that there is not a long period of folding required for CD45 to achieve a transport competent conformation. In fact, examining the rate of protein folding using non-reducing/denaturing conditions indicates CD45 achieves its fully folded conformation soon after synthesis (Figure 4-5).

Interestingly, the rapidly expressed, lower molecular weight pool of CD45 contained exclusively Endo H sensitive carbohydrate, while the higher molecular weight CD45 possessed its fully processed carbohydrate. This finding suggests that the rapidly expressed pool by-passes the Golgi complex where the enzymes required to convert N-linked carbohydrate from Endo H sensitive to resistant reside. Its has been suggested that the minimal time required for transit through the Golgi is ten minutes. (Williams et al., 1985) and the finding that the rapidly expressed pool appears after five minutes supports the hypothesis that this pool of CD45 may by-pass the Golgi. The steady state GIIα and grp94 expressed on the surface also contains entirely Endo H sensitive carbohydrate, which suggests these proteins by-pass the Golgi as well. In addition, treatment of cells with BFA, which disrupts the Golgi, still permits the trafficking of the lower molecular weight CD45 to the cell surface while the higher molecular weight form of CD45 and Class I MHC was completely prevented from trafficking to the plasma membrane. Finally, in experiments where a block at the TGN was imposed, this

resulted in the abrogation of the higher molecular weight CD45 and Class I MHC from the cell surface, but the lower molecular weight CD45 still appeared. Collectively, these data support the by-pass of the Golgi complex by the rapidly expressed, lower molecular weight cell surface CD45. Given the rapidity with which GIIα is expressed on the surface and the finding that surface GIIα and grp94 N-linked carbohydrate is Endo H sensitive suggests that the resident ER proteins found on the cell surface may also by-pass the Golgi complex.

There are a few examples of transport pathways which by-pass the Golgi including rotavirus production and cystic fibrosis transmembrane conductance regulator (CFTR) transport. Examination of CFTR trafficking demonstrated that there was a two-fold decrease of CFTR in the Golgi compared to the ER, with limited amounts in both the cis-Golgi and TGN (Bannykh et al., 2000). Subsequently, it was also observed that CFTR export required the machinery of the early secretory pathway, but after leaving the ER in COPII vesicles, the remaining transport utilized a non-conventional pathway (Yoo et al., 2002). In examining rotavirus particle release, it was observed that viral particle production was dependent on the ER, however disruption of the Golgi complex by treatment with monensin had no impact on viral particle release (Jourdan et al., 1997). Interestingly, cholesterol transport has recently been demonstrated to occur in a BFA insensitive manner. It reached the cell surface with a halftime of approximately ten minutes, but its expression was reduced by a 15°C chase (Heino et al., 2000). These characteristics are reminiscent of the transport kinetics and BFA sensitivity shown by the rapidly expressed pool of CD45.

Consistent with the model of CFTR trafficking we hypothesize that all CD45 utilizes the machinery of the early secretory pathway, but the rapidly expressed, newly synthesized lower molecular weight CD45 by-passes the Golgi on route to the cell surface, while the higher molecular weight form traffics through the Golgi on its way to the cell surface.

In examining the effect of BFA on the disruption of Golgi structure, it has been observed that different Golgi constituents re-localize to different areas of the cell post-BFA treatment (Klumperman, 2000). BFA prevents transport by inhibiting a guanine nucleotide exchange factor (GEF) for the ARF-1 GTPase which is thought to be required for the recruitment of COPI to the vesicle membrane. Interestingly, examination of the ER has revealed that BFA has little to no impact on the structure of ER exit sites (Ward et al., 2001). ER exits sites are believed to be the place where vesicles bearing cargo leave the ER for trafficking to the intermediate compartment. Since BFA has little impact on the structure of ER exit sites, it is conceivable that vesicles with no requirement for BFA sensitive COPI trafficking, containing cargo destined for the cell surface independent of Golgi, would be able to effectively reach the cell surface in the presence of BFA. In support of a COPI independent transport route from the ER to the ERGIC, work by Scales et al., demonstrated the sequential action of COPII and COPI for ER to ERGIC trafficking and ERGIC to Golgi trafficking, respectively (Scales et al., 1997). In the event that COPI is required for ER to ERGIC transport, a BFA-resistant GEF for ARF-1 was recently cloned (Claude et al., 1999). In support of either a COPI independent or BFA resistant COPI

dependent mode of transport, a member of the connexin family of gap junction proteins was also shown to traffic to the cell surface in a BFA insensitive manner (Martin et al., 2001). As well, with respect to CFTR trafficking, a dominant negative ARF-1 construct had no effect on the transport of CFTR (Yoo et al., 2002) Therefore, a BFA insensitive GEF, or a COPI independent mechanism may be responsible for the trafficking of the rapidly expressed pool of CD45 to the cell surface.

Another intriguing observation regarding the trafficking of CD45 is that only the higher molecular weight glycoform of CD45 is affected by BFA and therefore presumably uses the conventional trafficking pathway. This suggests that during ER export, CD45 is either actively directed, or sorted, into vesicles that utilize the BFA resistant pathway, or that entry into those BFA insensitive vesicles is random and based on the proximity of CD45 to those vesicles. Since GII and possibly other resident ER proteins utilize the non-conventional pathway, they may also be sorted into those vesicles or randomly be included in those transport vesicles. With respect to the active sorting of CD45, it is conceivable that GII may be involved in this selection processes, however experiments designed to demonstrate GII dependent sorting of CD45 did not reveal such a role for GII. It is possible that other proteins may be involved in this function such as CD45-AP since this protein appears to associate with CD45 primarily in the ER (Cahir McFarland et al., 1997). In fact, sorting within the ER has been reported in a number of different situations. One recent report examined the trafficking of Class I MHC out of the ER and found that the cargo receptor BAP 31 specifically

associated with Class I MHC and this interaction may be involved in directing peptide loaded Class I to ER exit sites (Spiliotis et al., 2000). Additionally, in studying cholesterol transport from the ER, it was observed that newly synthesized cholesterol was found in ER transport vesicles distinct from those carrying VSV cargo (Urbani and Simoni, 1990). These data provide support for cargo sorting within the ER.

To this point it is unclear if the rapidly expressed pool of CD45 traffics through the ERGIC before moving to the plasma membrane. It is entirely possible that once leaving the ER in a BFA insensitive manner, vesicles containing CD45 fuse directly with the plasma membrane. Since COPI has been implicated in transport at the level of the ERGIC and BFA does not prevent the rapidly expressed population of CD45 from reaching the cell surface, it is likely that the BFA resistant vesicles do not traffic through the ERGIC, rather they fuse directly with the plasma membrane. In support of this model, electron micrograph images of the internal architecture of the cell revealed a close juxtaposition of ER membranes with the plasma membrane (Ladinsky et al., 1999). Our data also agree with this model. First, given the rapidity with which CD45 reaches the surface, it seems unlikely that transport through the ERGIC would occur. Additionally, BFA treatment tends to disperse the ERGIC leaving matrix proteins normally localized to this pre-Golgi compartment in Golgi remnant bodies, so traffic through this compartment would be blocked. Finally, the 15°C blockade, which blocks protein transport at the ERGIC, is only partially effective at preventing the transport of this rapidly expressed population of CD45.

This partial block could simply be due to the decrease in metabolic processes which would accompany growth at 15°C, and has been observed with cholesterol transport as well (Heino et al., 2000). Therefore, upon exit from the ER, we posit that BFA insensitive vesicles containing CD45 fuse directly with the plasma membrane (Figure 4-12).

In addition to the possibility that an alternative vesicle mediated transport pathway exists, a direct ER to plasma membrane fusion event could explain the finding of rapidly expressed, unprocessed CD45 on the cell surface. Recently, Gagnon et al. described the direct fusion of the ER with the plasma membrane during phagocytosis (Gagnon et al., 2002). By a similar mechanism, fusion of ER containing newly synthesized CD45 with the plasma membrane could account for our observations. As well, this mechanism may explain the expression of putative resident ER proteins on the cell surface. To discriminate between a vesicular transport and direct fusion mechanism, introduction of a dominant negative COPII component, Sar1, which would block vesicular ER export, but likely not direct ER to plasma membrane fusion, could be performed.

The expression of cell surface CD45 containing completely unprocessed carbohydrate is somewhat unexpected, however this pool of CD45 may have important biological implications. First of all, this lower molecular weight form of CD45 can be detected on the cell surface at steady state levels, which suggests this is a constitutive pathway resulting in a stable pool of protein (Figure 4-3b). Because this glycoform contains completely unprocessed carbohydrate, it likely contains both terminal glucose and mannose residues on its glycans. These

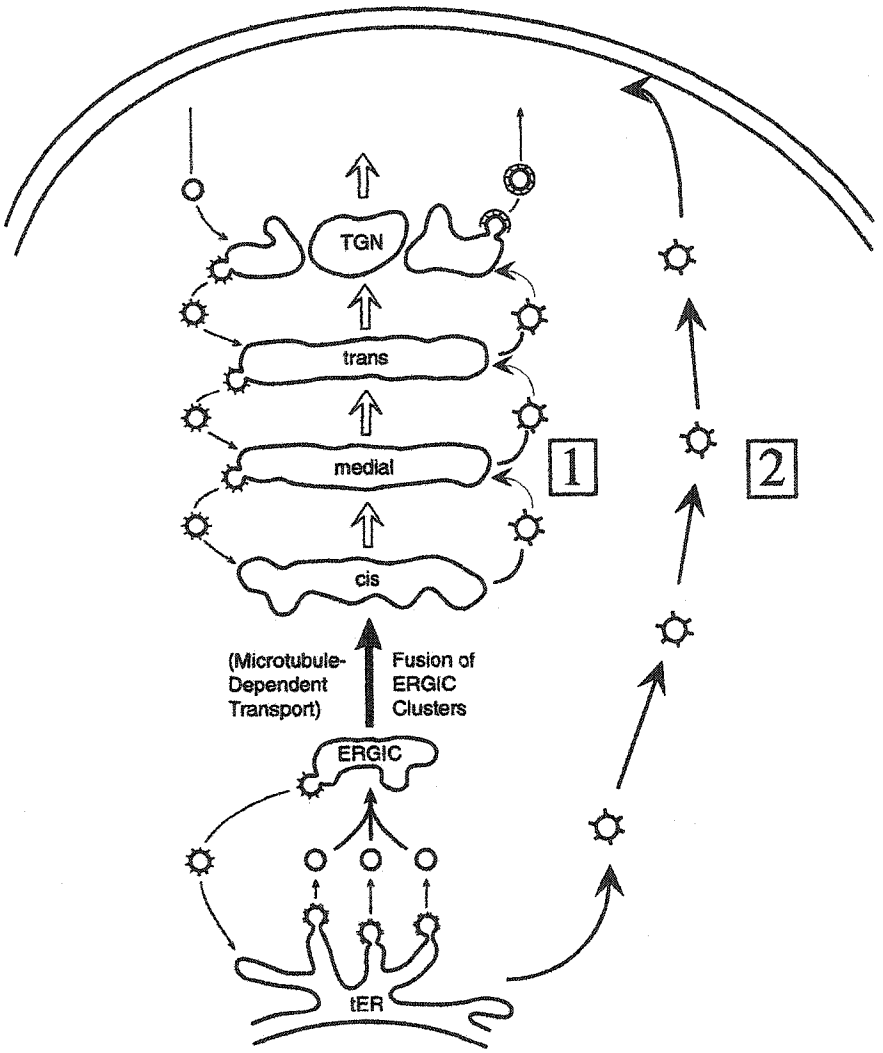


Figure 4-12: A model for the rapid expression of CD45 which by-passes both the ERGIC and Golgi and fuses directly with the plasma membrane.

A portion of newly synthesized CD45 is expressed rapidly on the cell surface. The mechanism for expression may be due to vesicular transport as depicted above, or involve the direct fusion of the ER with the plasma membrane. Other resident ER proteins such as GII and grp94 may also utilize either possibility (figure adapted from (Glick and Malhotra, 1998)).

carbohydrate residues on cell-surface CD45 have been demonstrated to be recognized by lectins such as MBL (Baldwin and Ostergaard, 2001). Lectin recognition of CD45 could have many implications in T cell biology including the modulation of adhesion, cellular migration, mobility of CD45 within the plasma membrane, as well as signaling thresholds.

Chapter 5: General Discussion

A. Summary of Results

The biochemical basis for the association between CD45 and GII is primarily lectin in nature. That is, high mannose containing carbohydrate on CD45 is bound by the active site of GII. Therefore, the active site of GII is providing the lectin binding function. However, the active site of GIIα is not sufficient to mediate stable binding, as an alternatively spliced sequence, Box A1, found within GIIα is necessary for the sustained interaction. The requirement for Box A1 indicates this association is specific and not simply a consequence of a glycoprotein interacting with an enzyme capable of modifying its carbohydrate. As well, mannose residues on CD45 carbohydrate play a key role in initiating the association which again demonstrates specificity of the lectin activity of Box A1 containing GIIα.

In addition to the specificity of the interaction dictated by both GII and the carbohydrate found on CD45, the association is developmentally regulated and appears to have the potential to modify the carbohydrate expressed on CD45. The association between CD45 and GII is readily detected in immature T cells, such as thymocytes, but is significantly reduced in mature T cells, including splenic T cells. In addition to the CD45-GII interaction found in immature cells, surface carbohydrate from immature cells is recognized by a mannose specific lectin, MBL. Importantly, surface CD45 from immature cells is among those glycoproteins recognized by MBL, even though surface CD45 from both mature

and immature cells contains high mannose carbohydrate. Finally, GII activity has the capability of regulating the expression of MBL ligands on the surface of cells, such that reduction in GII enzymatic activity in mature cells results in an increase in MBL surface ligands. CD45 is a member of those proteins whose surface carbohydrate is regulated by GII activity.

Surface glycoproteins capable of being recognized by MBL opposes the widely held views on carbohydrate processing during trafficking of glycoproteins to the cell surface. Generally, as proteins traffic to the plasma membrane, N-linked carbohydrate is processed in the Golgi to a form lacking terminal mannose. CD45 however appears to utilize two different routes during transport to the plasma membrane. One route involves the Golgi complex, while the other appears to by-pass the Golgi. This alternative route to the cell surface can account for at least one pool of surface CD45 containing high mannose carbohydrate.

B. Biochemical Features of the association between CD45 and GII

Bulk Carbohydrate on CD45

In comparing the carbohydrate content of surface CD45 from immature and mature cells, it is becoming increasingly clear that the glycans expressed by these two cell types are not equivalent. This heterogeneity represents yet another variable in the study of CD45. The bulk population of CD45 N-linked carbohydrate expressed by mature or immature populations of T cells is similar in that they both appear to possess equal amounts of Endo H sensitive and resistant

forms. This observation itself is quite unexpected considering that most carbohydrate expressed on surface glycoproteins is thought to be Endo H resistant. Although we suspect GII is involved in the expression of cell surface CD45 containing Endo H sensitive carbohydrate in immature cells, another mechanism must exist in mature T cells. Closer inspection of CD45 carbohydrate reveals that the fine structure of the glycans on surface CD45 from immature cells is different from those found on mature cells. This difference is illustrated by the ability of MBL to bind to surface CD45 and probably other glycoproteins from immature T cells but not from mature T cells. In fact, examining the carbohydrate found on one isoform of CD45 from immature cells reveals there are at least two different glycoforms of that isoform. This was demonstrated using differential sugar competition where MBL binding of only one glycoform was sensitive to glucose competition, while both forms were sensitive to mannose competition. It is unclear to this point whether or not GII exhibits a preference for either one of these glycoforms, but we do know that GII can bind to at least one of the forms. These subtle differences in carbohydrate structure, which can potentially influence biological outcomes, illustrate the need for closer inspection of glycans when studying CD45 or any other glycoprotein.

Terminal Glucose on CD45 Carbohydrate

Another interesting feature of the association between CD45 and GII is the ability of their interaction to be reconstituted *in vitro*. Since the association can be re-capitulated, it suggests that the carbohydrate components on CD45 required

to initiate binding with GII remain even after CD45 and GII have previously interacted. Because the association is dependent on the active site of GII and the active site recognizes glucose, this suggests that the initial binding does not cleave the glucose, rather this residue remains. A potential mechanism to support this hypothesis is that when bound to CD45, the enzymatic activity of GII may be reduced leaving a percentage of carbohydrate on CD45 with terminal glucose. A reduction in enzymatic activity of GII may also contribute to the stability of the CD45-GII association. It is also conceivable that because CD45 traffics so rapidly out of the ER to the Golgi, and because there are so many carbohydrate moieties on CD45, GII does not have sufficient time to process all the glycan additions leaving some with terminal glucose. Some of the carbohydrate moieties on CD45 are fully processed requiring the cleavage of glucose, and this cleavage could be performed by either GII or a post-ER endomannosidase (Lubas and Spiro, 1987; Zuber et al., 2000). In support of the maintenance of terminal glucose on CD45 carbohydrate, MBL recognition of one glycoform of CD45 can be inhibited with the inclusion of glucose. As well, the rapidly expressed population of surface CD45 contains entirely Endo H sensitive glycans, and because it is completely Endo H sensitive, it may contain terminal glucose residues.

C. Carbohydrate Protection Model

Given the ability of GII activity to regulate the expression of MBL ligands on the surface of cells and the correlation between the cell types that express

surface MBL ligands and have GII associated CD45, we have developed a model in an attempt to reconcile these data. Upon translation and addition of N-linked carbohydrate, GII can associate with CD45 in the ER as GII is thought to do with all glycoproteins. Some of these interations are likely productive and result in the cleavage of the terminal glucose moiety, while others, particularly those involving Box A1 positive forms of GIIα, remain stably associated (Figure 5-1). To this point it is unclear what features of this association result in a stable interaction, nonetheless, the binding remains intact. Once directed to leave the ER, the CD45-GII complex presumably travels through the Golgi and finally to the cell surface (Figure 5-1). Because of the associated GII, other resident ER and Golgi carbohydrate processing enzymes are prevented from accessing and modifying the carbohydrate bound by GII. The lack of processing results in the maintenance of the carbohydrate structure found on CD45 allowing a potential lectin such as MBL to bind. In agreement with this model, CD45 and GII associate rapidly after synthesis and remain stably associated as CD45 traffics to the surface. This protection provides one possible mechanism for the appearance of Endo H sensitive carbohydrate on surface CD45. There must be other mechanisms in place in mature cells to prevent the processing of carbohydrate on CD45 to an Endo H resistant form as surface CD45 from both immature and mature cells bear similar amounts of Endo H sensitive carbohydrate. This carbohydrate from mature cells however, contains different terminal sugars as illustrated by the lack of MBL recognition in mature T cells. In addition, this protection of carbohydrate

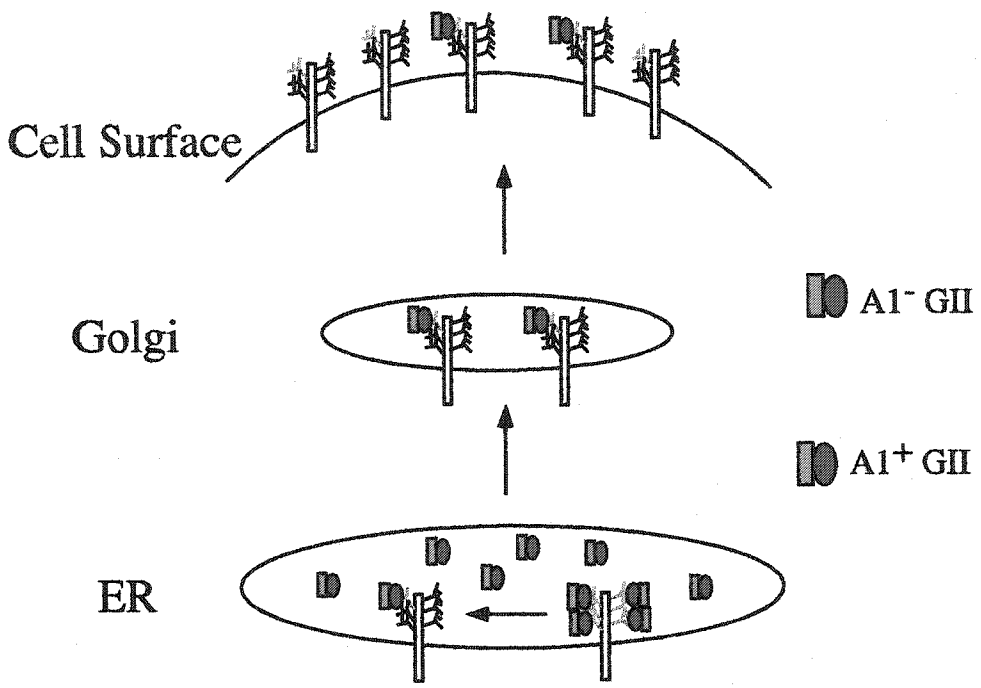


Figure 5-1: A model for the protection of CD45 carbohydrate in immature cells.

CD45 and GII associate in the ER. Box A1 containing GII remains stably associated with CD45, and the CD45-GII complex traffics together through the Golgi and finally to the cell surface. By remaining stably associated, the GII can protect the carbohydrate on CD45 from further processing by ER and Golgi enzymes. The carbohydrate on CD45 that is protected from processing by GII is shown in orange.

in immature cells probably applies to other glycoproteins as CD45 is not the only surface protein recognized by MBL. In fact, chains of the CD3 complex reach the cell surface with terminal glucose bearing carbohydrate (Wiest et al., 1997), and this carbohydrate is likely able to be recognized by MBL.

D. CD45 and Lectins

Because the binding of MBL to surface glycoproteins appears to be developmentally regulated, and thymocytes express MBL ligands, it is conceivable that these carbohydrate structures play a functional role in the thymus. In addition, although CD45 appears to be the major protein bearing the appropriate carbohydrate for MBL recognition, there are other proteins expressed with the required carbohydrate structure. Therefore, we believe there is an overall change in the carbohydrate expression pattern seen in thymocytes. This idea of developmentally regulated changes in carbohydrate structure is not a new hypothesis, but recently has been supported by a number of different studies (Lefrancois, 1987; Reed et al., 1998; Uemura et al., 1996; Wu et al., 1997). As well, because MBL ligands appear to be up-regulated at the double positive stage of thymocyte differentiation, it is likely that the influence of these carbohydrate structures occurs at the positive or negative selection checkpoints. We posit there are many possible ways lectin recognition of carbohydrate structures on thymocytes can impact T cell development. Additionally, the lectins may be soluble in nature, or expressed on the cell surface by thymic stromal cells. These

interactions may be specific for CD45, or include other glycoproteins bearing similar carbohydrate structures.

Lectins Expressed on Thymic Stromal Cells

The first proposed way in which carbohydrate can influence selective events is through lectins expressed by thymic stromal cells interacting with glycoproteins on thymocytes bearing the appropriate carbohydrate structure (Figure 5-2). This interaction may be important for adhesion between the thymocyte and stromal cell ultimately affecting the signal strength seen by the thymocyte. Since signal strength is thought to have a major effect on the process of positive selection (Sebzda et al., 1999), and changes in adhesion can impact signal strength, adhesion may influence selective events. In fact, there have recently been described a number of putative lectins expressed in the thymus and each of these may be a potential regulator of positive selection (Ariizumi et al., 2000a; Ariizumi et al., 2000b; Bates et al., 1999). Consistent with lectin interactions influencing positive selection, recent reports suggested that carbohydrate moieties on thymocytes do impact selective events. For example, sialic acid residues expressed on CD8 can mediate binding to non-cognate Class I MHC. This interaction has been demonstrated to affect adhesion of DP thymocytes to Class I MHC (Daniels et al., 2001), and mice deficient in the ability to sialylate CD8 showed a skewing in their TCR repetoire (Moody et al., 2001). Interestingly, CD45-deficient mice also have an altered TCR repetiore such that the T cells being positively selected are self-reactive (Trop et al., 2000).

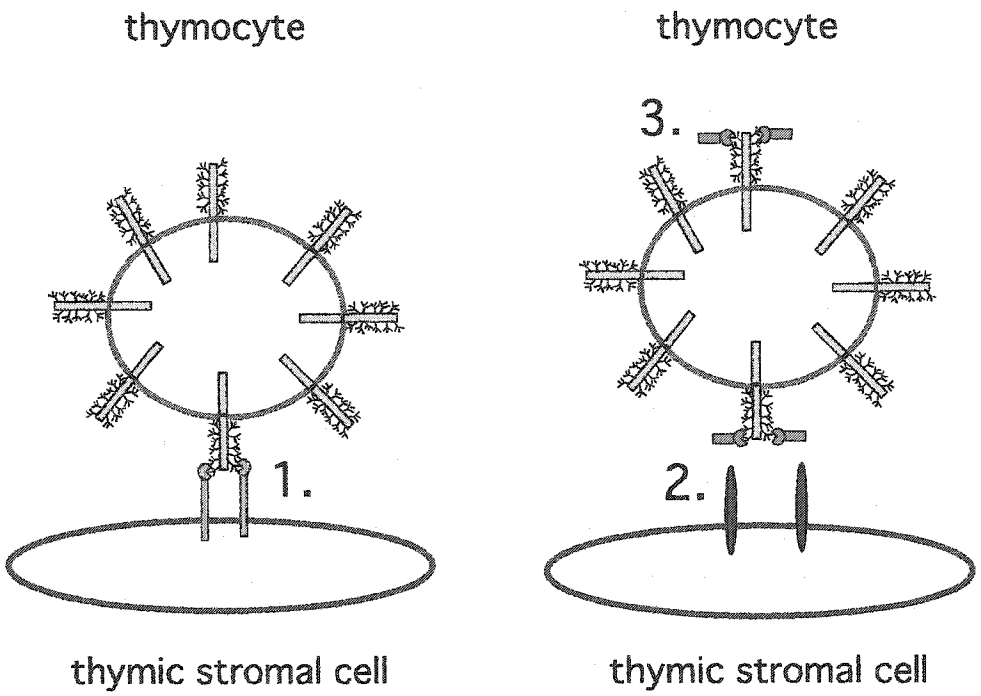


Figure 5-2: Possible modes of interaction between lectins within the thymus and thymocytes.

Depicted are three possible ways that lectins found in the thymus could interact with thymocytes and influence thymic events. Lectins in the thymus may be expressed either on the surface of stromal cells (1), or in soluble form (2,3). These interactions could affect adhesion between thymocytes and thymic stromal cells directly (1,2), or impact the biochemical properties of the glycoprotein on the thymocyte including plasma membrane mobility, or enzymatic activity (3). The outcome of these interactions may lead to changes in the signals received by the thymocyte, which could ultimately impact positive selection events.

Therefore, changes in carbohydrate on thymocytes can directly influence the peripheral T cell population. In addition to affecting adhesion, ligation of receptors on thymocytes by lectins may affect their enzymatic properties, thereby altering signaling thresholds and directly influencing selective events.

One role for mannose specific lectins on cell types such as macrophages and dendritic cells is to phagocytose bacteria and other particles containing high mannose carbohydrate. Since the majority of double positive thymocytes die by apoptosis after negative selection, there must be a mechanism in place to remove this cellular debris. Double positive thymocytes express the highest level of MBL ligands, so apoptotic bodies from DP thymocytes would likely contain a high level of high mannose carbohydrate. Since macrophages have been proposed to play a role in the clearance of these apoptotic bodies from the thymic environment (Surh and Sprent, 1994), this recognition of high mannose carbohydrate could be used as a potential mechanism by thymic macrophages to scavenge the apoptotic remnants from the large amount of death of DP thymocytes.

Soluble Lectins

It is also possible that soluble lectins found within the thymus could bind glycoproteins expessing the appropriate carbohydrate, eliciting a number of possible outcomes. Binding of a soluble lectin to thymocyte glycoproteins may decrease adhesion of the thymocyte to the stromal cell by preventing the binding to a cognate ligand, or affect the enymatic activity of the glycoprotein by preventing ligation by its ligand (Figure 5-2). This blockage could alter the signal

strength seen by thymocytes. A recent report examining the effect of galectin-3 on thymocyte adhesion supports this hypothesis (Villa-Verde et al., 2002). Recently, carbohydrate structures have been implicated in regulating receptor mobility within the plasma membrane (Demetriou et al., 2001). Given the finding of CD45 movement at the T cell-APC interface, and the fact that thymocytes undergoing positive selection form an immune synapse (Richie et al., 2002), carbohydrate structures may be important for regulating the movement of CD45 or other proteins into and out of the T cell-APC contact area. Finally, there may also be direct regulation of the receptor through the binding of a soluble lectin to carbohydrate on the receptor (Figure 5-2). This hypothesis has direct implications on the activity of CD45. If the dimerization model of CD45 regulation is correct, binding of CD45 by lectins, which are normally mulitmeric, could potentially dimerize or multimerize CD45 and therefore alter its enzymatic activity. In support of soluble lectins binding to CD45 having effects on thymic events, addition of soluble CD45 antibodies to fetal thymic organ culture (FTOC) has previously been demonstrated to impact selection events (Benveniste et al., 1994). Regulation of activity may again affect signal strength and therefore selection.

CD45, Lectins and Apoptosis

In addition to the effects of lectin binding on positive selection, ligation of CD45 in particular by any means mentioned above could induce an apoptotic program in the thymocyte. In fact, other lectins, namely galectin-1, have been shown to bind CD45 in a carbohydrate dependent manner and induce apoptosis in

thymocytes (Nguyen et al., 2001). As well, cross-linking of CD45 on double positive thymocytes with immobilized mAb can induce an apoptotic pathway (Lesage et al., 1997). Soluble lectins may be particularly effective for inducing apoptosis as they are generally multimeric in nature and could possibly cross-link a number of CD45 molecules. MBL is an important molecule in innate defenses due to its recognition of high mannose carbohydrate structures expressed on bacteria and the subsequent lysis of the bacteria via the complement system (Kawasaki, 1999). Given the ability of MBL to preferentially bind CD45 on double positive thymocytes, the role MBL plays in innate immunity and the propensity with which double positive thymocytes die, there is a possible role for CD45 carbohydrate recognition by MBL in apoptosis of DP thymocytes.

Future Directions

It will be of great interest to determine the effect CD45 carbohydrate and carbohydrate in general has on thymocyte development, particularly positive and negative selection. Of specific interest is the role of terminal glucose and mannose containing glycans. To examine the effects of carbohydrate on thymic development, possibly the best technique to use is fetal thymic organ culture (FTOC). With this technique, the architecture of the thymus remains intact, while various reagents can be added to the culture to observe their effect on thymic events. This approached has been used in the past to study the role of CD45 in thymic selection (Benveniste et al., 1994). For example, individual monosaccharides such as glucose or mannose can be added to FTOCs in an

attempt to block the interaction of a mannose or glucose specific lectin. As well, soluble lectins such as MBL can be added to block carbohydrate structures on the thymocyte and prevent ligand binding. MBL can also be used to cluster CD45 and observe the effects of lectin induced multimerization. Further, inhibitor compounds such as the GII specific inhibitor can be added in an attempt to globally modify the carbohydrate structure expressed by thymocytes. Changes in selective events can be monitored by standard FACS based assays for thymocyte differentiation markers. As for the possibility of changes in the removal of apoptotic thymocytes by scavenger receptors, FTOC can also be used, where the apoptotic thymocytes can be identified by a FACS based approach. To monitor the effect of MBL on apoptosis, a more in vitro system may be of greater utility. MBL can be immobilized, thymocytes allowed to interact with the lectin and the apoptotic outcome can ultimately be measured (Lesage et al., 1997). Overall, these *in vivo* and *in vitro* experiments can allow a further clarification of the role carbohydrate plays in thymic biology. The results can then focus attention on one particular aspect of thymic development for intensive study.

E. CD45 Trafficking

An unexpected, yet interesting finding was made when examining the trafficking of the CD45-GII complex. CD45 was found to utilize two different pathways during transit to the cell surface. One route was the well characterized Golgi and COPI dependent transport pathway, while the other appeared to by-pass the Golgi complex and not rely on a COPI dependent mechanism. This

alternative route allowed the rapid expression of CD45 possessing completely unprocessed carbohydrate.

CD45 Carbohydrate

Utilization of the non-conventional pathway for surface expression by CD45 provides another mechanism for the maintenance of carbohydrate in an unprocessed form. By-passing the Golgi complex eliminates the need for carbohydrate protection by associated proteins. The rapidly expressed form of CD45 possesses entirely Endo H sensitive carbohydrate, likely containing terminal glucose and mannose residues which could be recognized by glucose or mannose specific lectins, such as MBL. Lectin binding may influence any of the thymic events discussed above.

Rapid and Directed Expression of CD45

In addition to its positive effect on T cell activation, CD45 has also been suggested to have a negative effect on the interaction between a T cell and APC. One mechanism CD45 could use to exert a negative effect on the interaction between a T cell and APC is through steric interference. Another way CD45 could adversely affect T cell activation is through a global dephosphorylation by the phosphatase activity of CD45. Originally, CD45 was suspected to be excluded from the contact site between a T cell and APC. This hypothesis was mainly generated because of the potential steric effects CD45 could have on T cell-APC conjugation (Shaw and Dustin, 1997), but a global dephosphorylation

would also be detrimental to T cell-APC interactions as phosphorylation dependent cytoskeletal rearrangements are necessary for a sustained interaction. Therefore, if CD45 were localized to the contact site, or SMAC, the binding of the TCR to MHC could be negatively affected by the size of CD45 and signals required for T cell activation could be blunted due to global dephosphorylation. This exclusion hypothesis was tested and found to be somewhat incorrect. Early formation of the SMAC resulted in the loss of CD45 from the initial contact area, however, after a short period of time, CD45 was found to co-localize with the TCR in the centre of the SMAC (Johnson et al., 2000). Interestingly, upon engagement of the TCR a re-orientation of the microtubule organizing centre occurs such that intracellular traffic is directed toward the engaged TCR (Egen and Allison, 2002; Kupfer et al., 1987). Since the pool of CD45 that by-passes the Golgi is rapidly expressed, this trafficking pathway provides a possible mechanism for the expression of CD45 at the SMAC and a way for CD45 to down-regulate the immune response. Clearly, in the work by Johnson *et al.* (Johnson et al., 2000), the fluorescently labeled CD45 found in the central region of the synapse was derived from cell surface material, however this does not preclude the possible contribution of newly synthesized CD45. It is also possible that new CD45 expression at the T cell-APC interface is required to stabilize the SMAC by keeping Lck in an active conformation, allowing for sustained signaling.

CD45 Expression during Mitosis

During mitosis, the Golgi complex is disassembled. As a result, the trafficking of proteins to the cell surface is prevented during this stage. For T cells and thymocytes, some of the highest proliferative times occur either immediately before or after the cell has responded to an extracellular stimulus. It has been demonstrated quite conclusively that CD45 is required to respond to TCR engagement. This response could be due to challenge by a pathogen in the periphery, or during positive and negative selection in the thymus. Therefore, to maintain responsiveness to TCR stimulation, it may be essential to maintain a certain level of new expression of CD45 on the surface. With the Golgi complex disassembled, proteins using the Golgi for transport will not be able to effectively reach the surface. The non-conventional pathway offers a alternative to Golgi dependent trafficking and can allow the expression of proteins during or immediately after division. This pathway could then ensure a continuous level of CD45 expression at all times during the cycle of cell for a rapid response to TCR engagement.

Future Directions

Other than the demonstration of the existence of the Golgi independent pathway for rapid CD45 surface expression, the mechanism behind this transport is currently unknown. In a report examining the trafficking of CFTR, components of the traditional COPI mediated trafficking through the Golgi appeared to be

dispensable. It will be of interest to determine the factors important for this novel transport pathway. Techniques such as confocal microscopy as well as cellular fractionation can be employed in an attempt to directly identify the BFA resistant transport vesicles. Once the vesicles can be readily identified, standard biochemical approaches can be taken to characterize this pathway. It also appears as though a population of CD45 is directed to the non-conventional transport pathway. It is unclear which protein(s) is involved in this sorting function. One potential candidate protein is CD45-AP. CD45-AP is a small protein that associates with CD45 through transmembrane domain interactions. These characteristics are reminiscent of the interaction between Class I MHC and BAP-31. Therefore, it will be of interest to determine if CD45 sorting does take place, and what proteins are involved in the sorting process.

F. Final Conclusions

The work in this study has highlighted the importance of examining the carbohydrate structures expressed by glycoproteins, and illustrated possible mechanisms for achieving surface expression of glycoproteins bearing different carbohydrate structures. Not only are there significant differences in the glycans expressed by different glycoproteins, but within the same glycoprotein, different carbohydrate structures can be expressed. The differences in carbohydrate structure may have impacts on fundamental processes such as thymic development. Therefore, when studying a particular glycoprotein in the context

of biological process, it is necessary to examine differences in post-translation modifications such as carbohydrate addition.

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