CONSTRUCTION, EXPRESSION AND CHARACTERIZATION OF CD45-IMMUNOGLOBULIN FUSION PROTEINS

by

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ABSTRACT

The aim of this work was to create, express, and characterize fusion proteins consisting of different alternatively spliced exons of murine CD45, a protein tyrosine phosphatase, linked to the heavy chain constant regions of murine immunoglobulin G. CD45-immunoglobulin fusion proteins were secreted as dimers in a relatively pure form using serum free media at an approximate yield of 1.5-4.5 μg/ml, depending on the isoform of CD45 and the cell line in which it was expressed. Fusion proteins secreted by Cos 7 cells had a higher apparent molecular weight by approximately 5-10 kDa than those expressed by X63-Ag8.653 or T28 cells.

The interaction of CD45 with putative ligands may be mediated by specific carbohydrate residues on CD45, therefore, the carbohydrate residues expressed on CD45-immunoglobulin fusion proteins were characterized. O-glycosidase digestion and lectin analysis revealed that all fusion proteins were extensively O-glycosylated in a cell-specific manner. Neuraminidase digestion and analysis of subsequent Peanut agglutinin reactivity suggested that fusion proteins secreted by Cos 7 cells expressed more sialic acid when compared to that secreted by X63-Ag8.653 or T28 cells. Thrombin cleavage and PNGase F digestion revealed that the immunoglobulin portion was 34 kDa and the only site of N-linked carbohydrate addition.

All fusion proteins reacted with anti-CD45 exon-specific antibodies as predicted with the exception of RA3 6B2, a B220-specific antibody that reacted with CD45RABC-Ig expressed by Cos 7 cells but not with that expressed by X63-Ag8.653 or T28 cells. RA3 6B2 reacted with fusion proteins containing exons A, B, and C inclusive in addition to fusion proteins containing only exon A. RA3 6B2 binding was not affected by neuraminidase treatment, but did correlate to the binding of wheat germ agglutinin.

Once expressed and purified, CD45-immunoglobulin fusion proteins can be used as diagnostic tools in immunoadherence and adhesion assays in an attempt to further our understanding of T lymphocyte signalling via the identification an isoform-specific ligand(s) for murine CD45.

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
BSA	Bovine serum albumin
bp	Base pairs
DMEM	Dulbecco's Modified Eagle Medium
dNTP	Deoxynucleotides
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FCS	Fetal calf serum
gal	Galactose
galNAc	N-acetylgalactosamine
glc	Glucose
glcNAc	N-acetylglucosamine
HBS	Hepes buffered saline
HRP	Horseradish peroxidase
HS	Horse Serum
hu	Human
kb	Kilobase pairs
LA	Luria + ampicillin
LB	Luria Broth
man	Mannose
M	Molar
µg	Microgram
µl	Microlitre
µM	Micromolar
µmol	Micromole

mg	Milligram
ml	Millilitre
mM	Millimolar
neuNAc	N-acetylneuraminic acid
ng	Nanogram
nm	Nanometre
OD	Optical Density
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
pmol	Picomole
TBS	Tris buffered saline
term	Terminal
Tris	Tris (hydroxymethyl) amino methane
FACS	Fluorescence activated cell sorter
FITC	Fluorescein isothiocyanate
PTPase	Protein tyrosine phosphatase
PVDF	Polyvinylidene difluoride
SH2	Src homology 2
TCR	T cell receptor
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

DEFINITION OF FUSION PROTEIN NOMENCLATURE

CH2	Constant region heavy chain 2
CH3	Constant region heavy chain 3
Mu/mu	Murine
MuIgG	Murine immunoglobulin G
CD45 alternatively spliced exons	A, B, C = 4, 5, 6
muCD45R0:MuIgG	Fusion protein consisting of murine CD45 exons 3,7, and 8 linked to the hinge, CH2 and CH3 regions of murine IgG_{2a} heavy chain
muCD45RA:MuIgG	Fusion protein consisting of murine CD45 exons 3,4,7, and 8 linked to the hinge, CH2 and CH3 regions of murine IgG_{2a} heavy chain
muCD45RB:MuIgG	Fusion protein consisting of murine CD45 exons 3,5,7, and 8 linked to the hinge, CH2 and CH3 regions of murine IgG_{2a} heavy chain
muCD45RC:MuIgG	Fusion protein consisting of murine CD45 exons 3,6,7, and 8 linked to the hinge, CH2 and CH3 regions of murine IgG_{2a} heavy chain
muCD45RBC:MuIgG	Fusion protein consisting of murine CD45 exons 3,5,6,7, and 8 linked to the hinge, CH2 and CH3 regions of murine IgG_{2a} heavy chain
muCD45RABC:MuIgG	Fusion protein consisting of murine CD45 exons 3,4,5,6,7, and 8 linked to the hinge, CH2 and CH3 regions of murine IgG_{2a} heavy chain
Onco M	Oncostatin M

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DEDICATION

I would like to dedicate this body of work to my parents, Gary and June Awrey, whose continual love, guidance, and understanding have helped me to become the person I am today. And to Torsten, whose love and encouragement forced me to hold my head up and keep going, even when the tasks ahead seem insurmountable.

INTRODUCTION

CD45

CD45 (T200, B220, Ly-5, L-CA) is a protein tyrosine phosphatase (PTPase) that is expressed on all nucleated cells of hematopoietic lineage, reviewed in [1, 2]. Members of the leukocyte common antigen (L-CA) family are abundant as they can account for up to 10% of the protein on the lymphocyte cell surface [3]. PTPases such as CD45 are involved in the reversible process of tyrosine dephosphorylation, a key regulatory mechanism for controlling the growth and division of eucaryotic cells. CD45 is a transmembrane protein containing a large, heavily glycosylated, variable, amino-terminal external domain, a single transmembrane region, and a large carboxy-terminal cytoplasmic domain (Figure 1). The highly conserved cytoplasmic domain consists of two tandem PTPase domains of approximately 240 amino acids separated by a short spacer region. Research has demonstrated that the cytoplasmic domain of CD45 possesses intrinsic enzymatic activity [4] independent of the transmembrane and extracellular domains [5, 6]. Mutation of a conserved cysteine residue at position 817 of PTPase domain I resulted in a total loss of enzymatic activity whereas mutation of the comparable cysteine in domain II did not significantly affect enzymatic activity, suggesting that PTPase domain II is inactive [6]. However, using an *in vitro* rabbit reticulocyte translation system, it was found that deletion of PTPase domain II resulted in a total loss of enzymatic activity, thus its presence is required in order for domain I to be active [7]. Although the cytoplasmic function of CD45 has been studied extensively, the functional role of the external domain and the alternatively spliced exons remains largely unknown. The external domain of CD45 can undergo alternative splicing resulting in the creation of different isoforms that are expressed on various cell types and at different stages of immune cell development. Multiple isoforms of CD45 have been shown to bind

CD45 Isoforms

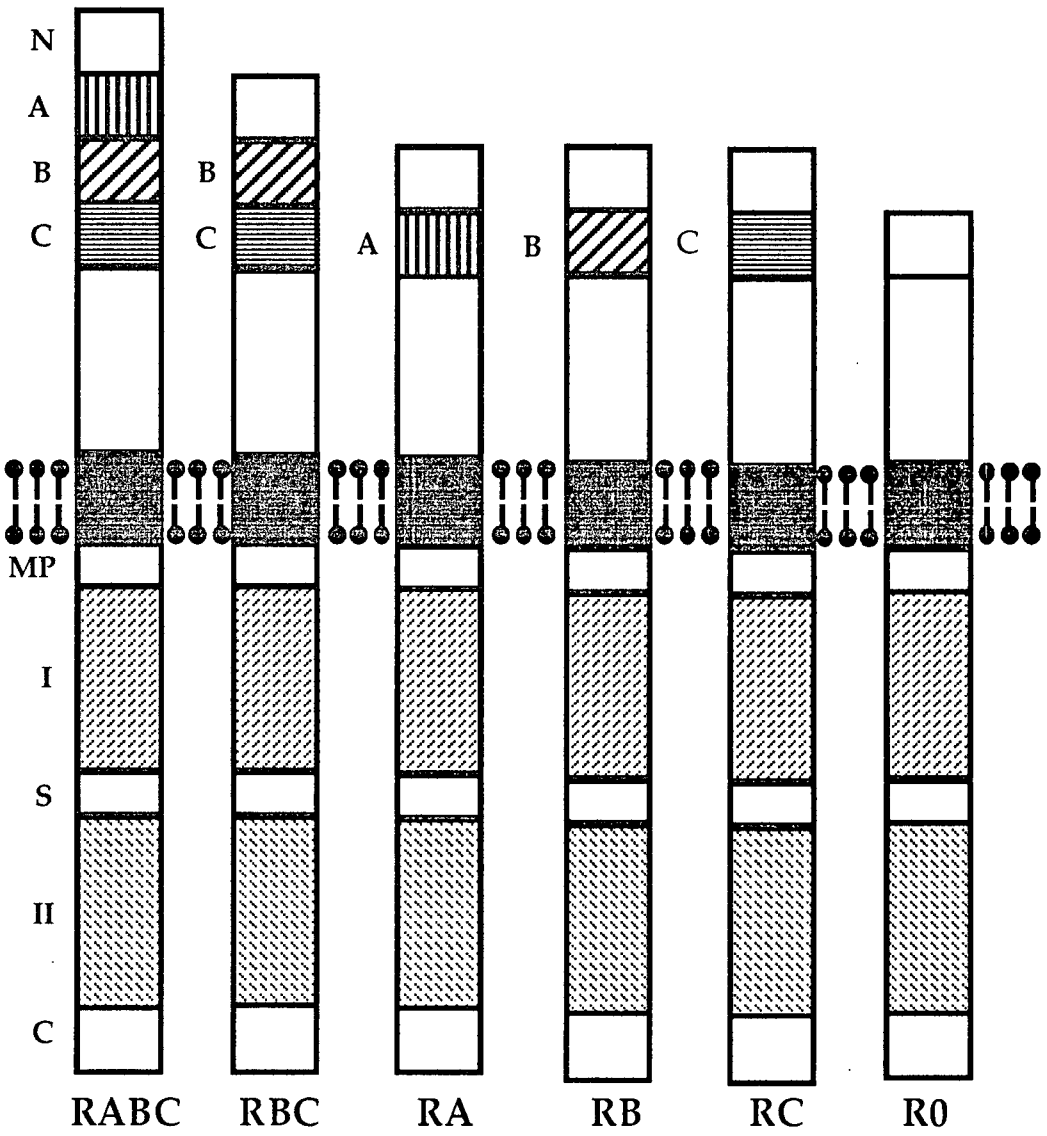


Figure 1: Schematic Diagram of Murine CD45 Isoforms.

Six different isoforms of murine CD45 containing different alternatively spliced exons are shown. N, amino terminus; A,B,C, alternatively spliced exons; MP, membrane proximal region; I, PTPase domain one; S, spacer region; II, PTPase domain two; C, carboxy terminus.

to a molecule expressed on B cells, CD22, via specific carbohydrate residues on CD45 [8, 9], but as yet, an isoform-specific ligand has not been identified.

Differential Expression of Isoforms

Different isoforms of CD45, ranging in molecular weight from 180 to 220 kDa, exist due to alternative splicing of at least three variable exons denoted A, B, and C (4, 5, and 6) located in the extracellular domain. The different isoforms vary not only in the exons expressed at the protein level but also in carbohydrate composition and antigenicity [3]. In addition, the expression of different isoforms varies with cell type, developmental stage and antigenic exposure. For example, B cells express predominantly the highest M_r 220,000 isoform, immature T cells or thymocytes express predominantly the lowest M_r 180,000 isoform and mature T cells can express multiple isoforms. In general, the maturation of T lymphocytes involves the acquisition of higher molecular weight forms of CD45, whereas antigen activation of T cells results in a down regulation to the lowest M_r 180,000 isoform.

The Extracellular Domain

The extracellular domain of CD45 can be divided into two structural domains: the variable region and the cysteine rich domain. Comparison of human and murine CD45 cDNA sequences indicated that although a high degree of sequence identity exists between the cytoplasmic, transmembrane, and signal sequences, the external domain identity between the two species is only 39% [10]. It may be possible that just the isoform-specific regions of the extracellular domain are conserved, thus providing conserved ligand binding epitope(s). At least eight different isoforms of CD45 can exist due to alternative splicing of three exons of approximately 50 amino

acids each (A, B, and C or 4, 5, and 6) located in the N-terminal region of the gene. The largest isoform of CD45, so called CD45RABC due to the presence of exons A, B, and C, has an extracellular domain of 542 amino acids. The smallest isoform of CD45, lacking exons A, B, and C, is denoted CD45R0, and has an extracellular domain of 404 amino acids. Of the 33 exons comprising the entire protein, exon 3 and exons 7-15 encode extracellular coding sequences common to all isoforms of CD45 [11]. Differential usage of the three alternatively spliced exons results not only in proteins of different length but also in isoforms with different carbohydrate content and antigenicity. The variable, N-terminal region is rich in serine and threonine residues which are potential sites for extensive O-linked glycosylation [12]. In addition, the variable region is highly negatively charged due to the addition of sialic acid residues [13, 14]. Furthermore, electron microscopy analysis by low-angle shadowing shows the external domain as an extended rod [15].

The cysteine-rich domain is approximately 360 amino acids long and is common to all isoforms of CD45. This region is thought to contain fibronectin type III modules similar to those found in tapeworm proteins and the fruit fly receptor protein tyrosine phosphatase DPTP[6, 16]. This region contains 15 potential N-linked glycosylation sites for the addition of carbohydrate residues during processing. Studies by Pulido and Sanchez-Madrid indicated that inhibition of N-linked glycosylation by treatment with tunicamycin resulted in decreased cell surface expression and decreased phosphatase activity of CD45 in K-562 erythroleukemic cells [17]. Hence, it appears that intact glycosylation is needed for correct transport, stability or expression of CD45 at the cell surface.

Glycosylation studies of CD45 expressed in K-562 erythroleukemic cells revealed the presence of complex, highly sialylated, O- and N-linked carbohydrate chains [13]. An independent structural study of the sugar chains on human CD45 on T cells was done by Sato et al., revealing that CD45 contains complex type carbohydrate chains containing poly-N-acetyl-lactosamine and exclusively a-2,6

linked sialic acid resides [18]. The fact that only certain isoforms of CD45 co-cap with certain T cell surface molecules suggests that isoforms-specific interactions are possible [19]. As different isoforms of CD45 express different alternatively spliced exons with distinct glycosylation sites, each isoform of CD45 has the potential for a differential glycosylation pattern. As alternate isoforms of CD45 are expressed on the surface of different cell types and at distinct stages of immune cell development, it is reasonable to hypothesize that the presence or absence of a certain isoform, hence the presence or absence of certain carbohydrate residues, at a particular time during development would have specific functional outcomes.

Role of CD45 in the Immune System

The role and function of CD45 in immune cell interactions has been investigated by many groups, in particular by studying CD45 deficient cell lines, reviewed in [2, 20, 21]. Evidence indicated that CD45 negative T cell clones reactive against pigeon cytochrome *c* proliferated in response to interleukin-2 but failed to proliferate in response to antigen. Flow cytometric analysis did not detect the absence of other molecules required for T cell activation and antigen responsiveness was restored in revertant cells that re-expressed CD45 [22]. This result was confirmed by another CD45 negative mutant of L3 CD8+ cytotoxic T cells, which was also unable to respond to antigenic stimulation while the expression of CD45 rescued the T cell response [23]. In addition, CD45 has been implicated in coupling of the T cell receptor (TCR) to the phosphatidylinositol second messenger pathway. Tyrosine phosphorylation and generation of soluble inositol phosphates was absent in spontaneous CD45 negative variants of a human T leukemic cell line, HPB-ALL. However, response to anti-CD3 stimulation, as measured by intracellular calcium increase and generation of inositol phosphates, was restored upon transfection of murine CD45, suggesting that expression of CD45 is required for efficient TCR

signalling and linkage to downstream phosphorylation events [24]. Moreover, in CD45 negative mutants of Jurkat T cells, expression of CD45 was required for tyrosine phosphorylation induced by anti-TCR antibodies [25]. Furthermore, there is evidence that CD45 is required in an analogous fashion for signalling via the B cell receptor [26, 27].

A chimeric molecule consisting of the external and transmembrane domains of MHC class I and the cytoplasmic domain of CD45 expressed in CD45 deficient Jurkat cells restored the TCR induced tyrosine phosphorylation of phospholipase Cγ1 [28]. Similarly, the expression of a chimeric molecule consisting of the extracellular and transmembrane domains of the epidermal growth factor receptor (EGFR) linked to the CD45 cytoplasmic domain, rescued T cell responses to antigenic stimulation in CD45 deficient HPB-ALL cells [29]. Hence, both of these experiments suggest that the extracellular and transmembrane regions of CD45 are not required for TCR mediated signalling. However, these experiments do not rule out the possibility of regulation of CD45 function by ligand binding to the external domain of CD45.

There is some evidence that specific isoforms of CD45 have differential effects on T cell signalling. In an experiment in which CD45RABC and CD45R0 were expressed as transgenes in murine T cells regulated by the thymocyte-specific *lck* promoter, the largest isoform, CD45RABC, was observed to increase thymic CD4+ CD8- T cell proliferation in response to anti-TCR antibodies, resulting in an increase in phosphotyrosine levels and intracellular calcium concentration [30]. In the same experiment, expression of the null isoform, CD45R0, resulted in a decrease in phosphotyrosine levels. This data is in contrast to other observations in which BW5147 CD4/TCR transfectants, expressing different isoforms of CD45, responded equally to stimulation via anti-TCR antibodies while stimulation via addition of antigen presenting cells (APC) and conalbumin peptide resulted in the lower molecular weight isoforms having the highest level of T cell response [31]. Despite

the seemingly contradictory data, a unifying theme exists that different isoforms of CD45 can have differential effects on T cell signalling events.

Potential Ligands

Binding of a ligand specific for an isoform of CD45 may result in a direct alteration in cellular activity or activation state. At this time, a CD45 isoform-specific ligand has not been clearly identified, although two molecules, CD22 [32] and galectin-1 [33], have been shown to be involved in the extracellular domain interactions of CD45.

CD22 is an adhesion molecule expressed exclusively on B cells that has been shown to bind to carbohydrate residues on CD45 expressed by T cells. A CD22-immunoglobulin fusion protein (CD22Rg) was reported to bind to many different ligands on cells of the T, B, and myeloid lineages. One of the T cell ligands was a sialylated glycoprotein with enzymatic activity identified as CD45R0 [32]. Subsequently, CD22 was shown to interact with multiple isoforms of CD45 [34] and that the interaction was dependent on the presence of N-linked carbohydrates containing α-2,6 linked sialic acids on CD45 [8, 9]. Recent studies have shown that the engagement of CD45 by soluble CD22Rg can modulate early T cell signalling events such as tyrosine phosphorylation of phospholipase C-γ (PLC-γ) resulting in the production of inositol triphosphate leading to an increase in intracellular calcium concentration [35]. Moreover, successive deletion of each of the seven extracellular immunoglobulin (Ig) domains of CD22 mapped the sialylated ligand-binding domain to Ig domains 1 and 2 of CD22 [36]. However, as CD22 binds to other molecules on cells of the hematopoietic lineage other than CD45, it is not defined as either a CD45-specific or an isoform-specific ligand.

Another potential ligand for CD45 is a β-galactoside-binding lectin molecule expressed on human thymic cells and stromal cells called galectin-1 [33]. Galectin-1

has been shown to bind to core 2 O-linked glycan structures on the surface of thymocytes and T lymphoblastoid cell lines. This interaction was inhibited not only by an antibody to galectin-1, but also by an antibody, 2B11, that recognizes a carbohydrate-dependent epitope of CD45, suggesting that carbohydrate residues on CD45 are mediating this interaction [37]. Interestingly, the degree of galectin-1 binding to thymocytes correlated with maturation stage with immature thymocytes binding more galectin-1 than mature thymocytes. Whether the developmentally regulated fashion of galectin-1 binding reflects the differential expression of CD45 isoforms during T cell development remains to be elucidated.

In addition, recent evidence indicates that although galectin-1 binds to both activated human T cells and resting T cells, apoptosis is induced only in CD45R0+, CD45RA- activated T cells and not in CD45RA+ resting T cells [38]. HPB-ALL cells expressing CD45 underwent apoptosis upon addition of galectin-1 whereas a CD45 negative variant of this cell line did not, suggesting that CD45 is integral in this response. In addition, inhibition of N-glycan processing by treatment with swainsonine as well as inhibition of O-glycan elongation by addition of benzyl-α-galNAc decreased binding of galectin-1 to T cells. Swainsonine treatment reduced galectin-1 mediated cell death whereas benzyl-α-galNAc treatment increased apoptosis due to galectin-1 binding, suggesting that N-linked glycans are involved in the apoptotic response while O-linked glycans may have a regulatory function, perhaps acting to mask the effect of N-linked glycan induced apoptosis.

Summary of Goals

The aim of this thesis was to further understand the role of the different isoforms of CD45 in immune cell function. As alternate isoforms of CD45 are expressed on the surface of different cell types and at distinct stages of immune cell development, it is reasonable to hypothesize that the presence or absence of a certain

isoform at a particular time during development would have specific functional outcomes. The identification of a ligand that could bind to specific isoforms of CD45 would contribute greatly to our understanding of the role of CD45 in immune cell interactions. The creation of isoform-specific CD45:MuIgG fusion proteins and use as a diagnostic tool in immunoadherence and fluorescent binding assays may prove instrumental in determining the identity of isoform-specific ligands for CD45.

As the interaction of CD45 with a potential ligand may be mediated by unique carbohydrate residues located on the variable exons of CD45, it is important that CD45:MuIgG fusion proteins are correctly glycosylated. Therefore, the cell line used for expression of the fusion proteins is critical. The fusion proteins were first transiently expressed in Cos 7 cells, a non-hematopoietic cell line commonly used to express recombinant proteins. Once transient expression was observed and stable clones were obtained in Cos 7 cells, the focus of future experiments turned to expression of CD45:MuIgG fusion proteins in functionally relevant hematopoietic cell lines, particularly those of the T and B cell lineages. Stable clones were expressed in X63-Ag8.653 cells, a murine myeloma cell line frequently used as a fusion partner in the production of monoclonal antibodies. In addition, transient expression was observed in the T28 T lymphoma cell line.

Once expressed, the CD45:MuIgG fusion proteins had to be fully characterized with respect to molecular weight, dimerization in non-reducing SDS-PAGE conditions, and reactivity with anti-CD45 exon-specific antibodies, as well as to the carbohydrate content by reactivity with various sugar-specific lectins, PNGase F digestion, O-glycosidase treatment, thrombin cleavage and neuraminidase digestion. Fusion proteins expressed by different cell lines were analyzed in an attempt to determine if CD45:MuIgG fusion proteins were differentially glycosylated by each cell line. The amount and type of carbohydrate added was analyzed by treatment with enzymes that specifically remove N-linked sugars or terminal sialic acids. It is important to fully characterize the expressed fusion proteins because if specific

binding is observed by the use of these fusion proteins in binding assays then one can argue in favor of one of the following two options. If the binding observed is the same between differentially glycosylated CD45:MuIg fusion proteins then the importance of correct glycosylation becomes secondary to correct protein sequence and folding patterns. However, if binding is different between fusion proteins expressed in different cell lines, thus having differential carbohydrate content, then correct carbohydrate addition becomes crucial in order for the interaction to occur with the putative ligand.

Hence, the three goals of this work were as follows: 1) to create soluble, isoform-specific, murine CD45:MuIgG fusion proteins containing different alternatively spliced exons, 2) to express these fusion proteins in lymphoid cell lines 3) to characterize the expressed fusion proteins with respect to molecular weight, antigenicity, dimerization, and glycosylation and to compare and contrast isoforms of CD45 produced in different cell lines with respect to these characteristics.

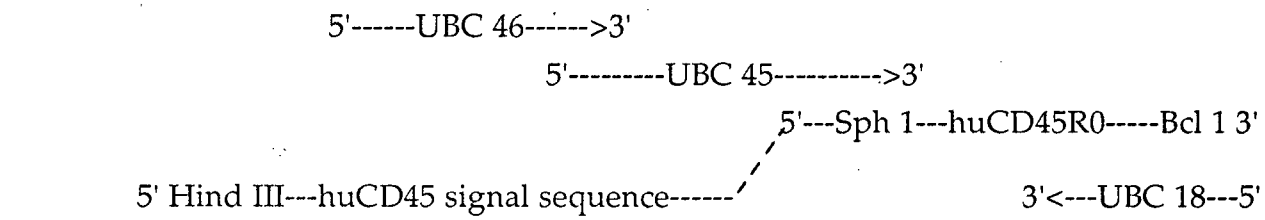
PCR of Isoform-specific muCD45 inserts

Murine CD45 (muCD45) isoform-specific inserts were created by the Polymerase Chain Reaction (PCR) using isoform-specific plasmid DNA as templates. A 5' primer corresponding to amino acid residues 1 to 6 of muCD45 exon 3 (UBC # 60: 5' GCG AGC ATG -->CAA ACA CCT ACA CCC AGT 3': Sph 1 site underlined, where --> indicates the beginning of muCD45) and a 3' primer corresponding to amino acid residues 171-177 of murine exon 8 (UBC 63: 5' ACA ACG AAG CAA ACA<-- GAT CTG GTT CCT CGT GGA TCC TCT GAT CAG GAG CCC 3': Bcl 1 site underlined, where <-- indicates the 3' end of exon 8) were used to generate muCD45 PCR products. Primer UBC #63 was engineered to contain a thrombin cleavage site, 5' CTG GTT CCG CGT GGA TCC 3', which would be incorporated at the junction of CD45 and murine IgG (muIgG) to allow for removal of the IgG portion if desired. Once isoform-specific plasmid DNA had been linearized by overnight digestion at 37°C with 5 units of Cla I (NEB: New England Biolabs, Mississauga, ON), PCR reactions were carried out using 1 ng of template, 10 µl of 10X Vent polymerase buffer (supplied by New England Biolabs), 2 µl of 10 mM dNTP (Pharmacia Biotech Inc., Piscataway, NJ), 50 pmol each primer and 2 Units of Vent DNA polymerase (New England Biolabs) in a final volume of 100 µl in an Ericomp Inc. Easy Cycler™ thermocycler. Samples were subjected to 30 cycles of 1 minute at 95°C for denaturation, 1 minute at 55°C for primer annealing, followed by 1 minute at 72°C for primer extension. PCR products were checked for correct size and purity on 2% agarose gels against 4 µl of 1 kb DNA ladder (Gibco BRL, Life Technologies, Burlington, ON), digested with Sph 1 and Bcl 1 (New England Biolabs), extracted from low melt agarose, and ethanol precipitated. Sph 1 does not cut at the end of DNA fragments with an acceptable efficiency so for each isoform, PCR products were

blunt end ligated using 400 Units of T4 DNA ligase (New England Biolabs) to create multimers, followed by Sph 1 and Bcl 1 digestion to create the correct size insert with the required 5' and 3' overhangs to facilitate subcloning into the modified murine IgG plasmid vector.

Creation of the Modified Oncostatin M/Murine IgG_{2a} Vector

The murine IgG (muIgG) fusion protein vector was kindly provided by Dr. Peter Linsley [39]. A plasmid vector containing the murine IgG_{2a} hinge, CH_2 and CH_3 regions and the Oncostatin M (Onco M) signal sequence [40] was constructed using a human CD45R0 Hind III/Bcl 1 fragment containing exons 3, 7, and 8 as a starting point (provided by Dr. Julie Deans). Firstly, the CD45 signal sequence was replaced with the Onco M signal sequence by three successive rounds of PCR (conditions described previously) using overlapping oligonucleotide 5' primers representing the Oncostatin M sequence (UBC #45, 46, or 47) and a 3' primer complementary to human CD45 exon 8 (UBC #18). A Hind III restriction enzyme site was created at the 5' end of Oncostatin M. Primers used for the first round of PCR were UBC #45 and UBC #18, for the second round UBC #46 and UBC #18, and the third round UBC #47 and UBC #18.



The Onco M/huCD45R0 Hind III/Bcl 1 PCR fragment was ligated to Bcl 1/Xba 1 digested murine IgG_{2a} DNA fragment and the Hind III/Xba 1 digested plasmid vector pBluescript SK (+/-) (pBS) (Strategene Cloning Systems, La Jolla, CA). Positive clones were grown up in GM48 dam^-, dcm^- *Escherichia coli* and digested

with Sph 1 and Bcl 1 (New England Biolabs; Bcl 1 restriction site is dam methylation sensitive) to remove the human CD45 fragment, followed by subsequent purification and dephosphorylation of the Onco M/MuIgG/pBS fragment using 1 µl (10 units) of calf intestinal alkaline phosphatase (CIP; New England Biolabs). The murine IgG_{2a} vector is hereafter referred to as the Ig vector. Care was taken to ensure the production of a pure preparation of the Ig vector absent of human CD45 contamination.

N-butanol Oligonucleotide Purification

Oligonucleotide primers were ordered from the Nucleic Acids Processing Unit (NAPS Unit, UBC, Vancouver, BC) and purified by the n-butanol method[41]. Primers were reconstituted in 100 µl of 30% ammonium hydroxide (NH_4OH) and 1 ml of n-butanol (BDH Inc., Vancouver, BC) After vigorous vortexing, the sample was centrifuged at 12,000 x g followed by removal of the single aqueous phase. The oligonucleotide pellet was resuspended in 100 µl of water and 1 ml of n-butanol and the above procedure was repeated. The resulting pellet was dried under vacuum and resuspended in 500 µl of distilled, deionized water. Optical Density (OD) readings at 260 nm and 280 nm were recorded and used to calculate the concentration of oligonucleotide recovered by using the equation: µmol/ml of oligonucleotide = OD 260/extinction coefficient. The value for the extinction coefficient was calculated as 10 times the length of the oligonucleotide. All oligonucleotides were heated to 80°C and rapidly transferred to -20°C to prevent self-hybridization.

Preparation of Competent Bacteria for Transformation

XL1 Blue and GM48 *Escherichia coli* were rendered competent for

transformation by resuspending a 500 ml log phase culture (OD_{600}= 0.4) in 200 ml of 30 mM potassium acetate, 100 mM rubidium chloride, 10 mM calcium chloride, 50 mM manganese chloride, 15% glycerol pH 5.8 on ice for 5 minutes. Following centrifugation, the pellet was resuspended in 20 ml of 10 mM 3-(N-Morpholino-propanesulphonic acid (MOPS), 75 mM calcium chloride, 10 mM rubidium chloride, 15% glycerol pH 6.5 on ice for 15 minutes. Prepared cells were frozen in a dry ice/ethanol bath and stored in 1 ml aliquots at -80°C until later use.

Ligation and Transformation

20 ng of muCD45 isoform-specific PCR product digested with Sph 1 and Bcl 1 was ligated to 20 ng of Ig vector using 1 µl of 10 X ligase buffer [50 mM Tris-HCl pH 7.8, 10 mM magnesium chloride, 10 mM dithiothreitol (DTT), 1 mM adenosine triphosphate (ATP), 50 µg/ml bovine serum albumin (BSA)], and 1 µl (400 Units) of T4 DNA ligase (New England Biolabs) in a final volume of 10 µl for 2-3 hours at 15°C. Ligation reactions were diluted 1/4 with pyrogen reduced water followed by addition of 100 µl of competent XL1-Blue *E. coli* and incubation on ice for 15 minutes. Samples were heated at 42°C for 90 seconds, followed by incubation on ice for 2 minutes. 100 µl of pre-warmed Luria Broth (LB) was added, and the mixture incubated at 37°C for 30 minutes to allow time for bacterial growth and acquisition of ampicillin resistance. Ligation mixtures (240 µl) were spread on LB agar plates supplemented with 50 mg/l ampicillin and incubated at 37°C overnight to allow for the formation of colonies.

Colonies were randomly selected and placed in 2 ml of LA broth and incubated overnight at 37°C in preparation for alkaline lysis miniprep analysis. 1 ml of culture was pelleted and resuspended in 100 µl of cold TEG lysis buffer (25 mM Tris pH 8.0, 10 mM ethylenediaminetetraacetic acid (EDTA), 50 mM D-glucose) and 200 µl of 0.2 M NaOH/1% sodium dodecyl sulfate (SDS) and left at room

temperature for 5 minutes. 150 µl of 3 M sodium acetate pH 5.2 was then added to the tube, mixed by inversion, and incubated on ice for 5 minutes to allow for precipitation of bacterial chromosomal DNA. After centrifugation at 12 000 x g for 5 minutes, the chromosomal pellet was discarded while the plasmid DNA within the supernatant was kept for further analysis. Excess protein was removed from the supernatant by extraction with an equal volume of phenol/chloroform (1:1) followed by precipitation of the plasmid DNA on ice for 5 minutes with 500 µl of isopropanol. The plasmid DNA pellet was washed once with 70% ethanol, dried briefly in a desiccator, and resuspended in 50 µl of TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA). 2 µl of miniprep DNA was digested with the appropriate restriction enzymes to determine if isoform-specific murine CD45 was present. Once fully sequenced[42], DNA from positive clones was digested with Xho 1 and Not 1, and the resulting Onco M/muCD45/MuIgG piece was subcloned into the Xho 1/Not 1 digested mammalian expression vector pBCMGSneo. Nucleobond AX 2000 column preps (Macherey-Nagel GmbH & Co.) were performed according to manufacturers instructions to obtain large amounts of pure DNA for transfection into eucaryotic cells.

Cell Culture

All eucaryotic cell lines were maintained in 90% Dulbecco's Modified Eagle Medium (DMEM), 10% fetal calf serum (FCS), 100 Units/ml penicillin G, 100 µg/ml streptomycin sulfate, and 0.25 µg/ml amphotericin B. Cos 7 adherent cells (American Type Culture Collection, Rockville, Maryland; CRL #1651) were washed once with 1X phosphate buffered saline (PBS; 154 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.5 mM KH_2PO_4) and then removed from 5 ml Nunc tissue culture dishes (Gibco BRL, Life Technologies, Burlington, ON) using 2X versene (0.7 mM EDTA, 0.14 M NaCl, 0.7 mM KCl, 8 mM Na_2HPO_4, 1.3 mM KH_2PO_4 pH 7.3) and

gentle pipet action. X63-Ag8.653 murine myeloma cells were obtained from Dr. Hung-Sia Teh. T28 T lymphoma cells were obtained from Dr. Fumio Takei. All incubations were at 37°C and 5% CO_2.

Antibodies

Exon-specific rat-anti-mouse CD45 antibodies used were the exon A-specific antibody 14.8 [43], the B220 isoform-specific antibody RA3 6B2 [44], the exon B-specific antibodies 23 G2 and MB 4B4 [45], and the exon C-specific antibody, DNL1.9 (Ly 5-B220) [46] (Table I). All antibodies with the exception of DNL1.9 were used as tissue culture supernatants whereas DNL1.9 was obtained in a purified form from Pharmingen (San Diego, CA). Goat-anti-mouse-Horseradish peroxidase (GAMIgG-HRP) was obtained from Biorad Laboratories, Mississauga, ON and was used to detect the Fc portion of CD45:MuIgG fusion proteins at a dilution of 1/5000. Secondary antibody goat-anti-rat-HRP (GARIgG-HRP) was obtained from Southern Biotechnology Associates, Inc., Birmingham, AL and used at a dilution of 1/10,000. Antibodies used for FACS analysis included the pan specific rat-anti-mouse CD45 antibody, I3/2 [47] (gift from Dr. Ian Trowbridge), the anti-CD45 exon A-specific antibody RA3 2C2 [48], fluorescein isothiocyanate (FITC) conjugated goat-anti-rat immunoglobulin (GARIgG-FITC) (Pierce, Rockford, IL), and anti CD45 exon-specific antibodies as indicated previously.

Transient Transfection of Cos 7 cells with CD45-Immunoglobulin Constructs

Transient expression of CD45:MuIgG isoform constructs was obtained by the DEAE-dextran method. Briefly, 30 µg of plasmid DNA was mixed with 68 µl of 1X PBS, 20 µl of 50 mg/ml DEAE-dextran, and distilled deionized H_20 to a total volume of 100 µl. The DNA solution was then added to 1.6 ml of transfection media (10 ml

Table I

Antibody	Isotype	Specificity	Reference
I3/2	IgG_{2b}	all isoforms	[47]
14.8	IgG_{2b}	CD45 exon A	[43]
RA3 2C2	IgM	CD45 exon A	[48]
MB 4B4	IgG_{2a}	CD45 exon B	[45]
23 G2	IgG_{2a}	CD45 exon B	[45]
DNL1.9	IgG_{2a}	CD45 exon C	[46]
RA3 6B2	IgG_{2a}	B220 isoform	[44]

Table I: Description of Exon-specific Antibodies

The isotype and specificity of each anti-CD45 exon-specific antibody used in the characterization of expressed CD45:MuIgG fusion proteins is indicated.

of DMEM plus 10 µl of 0.1 M chloroquine), mixed by pipetting, and then added dropwise to a confluent 60 mm plate of adherent Cos 7 cells (1 x 10^6 cells). Plates were rocked gently to ensure complete coverage of cells with the DNA/media solution and then incubated at 37°C, 5% CO_2 for 4 hours. After 4 hours, the media was removed and the cells were gently washed in 3 ml of 90% PBS, 10% dimethylsulfoxide (DMSO) for 2-3 minutes, followed by one wash in 4 ml of DMEM and subsequent incubation in 3 ml of DMEM, 10% FCS for 72-96 hours at 37°C. After 3 days, the culture supernatant was harvested and tested for the presence of secreted CD45:MuIgG isoform-specific fusion proteins. In addition, the transfected cells were removed from the tissue culture dish using 3 ml of 2X versene, centrifuged and the cell pellet lysed in 0.5 ml of 1% Triton/TNE lysis buffer (25 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton-X-100) for 20 minutes on ice. Lysates were centrifuged at 12,000 x g for 20 minutes and the supernatant removed and kept for further analysis.

Stable Transfection of Cos 7 cells with CD45-Immunoglobulin Constructs

Stable expression of CD45:MuIgG isoform constructs was obtained by the calcium phosphate method [49]. 30 µg of each plasmid DNA fusion construct was ethanol precipitated and resuspended in 220 µl of distilled, deionized H_20 and 250 µl of 2X Hepes buffered saline (HBS; 280 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, 10 mM dextrose) in a 1.7 ml microcentrifuge tube (Island Scientific, Bainbridge Island, WA). 30 µl of 2 M $CaCl_2$ was added slowly, 5 µl at a time, over 30 seconds, and left at room temperature for 15 minutes to allow the DNA to precipitate. The precipitated DNA solution was added to a 50% confluent monolayer of Cos 7 cells in a 60 mm Nunc tissue culture dish containing 5 ml of DMEM/10% FCS. The cells were incubated at 37°C, 5% CO_2 for 3 to 4 hours at which time the DNA containing media was removed and replaced with fresh DMEM/10% FCS. At 48 hours after the

addition of DNA, selection of neomycin resistant clones was achieved by removing cells from the dish using 4 ml of 2X versene and placing 1 ml into each of four 60 mm tissue culture dishes and adding 4 ml of DMEM/10% FCS and 350 μg/ml of active G418 (Gibco BRL Life Technologies, Burlington, ON). The four dishes were then incubated at 37°C, 5% CO_2 for 13 days until small colonies started to appear on the surface of the dish. Single colonies were removed from the plate using 2 μl of 2X versene and transferred to one well of a 96 well plate containing 200 μl of DMEM/10% FCS and allowed to grow to confluency. At that time, the culture supernatant was removed for analysis and detection of secreted isoform-specific CD45:MuIgG fusion proteins.

Transfection of X63-Ag8.653 and T28 cells by Electroporation

Stable CD45RABC:MuIgG, CD45RB:MuIgG, and CD45R0:MuIgG fusion protein producing clones were obtained by the following method. Briefly, 1 X 10^7 X63-Ag8.653 cells or 5 X 10^6 T28 cells were harvested and resuspended in 800 μl of DMEM with 20 μg of ethanol precipitated plasmid DNA in a Biorad electroporation cuvette. Samples were electroporated at 250 volts and 960 μF using a Biorad Gene Pulser Electroporator (Biorad Laboratories, Mississauga ON). After electroporation, the cells were placed on ice for 10 minutes, then removed from the cuvette and placed in a 100 mm tissue culture dish (Fisher Scientific, Vancouver, BC) with 10 ml of DMEM/10% FCS and incubated at 37°C, 5% CO_2 for 48 hours. At this time, cells containing the transfected gene for neomycin resistance were selected by the addition of 500 μg/ml (X63-Ag8.653) or 1 mg/ml (T28) of active G418. Culture supernatants were harvested at 48 hours and tested for transient expression of CD45:MuIgG fusion protein while the transfected cells were resuspended in 20 ml of DMEM/10% FCS, 500 μg/ml to 1 mg/ml of active G418 and cloned into 96 well plates and incubated at 37°C for 2-3 weeks until discrete colonies appeared. At that

time, 150 µl of supernatant was removed from each well containing an individual colony and tested for the presence of secreted CD45:MuIgG fusion protein. Cells that tested positive for stable fusion protein expression were grown to confluency and aliquots were frozen down in liquid nitrogen.

Precipitation

CD45:MuIgG fusion proteins were precipitated from 100-500 µl of tissue culture supernatant (after a 3 day incubation with 1 X 10^6 cells) using 20 µl of a 10% solution of protein A produced by *Staphylococcus aureus* for 2 hours at 4°C on a rotator. Protein A producing Pansorbin cells were obtained from Calbiochem (San Diego, CA) and were washed once in 1% Triton/TNE lysis buffer prior to use. After 2 hours, the precipitate was washed once with lysis buffer, boiled for 5 minutes at 100°C in 20 µl of 1X reducing SDS sample buffer containing 0.125 M Tris-HCl pH 7.5, 10% glycerol, 100 mM dithiothreitol (DTT), 2% SDS and 0.2% bromophenol blue. The supernatant was loaded onto 7.5% SDS-polyacrylamide (SDS-PAGE) minigels and electrophoresed in running buffer (25 mM Tris pH 7.5, 192 mM glycine, 0.1% SDS) for 45-50 minutes at 200 volts. The resulting SDS-PAGE gel was transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) in 0.3 M Tris pH 7.5, 0.23 M Glycine, 20% methanol for 1 hour at 100 volts.

Western Blots

Western blots were performed by wetting PVDF membranes in methanol, rinsing once in Tris buffered saline consisting of 25 mM Tris pH 7.5, 150 mM NaCl (TBS) followed by a blocking step using either 5% bovine serum albumin fraction V (BSA) (Gibco BRL, Life Technologies) or 5% skim milk powder in TBS for 1 hour. Anti-CD45 tissue culture supernatants were diluted 1/10 for 14.8 and 1/20 for MB

4B4, 23 G2, RA3 6B2 in TBS plus 0.1% Tween-20 (TTBS) and incubated with the membranes for 1.5 hours. Membranes were then washed three times in TTBS, followed by subsequent incubation with 1/10,000 GARIgG-HRP in TTBS for 45 minutes. All incubations were at room temperature on a rotary shaker. After washing membranes for 35 minutes with multiple changes of TTBS, blots were developed by incubation with ECL reagents (Enhanced Chemiluminescence, Amersham Life Science, Oakville, ON) according to manufacturers instructions and exposure to film (Eastman Kodak Biomax MR, Rochester, New York).

Flow Cytometry

Cell lines used for transfection of CD45:MuIgG isoform fusion constructs were tested for the expression of different isoforms of murine CD45. Briefly, 2 X 10^5 cells were harvested in log phase growth, washed once in 1X PBS, resuspended in 200 µl 1X PBS/2.5% horse serum (HS) and placed in one well of a 96 well plate. The plate was centrifuged at 1000 rpm for 2 minutes to pellet the cells. The supernatant was removed by sharply inverting the plate and blotting on paper to remove excess liquid. After vortexing to resuspend the cell pellet, 100 µl of primary anti-CD45 antibody was added (undiluted tissue culture supernatant) and the plate was incubated on ice for 20 minutes. Following a wash step using 100 µl of 1X PBS/2.5% HS, 100µl of a 1/100 dilution of GARIgG-FITC was added and incubated in the same manner. The cells were washed once in 1X PBS/2.5% HS, followed by transfer to a #2052 Falcon tube (Fisher Scientific) containing 150 µl of 1X PBS/2.5% HS and 10 µg/ml propidium iodide (PI). Immunocytometry was performed using a Becton Dickinson FACSCAN. Analysis of data was carried out using Lysis II software .

Lectin Analysis

CD45:MuIgG fusion proteins were precipitated as described previously. Membranes were blocked for one hour using 5% BSA fraction V in TBS, washed three times, 5 minutes each, in TTBS, followed by incubation with 0.05 to 0.2 μg/ml of biotinylated lectin in 5% BSA/TTBS for one hour. The secondary antibody used was streptavidin-HRP (Pierce, Rockford, IL) in 5% BSA/TTBS diluted 1/10,000. Finally, membranes were washed 6 times in TTBS, 5 minutes each, and then developed by ECL as previously described. Lectins used are as follows: RCA, *Ricinus communis* 120; PNA, *Arachis hypogaea* (Peanut agglutinin); VVA, *Vicia villosa*; Con A, Concanavalin A; WGA, *Triticum vulgaris* (Wheat germ agglutinin); SNA; *Sambucus nigra;* MAA, *Maackia amurensis*. The SNA [50] and MAA [51] lectins were obtained from Oxford GlycoSystems, Rosedale, NY. All other lectins were purchased from the Sigma Chemical Company, St. Louis, MO.

PNGase F Digestion and Thrombin Cleavage

Precipitated CD45:MuIgG fusion proteins were digested with PNGase F (New England Biolabs) as follows. Immunoprecipitates were resuspended in 10 μl of 1% Triton/TNE lysis buffer and 1 μl of 10X denaturation buffer (supplied by NEB; 5% SDS, 10% B-mercaptoethanol) and boiled for 10 minutes at 100°C. 10X reaction buffer (supplied by NEB; 0.5 M Na_2PO_4 pH 7.5) and 10% Nonidet P-40 (NP-40) were then added to final concentrations of 50 mM and 1% respectively, followed by addition of 1000 units of PNGase F and incubation at 37°C for 90 minutes. In addition, in separate experiments, CD45:MuIgG fusion proteins were digested with 12.5 cleavage units of thrombin (Sigma Chemical Company, St. Louis, MO) for two hours at room temperature. All samples were then electrophoresed under reducing conditions on SDS-PAGE gels as previously outlined.

Neuraminidase Digestion

Precipitated CD45:MuIgG fusion proteins were digested with 2 mUnits of neuraminidase (sialidase) isolated from *Vibrio cholerae* (Boehringer Mannheim GmbH, Laval, Quebec) at 37°C for one hour in 50 mM sodium acetate, 4 mM calcium chloride pH 7.8, followed by SDS-PAGE and western blot analysis as previously described.

O-Glycosidase Digestion

Precipitated CD45:MuIgG fusion proteins were digested with 0.5 mUnits of BSA-free O-glycosidase isolated from *Diplococcus pneumoniae* (Boehringer Mannheim GmbH, Laval, Quebec) for 16-18 hours at 37°C in 20 µl of 20 mM sodium cacodylate, 20 mM sodium dihydrogen phosphate pH 6.5, followed by SDS-PAGE and western blot analysis as previously described. To optimize the removal of O-linked sugars, samples were treated with neuraminidase as previously described prior to digestion with O-glycosidase.

RESULTS

1.0 Generation of Murine CD45-Immunoglobulin Isoform-specific Fusion Constructs

The objective of this work was to generate recombinant, secreted CD45-Immunoglobulin (CD45:MuIgG) fusion proteins containing isoform-specific regions of murine CD45. Previous attempts at expression of human CD45:MuIgG fusion constructs containing the original CD45 signal sequence were unsuccessful (Dr. Julie Deans, unpublished). Therefore, in an attempt to increase the expression of fusion proteins to a level that could be detected by precipitation of secreted culture supernatant with protein A, SDS-PAGE analysis and western blotting with anti-murine immunoglobulin G (anti-muIgG), a new expression strategy was devised. The plasmid vector previously used for expression of human CD45:Ig constructs, pCDM8, was replaced in favor of an alternate mammalian expression vector, pBCMGSneo [52]. As well, the CD45 signal sequence was removed and replaced with that of the growth factor, oncostatin M (Onco M) [40].

Transient expression of CD28Ig and B7Ig fusion constructs (containing the Onco M signal sequence) into Cos 7 cells by the DEAE-dextran method resulted in the secretion of Ig fusion proteins that were easily purified from culture supernatants by affinity chromatography on immobilized protein A columns to concentrations of 1.5-4.5 mg/l [39]. Purified fusion proteins are a soluble form of recombinant protein that can be used in subsequent applications such as adhesion and binding assays. In the past, Ig fusion proteins have been instrumental in the identification of molecules that bind to CD44, [53] CD22, [8, 9] CTLA-4, [54] and CD28 [39]. In this body of work, transient expression of modified human CD45:MuIgG constructs in pCDM8 resulted in a detectable level of expression by precipitation of culture supernatant with protein A and subsequent western blotting with anti-

muIgG (data not shown). As the inclusion of the Onco M signal sequence increased the levels of expression of human CD45 fusion constructs, it was reasonable to hypothesize that the expression of murine CD45:MuIgG fusion proteins would also be favorably affected by this modification.

Different isoforms of CD45 exist due to alternative splicing of at least three exons located in the variable region of the extracellular domain. CD45 isoform-specific Ig fusion proteins were designed to include extracellular exon 3 (the first exon encoding mature protein), exon 7, and the majority of exon 8 as well as one or more of the variable, alternatively spliced exons 4, 5, and/or 6. Exons 1 and 2 of the CD45 gene encode 5' untranslated regions and the CD45 signal sequence, hence they were not included in fusion protein constructs. The exons encoding the remainder of the extracellular domain of CD45 were omitted from fusion protein constructs, thus allowing the focus to remain on the binding of the alternatively spliced, isoform-specific exons. As well, inclusion of additional invariable exons could result in potentially confusing interactions occurring with the invariable region of CD45. Moreover, as the invariable region contains 16 cysteine residues, the inclusion of this domain could cause incorrect disulfide linkages to occur during expression that may cause the fusion protein to fold incorrectly. The cysteine residue in exon 8 at amino acid position 178 was not included for the same reason.

A schematic diagram of a secreted CD45Ig fusion protein is shown in Figure 2. Expressed fusion proteins are predicted to mimic the structure of an antibody heavy chain, except with the conventional variable, antigen binding region replaced by isoform-specific regions of CD45. In addition, Ig fusion protein constructs contain the Onco M signal sequence as well as the hinge, CH2 (constant region heavy chain 2), and CH3 (constant region heavy chain 3) regions of murine IgG_{2a}. The inclusion of the CH2 and CH3 domains of the Ig heavy chain allows for rapid purification of Ig fusion proteins by binding to protein A-sepharose. In order to investigate all possible isoform-specific interactions, six plasmid DNA constructs were created, each

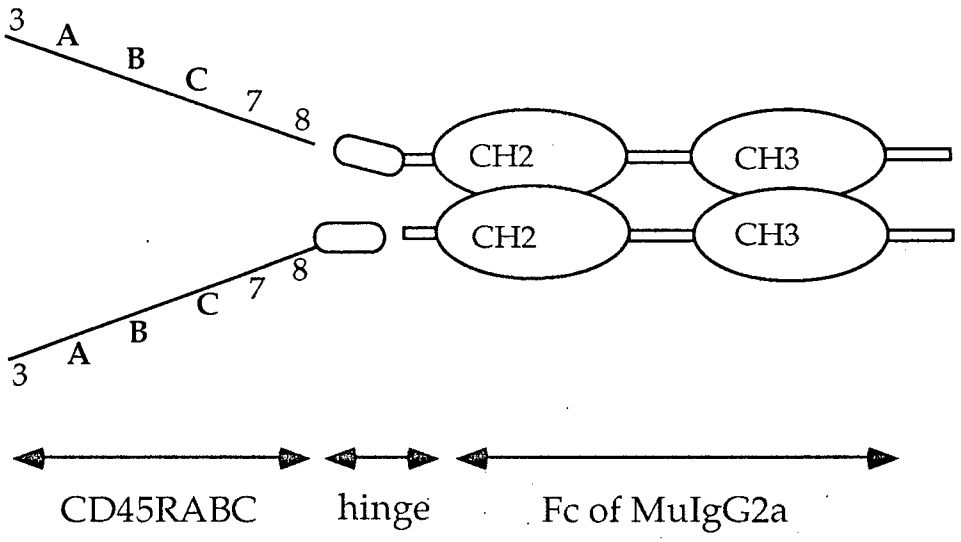


Figure 2: Schematic Representation of CD45RABC-Immunoglobulin Fusion Protein.

CD45RABC:MuIgG Fusion Protein is shown in dimerized form. Numbers indicate the exon of murine CD45 incorporated. Letters indicate alternatively spliced exons of murine CD45. CH2 and CH3 denote constant (Fc) regions two and three of murine immunoglobulin G heavy chain.

containing a different alternatively spliced isoform of murine CD45: CD45RABC, CD45RBC, CD45RA, CD45RB, CD45RC and CD45R0.

1.0.1 Reconstruction of the Oncostatin M signal sequence by Polymerase Chain Reaction

The Onco M signal sequence was reconstructed at the 5' end of the Hind III/Bcl 1 fragment containing exons 3, 7 and 8 of human CD45R0. This was achieved by three successive rounds of PCR using overlapping oligonucleotides corresponding to the Onco M signal sequence. The first round used two oligonucleotide primers: at the 5' end, UBC #45, which corresponds to the 3' 33 nucleotides of the Onco M signal sequence and the 5' 18 nucleotides of human CD45 exon 3, and at the 3' end, antisense primer UBC #18, which anneals to the 3' end of human CD45 exon 8. The expected DNA fragment size for the first round of PCR was 180 base pairs (bp): the 3' 33 nucleotides of the Onco M signal sequence in addition to 145 nucleotides of human CD45 exons 3, 7, and 8. Analysis of PCR products from the first round by agarose gel electrophoresis revealed a 180 bp band at an approximate concentration of 20 ng/μl as well as a minor contaminating band of lower concentration at approximately 300 bp (data not shown). As the total volume of the PCR reaction was 100 μl, approximately 2 μg of the 180 bp product was created from 1 ng of template. The second round of PCR, using the 180 bp product from the first round as the template, the 5' primer UBC #46 which annealed to the sequences created in round one by UBC #45, and the same 3' primer as in round one, yielded a DNA fragment of approximately 207 bp, consistent with the addition of an additional 27 nucleotides of the Onco M signal sequence to the 5' end of the DNA strand. The third round of PCR, using the 207 bp product from round two as the template, the 5' primer UBC #47, and the same 3' primer as in previous rounds, resulted in a 234 bp band, consistent with the addition of the final 15 nucleotides of

the Onco M signal sequence and a further 12 nucleotides creating a Hind III restriction enzyme site at the 5' end of the DNA strand. The 234 bp product, representing the complete Onco M signal sequence linked at the 5' end to human CD45 exons 3, 7 and 8, was digested with Hind III and Bcl 1 and purified to a concentration of 20 ng/μl (approximately 1.2 μg recovered) in preparation for 3-way ligation to the Bcl 1/Xba 1 digested murine heavy chain sequences (700 bp) and the Hind III/Xba 1 digested plasmid vector pBluescript (pBS; 3 kb). The resulting 3.9 kb plasmid was expressed in the *E. coli* strain XL1 Blue, fully sequenced [42] and named clone #30.

1.0.2 Creation of the Modified Murine IgG_{2a} Vector with the Oncostatin M signal sequence

The modified Ig vector was created by using the Onco M/huCD45R0/MuIgG/pBS plasmid (#30) created previously as a starting point (Figure 3). This plasmid was digested separately with Sph 1 or Bcl 1 to yield a linearized 3.9 kb band upon agarose gel analysis. In addition, bands at approximately 6 kb and 7 kb were observed after 3 hours of digestion, indicating uncut plasmid DNA was present. Therefore, in order to allow for complete digestion by both enzymes at the same time, it was necessary to cleave the 3.9 kb plasmid #30 with Bcl 1 overnight followed by addition of an aliquot of Sph 1 every hour for 5 hours. The resulting fragments were the desired 3.75 kb Ig vector and the 150 bp fragment corresponding to huCD45 exons 3, 7, and 8. Whereas the huCD45 portion was discarded, the resulting vector containing the murine IgG CH2 and CH3 domains as well as the Onco M signal sequence was dephosphorylated to remove the 5' phosphate to prevent self re-ligation, followed by purification to 60 ng/μl by extraction from low melt agarose and ethanol precipitation. Approximately 1.2 μg of digested, dephosphorylated, and purified Ig vector was recovered. The Ig vector

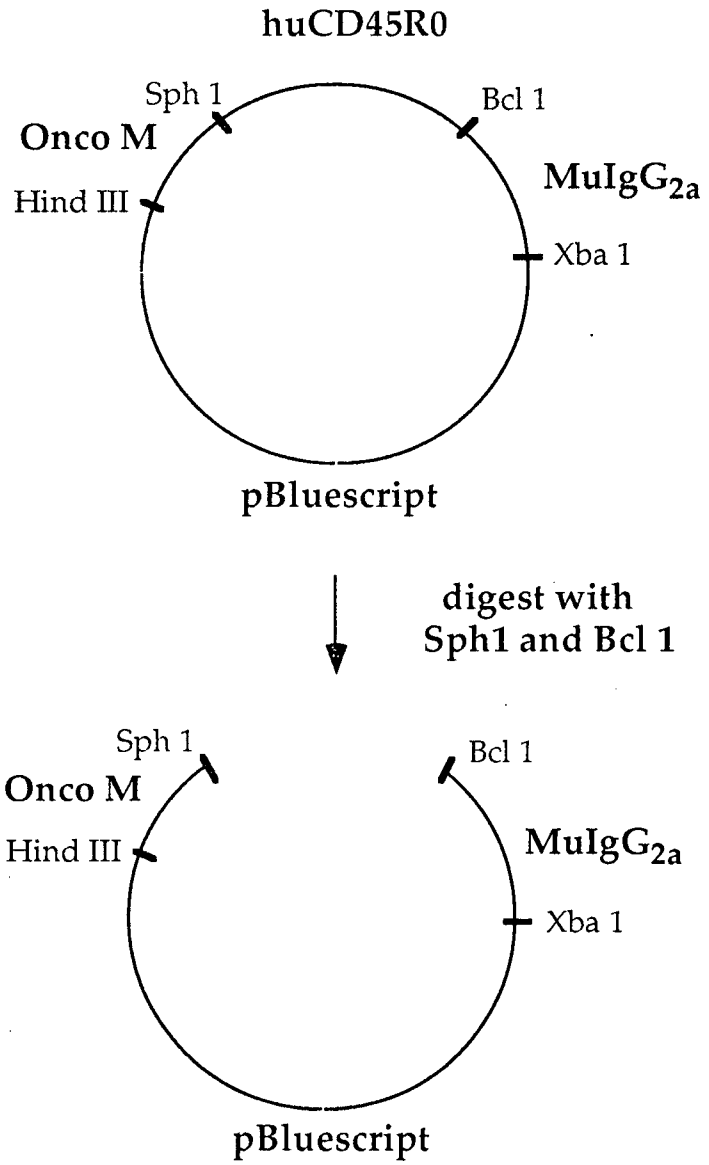


Figure 3: Creation of the Oncostatin M/ Murine IgG_{2a} Vector

The 3.9 kb pBluescript plasmid construct containing huCD45R0/Onco M /MuIgG2a (#30) was digested overnight with Bcl 1 followed by 5 hours digestion with Sph 1 to remove huCD45. The 3.75 kb Ig vector portion was subsequently purified by low melt agarose extraction in preparation for cloning in of murine CD45 inserts.

was then ligated to muCD45 isoform-specific PCR inserts, created as described in the next section, to create muCD45:MuIgG fusion constructs.

1.0.3 Creation of Isoform-specific murine CD45 inserts by Polymerase Chain Reaction

The predicted size of each isoform-specific PCR product is shown in Table II. Primers were designed to incorporate muCD45 exon 3, exon 7, and the majority of exon 8 (3' primer was designed to omit the cysteine residue at position 178) into all six isoform-specific PCR products. In addition, one or more of the alternatively spliced exons, 4, 5, and 6, was incorporated into the final product depending on the isoform-specific template used. Upon using the oligonucleotide primers indicated in Table II, PCR products of the predicted size for each of the six isoforms were observed upon agarose gel analysis (Figure 4, Panel A). For CD45R0, in addition to the band at 159 bp, a band was observed at roughly 400 bp. However, the 400 bp band was present at a much lower concentration than the desired 159 bp band. For CD45RBC, a 200 bp band was observed in addition to the desired 447 bp product, but again this non-specific band was present at a much lower concentration. In addition to non-specific priming, some smearing and degradation was observed for CD45RA and CD45RB PCR products. All isoform-specific PCR products were digested with Sph 1 and Bcl 1 and then purified to a single, sharp band by extraction from low melt agarose and ethanol precipitation (Figure 4, Panel B). Approximately 800 ng of each purified isoform-specific PCR fragment was available for ligation to the Ig vector.

Successive attempts at ligation of the Sph 1/Bcl 1 digested PCR products to the Ig vector containing the Onco M and murine IgG_{2a} sequences were not immediately successful. Further analysis revealed that Sph 1 cuts at the ends of linear strands of DNA with a very low efficiency, approximately 25% in 20 hours when there are 8 nt 5' to the restriction site. Using the 5' oligonucleotide primer UBC #60 for PCR

Table II

CD45 Isoform	Exons Incorporated	5' Primer Used	3' Primer Used	Predicted Size in base pairs
CD45R0	3,7,8	#60	#63	159
CD45RA	3,4,7,8	#60	#63	280
CD45RB	3,5,7,8	#60	#63	300
CD34RC	3,6,7,8	#60	#63	300
CD45RBC	3,5,6,7,8	#60	#63	447
CD45RABC	3,4,5,6,7,8	#60	#63	547

Table II: Results of Polymerase Chain Reaction of murine CD45 isoform-specific Inserts.

The exons of the variable, extracellular domain incorporated by PCR using isoform-specific plasmid DNA templates and the indicated primers is shown. The predicted size of each isoform-specific product is also shown.

reactions yielded products that had only 5 nucleotides 5' to the Sph 1 restriction site. In order to create an isoform-specific muCD45 insert that Sph 1 could digest with an acceptable efficiency, each PCR product was blunt end ligated to itself to create multimers. As the Sph 1 site was no longer at the end of the fragment in a multimer, digestion was able to occur to create an isoform-specific product with the required sites for ligation to the Ig vector.

Once the muCD45 isoform-specific inserts contained the appropriate cloning sites, ligation to the Ig vector was successful (Figure 5). All six fusion protein constructs were sequenced [42] to confirm accurate amplification of isoform-specific templates by PCR. All isoform-specific constructs were 100% confirmed with the exception of CD45RC, which was only 97% confirmed. A 100 ml mini plasmid prep of one positive clone for each isoform of CD45 was prepared in order to produce sufficient amounts of DNA for subcloning. The muCD45:MuIgG portion was cut out of the pBluescript plasmid vector using restriction enzymes Xho 1 and Not 1 and the purified fragment subcloned into the Xho 1/Not 1 digested and dephosphorylated mammalian expression vector, pBCMGSneo (Figure 6), in preparation for transient and stable expression in eucaryotic cells.

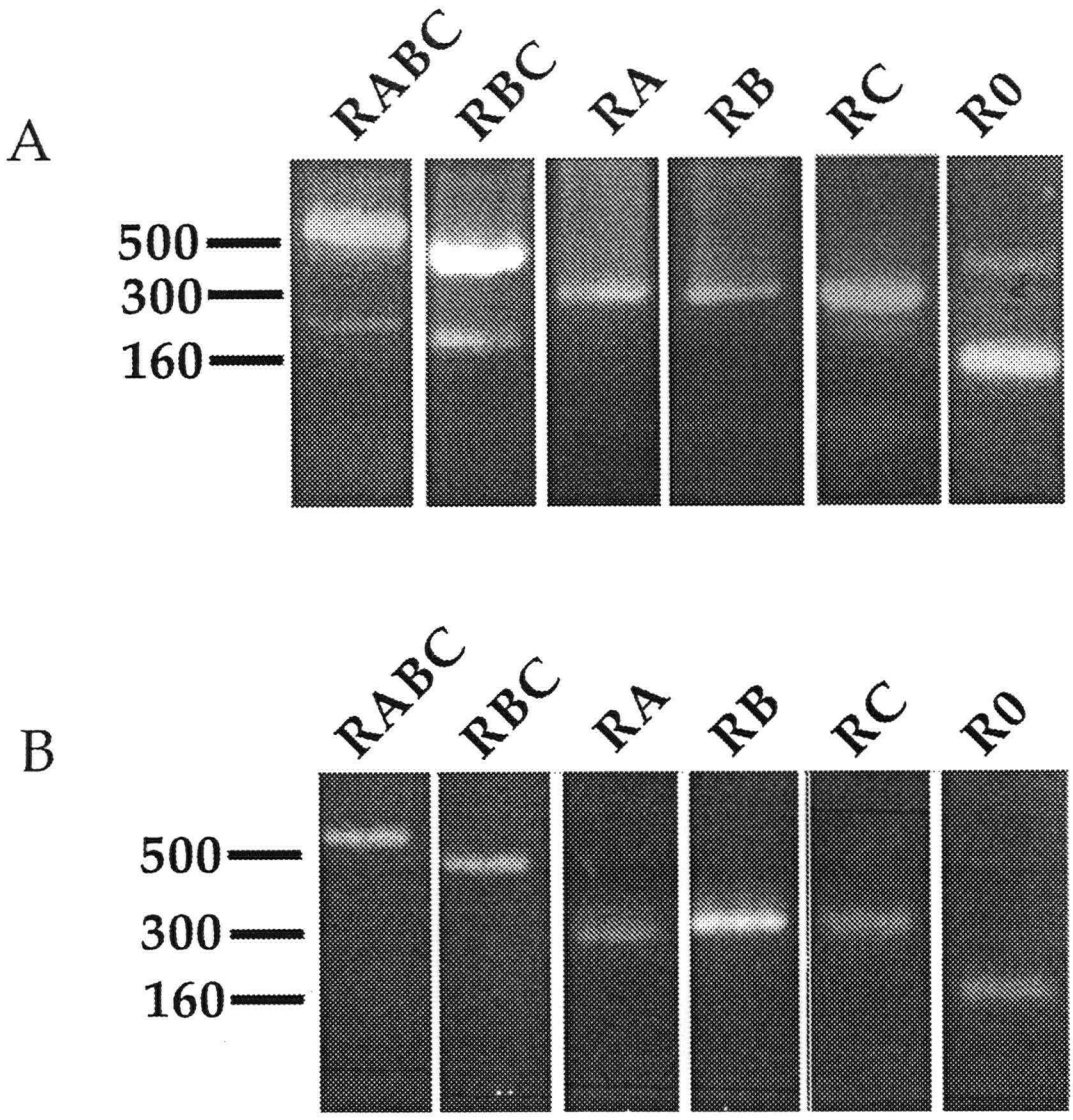


Figure 4: CD45 Isoform-specific PCR products

Isoform-specific inserts were created by PCR using isoform-specific plasmid DNA templates, 5' primer UBC#60 and 3' primer UBC#63. PCR products of the correct size were analyzed by 2% agarose gel electrophoresis and ethidium bromide staining followed by purification by extraction from low melt agarose and ethanol precipitation. A; unpurified PCR products, B; purified PCR products. Isoforms of CD45 are as indicated. Markers indicate size in base pairs.

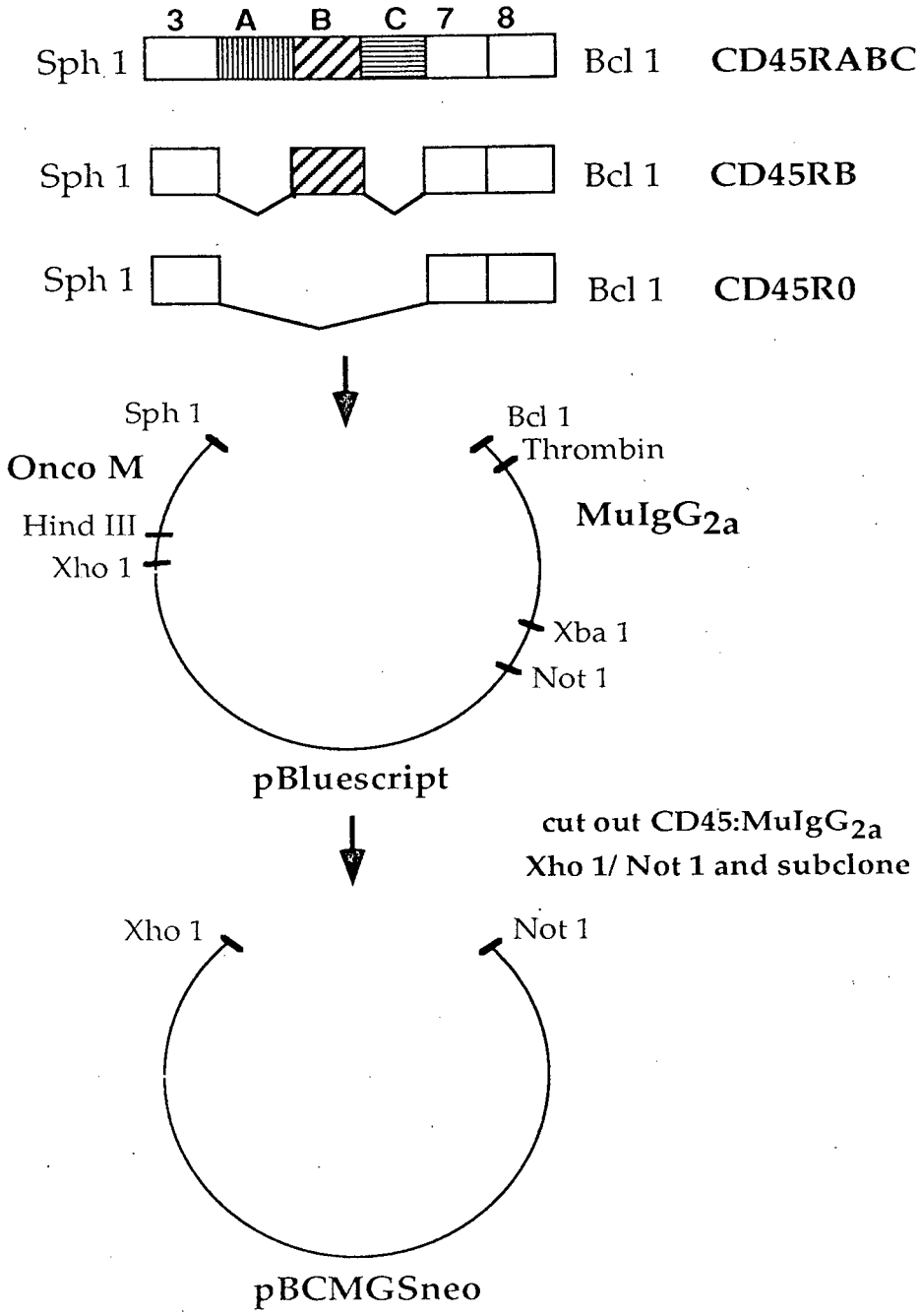


Figure 5: Subcloning of murine CD45 Isoform-specific Inserts into the murine Ig Vector.

Isoform-specific muCD45 inserts were created by PCR using isoform-specific plasmid DNA templates, an upstream 5' primer corresponding to muCD45 exon 3, and a downstream 3' primer corresponding to muCD45 exon 8. Restriction enzyme sites (Sph 1 upstream and Bcl 1 downstream) are present to allow for cloning into the Ig vector. The thrombin cleavage site is shown. 3,7,8, muCD45 exons incorporated;A,B,C, alternatively spliced exons incorporated; Onco M, Oncostatin M signal sequence.

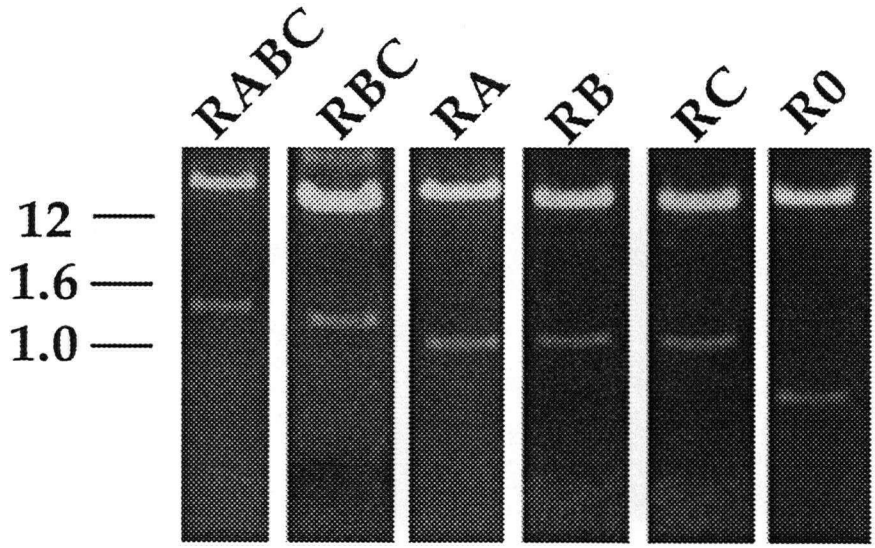


Figure 6: CD45:MuIgG Fusion Constructs in Mammalian Expression Vector pBCMGSneo

Fusion protein constructs in pBCMGSneo were digested with Xho 1 and Not 1 followed by 2% agarose gel electrophoresis and ethidium bromide staining to determine if isoform-specific muCD45:MuIgG was present. Isoforms of CD45 are as indicated. Markers indicate size is kilobase pairs.

RESULTS

2.0 Expression of murine CD45-Immunoglobulin Isoform-specific Constructs in Cos 7 cells

The goal of this series of experiments was to determine if CD45:MuIgG fusion proteins could be expressed by Cos 7 cells, a non-hematopoietic monkey kidney cell line that has been used successfully in the past for transient expression of recombinant proteins [9]. Cos 7 cells were used as an efficient transient expression system to rapidly establish if isoform-specific fusion proteins could be expressed and secreted into the culture supernatant. If isoform-specific fusion proteins are expressed by Cos 7 cells, it is reasonable to think that expression in more functionally relevant myeloid and lymphoid cell lines would also be successful. Attempts were made to transiently express six murine CD45 isoform-specific fusion protein constructs in Cos 7 cells by the DEAE-dextran transfection method. In theory, if an isoform-specific ligand interacts with the fusion protein consisting of the largest isoform of muCD45, CD45RABC:MuIgG, via the product of the three alternatively spliced exons, the same ligand will not bind to the smallest isoform of muCD45, CD45R0:MuIgG, due to the absence of exons A, B, and C. In addition, the putative ligand may or may not bind to a fusion protein containing only one or two of the alternatively spliced exons. Furthermore, CD45RB is a isoform commonly expressed by murine cell types, thus it is likely that this one-exon isoform will have a physiological ligand on murine cells. Therefore, in order to maximize the possibility of determining the identity of an isoform-specific ligand, stable clones of three isoform-specific fusion constructs, CD45RABC:MuIgG, CD45RB:MuIgG, and CD45R0:MuIgG were obtained by calcium phosphate transfection.

2.0.1 Transient Expression

Transient expression of five isoform-specific CD45:MuIgG fusion proteins was obtained by the DEAE-dextran method. Culture supernatants from 1 X 10^6 Cos 7 cells were harvested 3 days post-transfection and analyzed by precipitation with protein A followed by SDS-PAGE and western blotting with GAMIgG-HRP. Secreted fusion proteins were visualized as one distinct band (Figure 7, Panel A). The predicted molecular weight of unglycosylated fusion proteins and the apparent molecular weight as determined by precipitation and SDS-PAGE analysis of each isoform-specific fusion protein is shown in Table III. CD45RABC:MuIgG had an apparent molecular weight of 125 kDa, CD45RB:MuIgG was 70 kDa, and CD45R0:MuIgG was 55 kDa. In addition, fusion proteins containing two other isoforms of CD45, CD45RA:MuIgG, and CD45RBC:MuIgG, were expressed at the transient level and were observed as one band at 70 kDa and 85 kDa respectively. Although multiple attempts were made, transient expression of CD45RC:MuIgG was not observed. As the CD45RC PCR product was only 97% confirmed by DNA sequencing, this sequence should be reconfirmed before subsequent attempts at transient expression are made. Analysis of Cos 7 cell lysates for all expressed isoforms after allowing three days for secretion of fusion proteins revealed the existence of one or two bands at lower molecular weights than observed for the mature, glycosylated, secreted fusion protein. Lower molecular weight protein bands at approximately 60 kDa were observed in lysates of Cos 7 cells expressing CD45RA:MuIgG and CD45RB:MuIgG, at 73 kDa for CD45RBC:MuIgG, and at 75 kDa for CD45RABC:MuIgG. In addition, a band at approximately 35-40 kDa was observed in lysates of CD45RB:MuIgG and CD45RBC:MuIgG, indicating possible cleavage of proteins at the thrombin site. At least two independent clones of each isoform were analyzed by transient transfection and comparable results were obtained with respect to apparent molecular weight, purity, and protein bands in lysates. Figure 7, Panel B

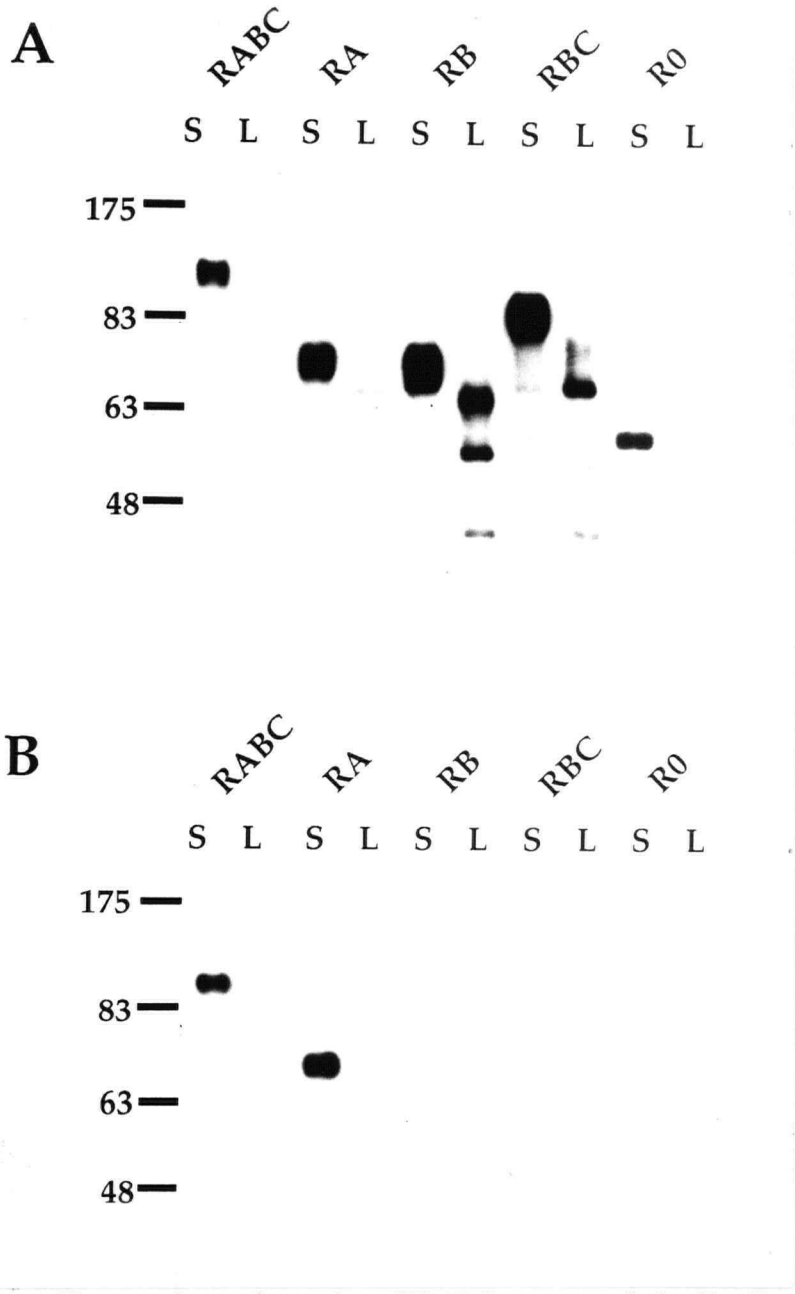


Figure 7: Transient Expression of murine CD45-Immunoglobulin Fusion Proteins in Cos 7 cells.

1 X 10^6 Cos 7 cells were transfected 30 µg of plasmid DNA by the DEAE-dextran method. Transfected cells were incubated in 3 ml of DMEM/10% FCS for three days prior to precipitation of 100 µl of supernatant (S) or 100 µl of lysate (L) with 20 µl of a 10% solution of protein A producing Pansorbin cells. Each sample was subjected to 7.5% SDS-PAGE and western blotting with: A, 1/5000 GAMIgG-HRP; B, 1/10 RA3 6B2. Cos 7 cells were lysed in 0.5 ml of 1% Triton/TNE lysis buffer for 20 minutes on ice.

Table III

Fusion Protein	Predicted Unglycosylated Molecular Weight	Apparent Molecular Weight in each cell line		
		Cos 7	X63-Ag8.653	T28
CD45R0:MuIgG	34	55	50	50
CD45RA:MuIgG	39	75	nd	nd
CD45RB:MuIgG	40	70	65	70
CD45RC:MuIgG	40	no	no	no
CD45RBC:MuIgG	46	90	nd	nd
CD45RABC:MuIgG	50	125	110	120

Table III: Apparent Molecular Weight of CD45-Immunoglobulin Isoform Fusion Proteins Expressed in Three Cell Lines.

The apparent molecular weight as determined by precipitation of 100 µl of cell culture supernatant with 20 µl of a 10% solution of protein A producing Pansorbin cells. The entire sample was subjected to 7.5% SDS-PAGE analysis against NEB unstained molecular weight standards and western blotting with 1/5000 GAMIgG-HRP. Values reflect an average of four experiments. nd; not determined. no; not obtained.

indicates that RA3 6B2, a B220 isoform-specific antibody, reacted with both CD45RABC:MuIgG and CD45RA:MuIgG. Fusion proteins containing no alternatively spliced exons or exons B and/or C did not react with RA3 6B2 as predicted.

2.0.2 Stable Expression

As attempts at stable expression of CD45:MuIgG fusion proteins by the DEAE-dextran method were unsuccessful, stable expression of three isoform fusion constructs, CD45R0:MuIgG, CD45RB:MuIgG, and CD45RABC:MuIgG, was obtained by the calcium phosphate method. Approximately 5% of neomycin resistant colonies tested positive for secretion of isoform-specific fusion proteins into the culture supernatant (Table IV). As with transient expression experiments, fusion proteins expressed in culture supernatants were observed as a single, distinct band upon precipitation with protein A, SDS-PAGE analysis, and western blotting with GAMIgG-HRP (Figure 8). In addition, comparison of apparent molecular weight between transient and stable expression systems revealed similar results (Figure 7 versus Figure 8). However, slight clonal variation in the apparent molecular weight of CD45RABC:MuIgG, and to some extent of CD45RB:MuIgG was observed.

CD45:MuIgG fusion proteins were produced in relatively pure amounts using serum free hybridoma media (Figure 9). Incubation of fusion protein-secreting cells in DMEM/10% FCS followed by precipitation of culture supernatant with protein A, SDS-PAGE analysis, and Coomassie brilliant blue staining resulted in the presence of contaminating proteins at approximate molecular weights of 55 kDa, 65 kDa and > 175 kDa. In contrast, incubation of fusion protein-secreting cells in serum free hybridoma media resulted in the appearance of only one protein band at the molecular weight previously observed to be that of either CD45R0:MuIgG or CD45RABC:MuIgG. Fusion protein yield appeared to be less in serum free media.

Table IV

Cell Line	CD45 Isoform	Total Number neo^r colonies	Number of neo^r colonies analyzed	Number of CD45:MuIgG secreting colonies
Cos 7	RABC	96	54	2
	RB	100	26	2
	R0	110	26	3
X63-Ag8.653	RABC	20	12	4
	RB	6	6	3
	R0	30	16	13
T28	RABC	80	24	3
	RB	83	23	19
	R0	90	4	4

Table IV: Results of Stable Transfection of CD45-Immunoglobulin Isoform Fusion Constructs into Three Cell Lines.

Colonies were screened by precipitation of 150 µl of culture supernatant with 20 µl of a 10% solution of protein A producing Pansorbin cells followed by 7.5 % SDS-PAGE analysis of the entire sample and western blotting with 1/5000 GAMIgG-HRP for 1 hour in 5 % BSA/TBS.

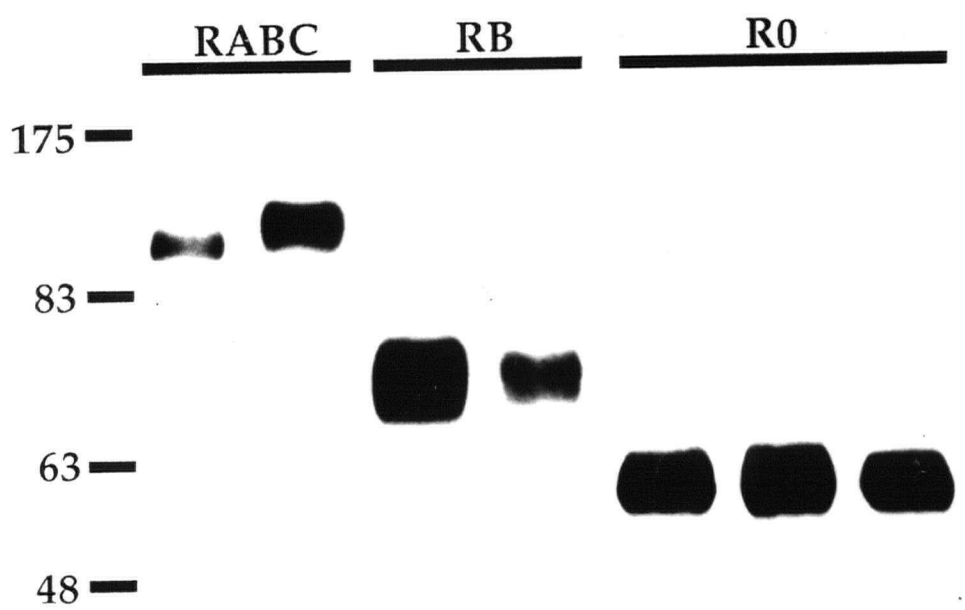


Figure 8: Western Blot of CD45-Immunoglobulin Fusion Proteins Expressed in Cos 7 cells.

100 µl of cell culture supernatant was precipitated with 20 µl of a 10% solution of protein A producing Pansorbin cells followed by 7.5% SDS-PAGE analysis of the entire sample and western blotting with 1/5000 GAMIgG-HRP. Duplicate lanes represent samples from different clones.

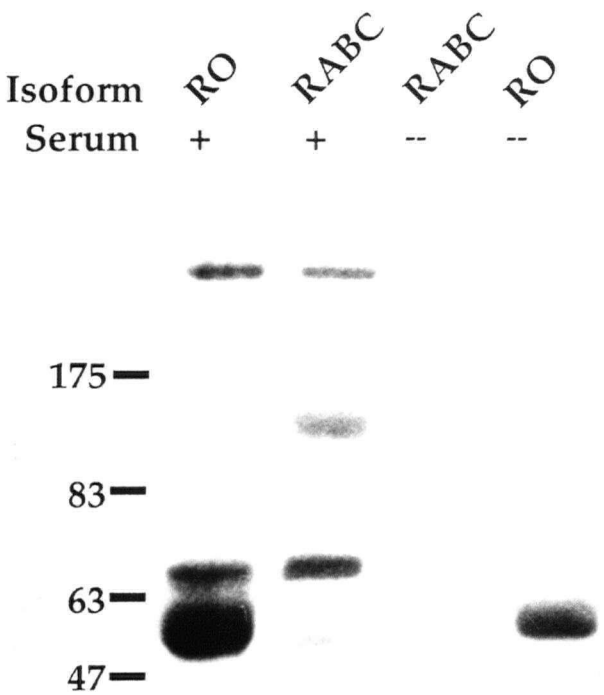


Figure 9: Relative Yield and Purity of CD45-Immunoglobulin Fusion Proteins Expressed in Cos 7 cells.

1.8 ml of cell culture supernatant was precipitated with 150 µl of a 10% solution of protein A producing Pansorbin cells followed by 7.5% SDS-PAGE and staining with Coomassie brilliant blue-R250. Serum +, supernatant from 1 X 10^6 cells grown in 10 ml of DMEM/10% FCS for three days; Serum -, supernatant from 1 X 10^6 cells grown in 10 ml of Hybridoma serum free media for three days.

Scanning densitometry and comparison to standards of known amount established that 1 X 10^6 Cos 7 cells secreted approximately 1.5-2.0 μg/ml CD45RABC:MuIgG or 3.0-4.0 μg/ml CD45R0:MuIgG over a three day period in 10 ml of serum free media (Table V). IgG standards of 1 μg, 5μg, and 8μg corresponded to OD readings of 4.78, 22.8, and 32.0 respectively. Therefore, upon calculation of the standard curve, the OD readings of 12.3 for CD45RRABC:MuIgG and 30.5 for CD45R0:MuIgG corresponded to 2.7 μg and 7.4 μg of protein respectively (per 1.8 ml of supernatant precipitated, therefore, 1.5 μg/ml CD45RABC:MuIgG and 4.0 μg/ml CD45R0;MuIgG). Limitations of this method include the following: protein A precipitated fusion proteins may not bind coomassie blue with the same affinity as unprecipitated murine IgG standards, or alternatively, the presence of carbohydrate residues may differentially affect the binding of coomassie blue.

The results obtained using Cos 7 cells as a model expression system serve to establish four important points. Firstly, it is clear from both transient and stable expression experiments that CD45:MuIgG fusion proteins are secreted into the culture supernatant. In addition, expressed fusion proteins are observed to have an apparent molecular weight significantly higher than the predicted molecular weight of unglycosylated proteins, suggesting that fusion proteins are extensively glycosylated before secretion. Thirdly, it has been established that by using the expression vector, pBCMGSneo, fusion proteins are secreted at sufficient levels to be detected in culture supernatants by precipitation with protein A followed by SDS-PAGE and Coomassie brilliant blue staining or western blotting with an anti-murine IgG antibody. Finally, in addition to transient expression, stable fusion protein secreting clones can be obtained by calcium phosphate transfection.

It has now been determined that this strategy for expression of murine CD45:MuIgG fusion proteins results in the secretion of detectable amounts of protein by the methods described. Thus, it is now important to express these fusion proteins in functionally relevant myeloid and lymphoid cells. If the interaction of

Table V

Cell Line	CD45RABC:MuIgG	CD45RO:MuIgG
Cos 7	1.5-2.0 μg/ml	3.0-4.0 μg/ml
X63-Ag8.653	1.5-2.5 μg/ml	3.5-4.2 μg/ml

Table V: Estimated Yield of CD45-Immunoglobulin Isoform Fusion Proteins Expressed in Two Cell Lines.

Estimated yield of secreted CD45:MuIgG fusion proteins was determined by precipitation of 1.8 ml of cell culture supernatant with 150 μl of a 10% solution of protein A producing Pansorbin cells followed by 7.5 % SDS-PAGE and staining with Coomassie brilliant blue-R250. Band intensity was determined by scanning densitometry. Values reflect the concentration of fusion protein produced by 1 X 10^6 cells in 10 ml of media over a three day period. Results are a range over three experiments.

CD45 with a putative ligand is indeed mediated by specific carbohydrate residues on CD45, it is crucial to express CD45:MuIgG fusion proteins in a cell line that will mimic endogenous CD45 glycosylation. If glycosylation of CD45:MuIgG fusion proteins is not comparable to that of endogenous CD45, a ligand that binds to endogenous CD45 would not bind to a CD45:MuIgG fusion protein or vice versa. As CD45 is expressed on the surface of all nucleated cells of hematopoietic lineage, the use of myeloid and lymphoid cells in place of Cos 7 cells to express CD45:MuIgG fusion proteins would most likely result in a closer, if not identical, glycosylation pattern to that of endogenous CD45. Characterization of carbohydrate residues on endogenous CD45 as well as on CD45:MuIgG fusion proteins is necessary to determine if secreted fusion proteins are mimicking endogenous CD45.

RESULTS

3.0 Expression of murine CD45-Immunoglobulin Isoform-specific Constructs in Myeloid and Lymphoid Cell Lines

As a detectable level of expression of muCD45:MuIgG fusion proteins was observed in Cos 7 cells using the methods previously described, the focus of the next series of experiments was to obtain stable, fusion protein-secreting clones in functionally relevant cells, specifically myeloid and lymphoid cell lines. The cell lines transfected with fusion construct DNA were X63-Ag8.653, a murine myeloma, and T28, a murine T cell lymphoma. As with stable clones expressed by Cos 7 cells, three isoforms of muCD45 linked to heavy chain constant regions 2 and 3 of muIgG were expressed: CD45RABC:MuIgG, CD45RB:MuIgG, and CD45R0:MuIgG. The expression of endogenous CD45 by X63-Ag8.653 myeloma and T28 lymphoma may reflect the ability of these cell types to express CD45:Ig fusion proteins in a manner that would mimic endogenous CD45 expression. FACS analysis indicates that X63-Ag8.653 cells do not express any isoform of endogenous CD45 whereas T28 cells express the common epitope of CD45, as recognized by a pan-specific antibody I3/2, as well as one isoform of CD45, CD45RB, as recognized by exon B-specific antibodies MB 4B4 and 23 G2 (Figure 10). Although the goat-anti-rat IgG negative control for X63-Ag8.653 cells appears to be slightly positive, indicating possible heavy chain Fc receptor binding, the binding of subsequent exon-specific antibodies did not increase significantly when compared to the negative control.

3.0.1 Stable Expression in the X63-Ag8.653 Murine Myeloma Cell Line

The results of transfection of muCD45:MuIgG fusion proteins into X63-Ag8.653 murine myeloma cells by electroporation is shown in Table IV.

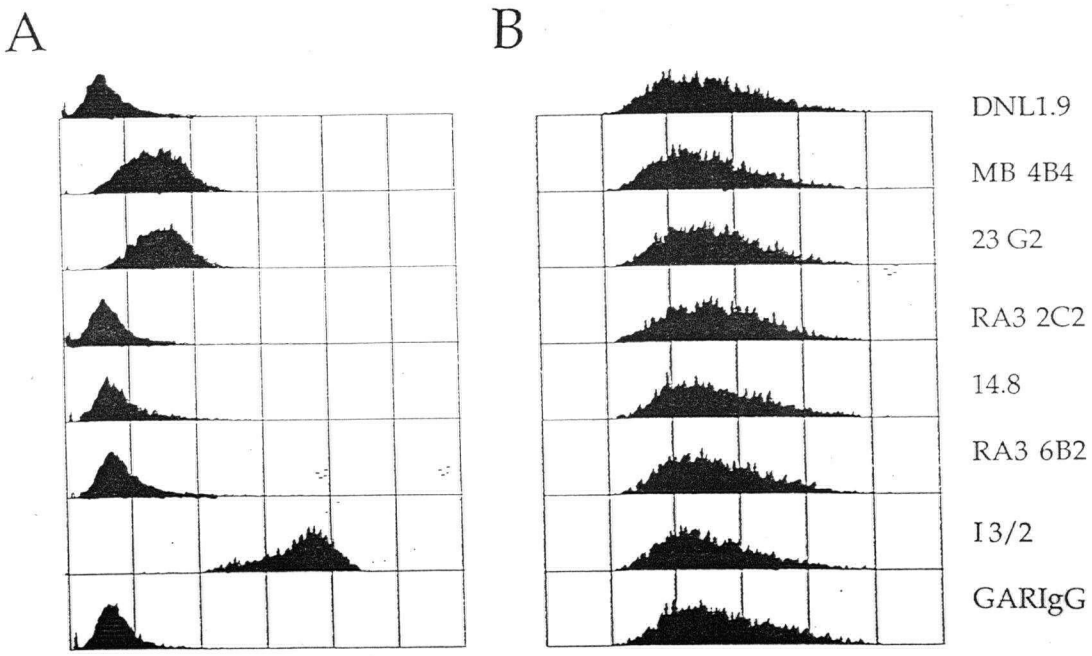


Figure 10: FACSCAN of Cell Lines Transfected with CD45-Immunoglobulin Isoform Constructs.

Expression of muCD45 isoforms as determined by Flow Cytometry. 2 X 10^5 cells were incubated with 100 µl of exon-specific tissue culture supernatant and 1/100 Goat-anti-rat-FITC. A, T28 cells; B, X63-Ag8.653 cells. Exon-specific antibodies: RA3 6B2, B220 isoform; 14.8 and RA3 2C2, CD45 exon A; 23 G2 and MB 4B4, CD45 exon B; DNL1.9, CD45 exon C, I 3/2, pan-specific anti-CD45.

Approximately 30% of neomycin resistant clones, as determined by precipitation with protein A followed by western blot analysis using an anti-muIgG antibody, tested positive for secretion of CD45RABC:MuIgG whereas 50% and 80% of clones tested positive for secretion of CD45B:MuIgG and CD45R0:MuIgG respectively. The apparent molecular weight of CD45:MuIgG fusion proteins expressed by X63-Ag8.653 cells as determined by precipitation with protein A and western blot analysis with anti-muIgG antibody is shown in Figure 11 and summarized in Table III. CD45RABC:MuIgG secreted by X63-Ag8.653 cells had an apparent molecular weight of 110 kDa, CD45RB:MuIgG was 65 kDa and CD45R0:MuIgG was 50 kDa. In general, the apparent molecular weight of fusion proteins expressed by X63-Ag8.653 cells was 5-10 kDa lower than that of the equivalent fusion protein expressed by Cos 7 cells. In addition, as with fusion proteins secreted by Cos 7 cells, the apparent molecular weight of fusion proteins expressed by X63-Ag8.653 cells was significantly higher than the predicted molecular weight of unglycosylated fusion protein. Scanning densitometry established that 1 X 10^6 X63-Ag8.653 cells in 10 ml of DMEM/10% FCS secreted 1.5-2.5 μg/ml of CD45RABC:MuIgG and 3.5-4.2 μg/ml of CD45R0:MuIgG over a three day period (Table V). IgG standards of 1μg, 5μg, and 8μg corresponded to OD readings of 8.0, 23.1, and 30.0 respectively. Upon calculation of a standard curve, the OD reading of 20.1 for CD45RABC:MuIgG corresponded to 4.5 μg of protein (in 1.8 ml of supernatant precipitated, therefore, 2.5 μg/ml). Likewise, calculation of a standard curve for CD45R0:MuIgG revealed that the OD reading of 33.2 corresponded to 7.6 μg of protein (in 1.8 ml, therefore, 4.2 μg/ml). These values are comparable to those obtained using Cos 7 cells.

3.0.2 Transient Expression in the T28 Murine T lymphoma Cell Line

Unfortunately, stable fusion protein secreting clones were not obtained by electroporation of T28 cells. As a result, transient expression of muCD45:MuIgG

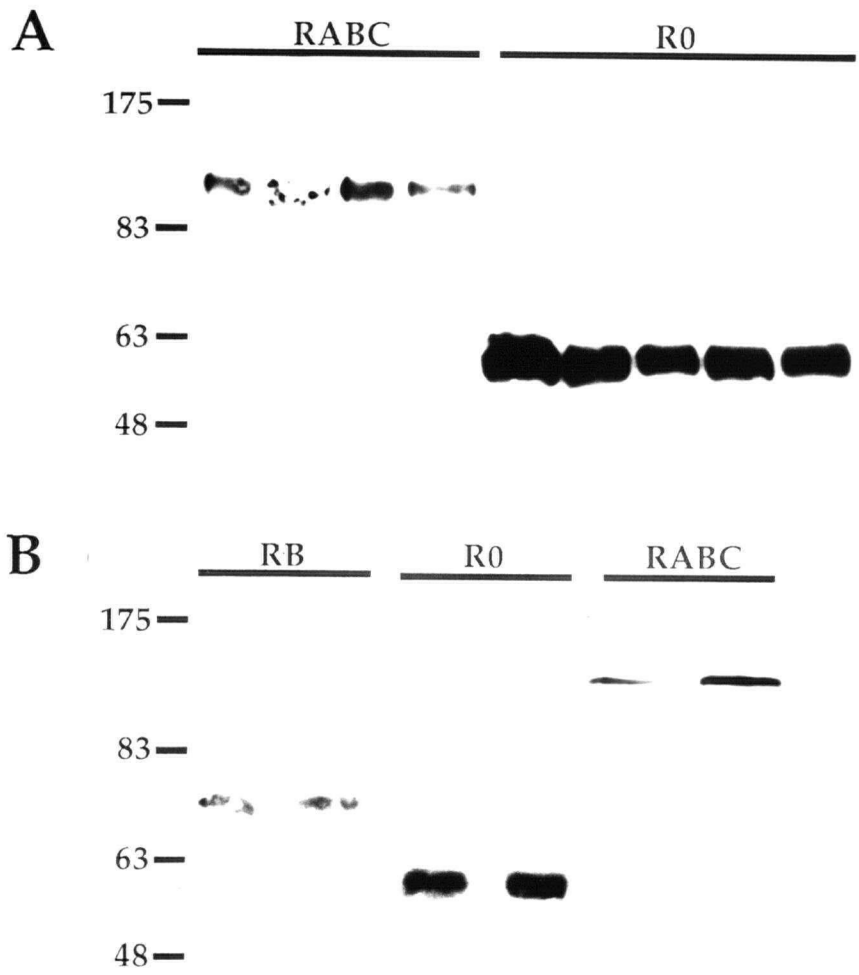


Figure 11: Western Blots of CD45-Immunoglobulin Fusion Proteins Expressed by X63-Ag8.653 and T28 cells.

100-400 µl of cell culture supernatant was precipitated with 20 µl of a 10% solution of Pansorbin cells followed by 7.5% SDS-PAGE analysis of the entire sample and western blotting with 1/5000 GAMIgG-HRP. A, fusion proteins expressed by X63-Ag8.653 cells; B, fusion proteins expressed by T28 cells.

fusion proteins by T28 cells was observed before levels of fusion protein expression decreased below the limit of detection possible by precipitation with protein A and western blotting with anti-muIgG antibody. The results of transfection of fusion construct DNA into T28 cells are shown in Table IV. CD45RABC:MuIgG had an apparent molecular weight of 120 kDa, CD45RB:MuIgG was 70 kDa and CD45R0:MuIgG was 50 kDa (Figure 11, Table III). As with CD45:MuIgG fusion proteins expressed by Cos 7 and X63-Ag8.653 cells, the apparent molecular weight of fusion proteins expressed by T28 cells was significantly higher than the predicted molecular weight of unglycosylated fusion protein. In addition, apparent molecular weight of fusion proteins expressed in T28 cells was in the same range as that observed with Cos 7 and X63-Ag8.653 cells (Figure 8 versus Figure 11, Table III). Initially, expression of fusion proteins by T28 cells was comparable to that observed for Cos 7 and X63-Ag8.653 cells, but within 3 days, T28 expression levels decreased to below detectable limits by SDS-PAGE and Coomassie brilliant blue staining or western blot.

4.0 Characterization of Expressed murine CD45-Immunoglobulin Fusion Proteins

Once expressed, muCD45:MuIgG fusion proteins were fully characterized not only as to apparent molecular weight, dimerization in non-reducing SDS-PAGE conditions, and reactivity with anti-CD45 exon-specific antibodies, but also as to N-linked and O-linked carbohydrate content by reactivity with carbohydrate residue-specific lectins, PNGase F digestion, O-glycosidase treatment, thrombin cleavage, and neuraminidase digestion. As the interaction with a putative ligand may be mediated by specific carbohydrate residues on CD45, it is important to fully characterize the amount and type of carbohydrate added by each cell line.

4.0.1 Reactivity with anti-CD45 Exon-specific Antibodies

Transient expression in Cos 7 cells of five isoforms of CD45 linked to the heavy chain constant regions of murine IgG is shown in Figure 7. All secreted fusion proteins reacted strongly with an antibody that recognizes the constant region of murine IgG_{2a} upon precipitation with protein A and western blot analysis. In addition, the anti-muIgG antibody reacted with lower molecular weight proteins in the lysates of Cos 7 cells transfected with fusion construct DNA (Figure 7, Panel A). RA3 6B2, an antibody specific for the B220 isoform of CD45, reacted not only with CD45RABC:MuIgG but also with the fusion protein containing only one alternatively spliced exon (4,A), CD45RA:MuIgG (Figure 7, Panel B). This is the first demonstration that RA3 6B2 reacts with an epitope dependent on the expression of exon A. All transiently expressed fusion proteins reacted with anti-CD45 exon-specific antibodies MB 4B4, 23 G2 (exon B dependent), 14.8 (exon A dependent), and

DNL1.9 (exon C dependent) as predicted depending on the presence of alternatively spliced exons A, B, and/or C (data not shown). In addition, anti-CD45 exon-specific antibodies did not react with the proteins in Cos 7 cell lysates (Figure 7) with the exception of the exon C-specific antibody, DNL1.9 (data not shown).

In addition to transient Cos 7 clones, stable clones produced by Cos 7 and X63-Ag8.653 cells secreting either CD45RABC:MuIgG, CD45RB:MuIgG, or CD45R0:MuIgG were analyzed for reactivity with anti-muIgG and anti-CD45 exon-specific antibodies. The results are summarized in Table VI. Moreover, antibody reactivity analysis was conducted on fusion proteins expressed transiently by T28 cells.

Antibody reactivity analysis of CD45:MuIgG expressed in a stable fashion by Cos 7 cells is shown in Figure 12. All isoform-specific fusion proteins reacted strongly with the anti-muIgG antibody. In addition, all fusion proteins reacted with exon-specific antibodies as expected depending on the presence of exons A, B, and/or C. CD45RABC:MuIgG reacted faintly with exon A-specific 14.8 and strongly with B220 isoform-specific RA3-6B2, although variation in RA3-6B2 reactivity was observed between the two clones obtained. Exon B-specific MB 4B4 reacted very strongly with CD45RABC:MuIgG and CD45RB:MuIgG but not with CD45R0:MuIgG as expected. An alternate exon B-specific antibody, 23 G2, reacted with all fusion proteins containing exon B, but to a lesser extent and lower intensity than that observed with MB 4B4. Variation in 23 G2 reactivity was observed for CD45RB:MuIgG in that one clone reacted with a significantly higher intensity than the other. Exon C-specific DNL1.9 reacted with CD45RABC:MuIgG and not CD45RB:MuIgG and CD45R0:MuIgG as expected.

Antibody reactivity analysis of CD45:MuIgG fusion proteins expressed by X63-Ag8.653 murine myeloma cells is shown in Figure 13. Four independent fusion protein secreting clones were analyzed for CD45RABC and five independent clones were analyzed for CD45R0. All fusion proteins reacted with anti-muIgG as expected. As observed with fusion proteins expressed by Cos 7 cells, fusion

Table VI

	CD45RABC		CD45R0		CD45RB	
	Cos 7	X63-Ag8.653	Cos	X63-Ag8.653	Cos 7	X63-Ag8.653
Anti Ig	+	+	+	+	+	+
RA3 6B2	+	-	-	-	-	-
MB 4B4	+	+	-	-	+	+
23 G2	+	+	-	-	+	+
14.8	+	+	-	-	-	-
DNL1.9	+	+	-	-	-	-

Table VI: Reactivity of CD45-Immunoglobulin Fusion Proteins with Anti-CD45 Exon-Specific Antibodies.

CD45:MuIgG Fusion proteins were precipitated from 100 µl of cell culture supernatant using 20 µl of a 10% solution of protein A producing Pansorbin cells. The entire sample was subjected to 7.5 % SDS-PAGE analysis and subsequent western blotting using anti-murine CD45 exon-specific antibody tissue culture supernatants. Antibody specificity is as follows: Anti Ig, heavy and light chains of mouse IgG_{2a}; RA3 6B2, B220 isoform; MB 4B4 and 23 G2, CD45 exon B; 14.8, CD45 exon A; DNL1.9, CD45 exon C. +, positive reaction; - , negative reaction.

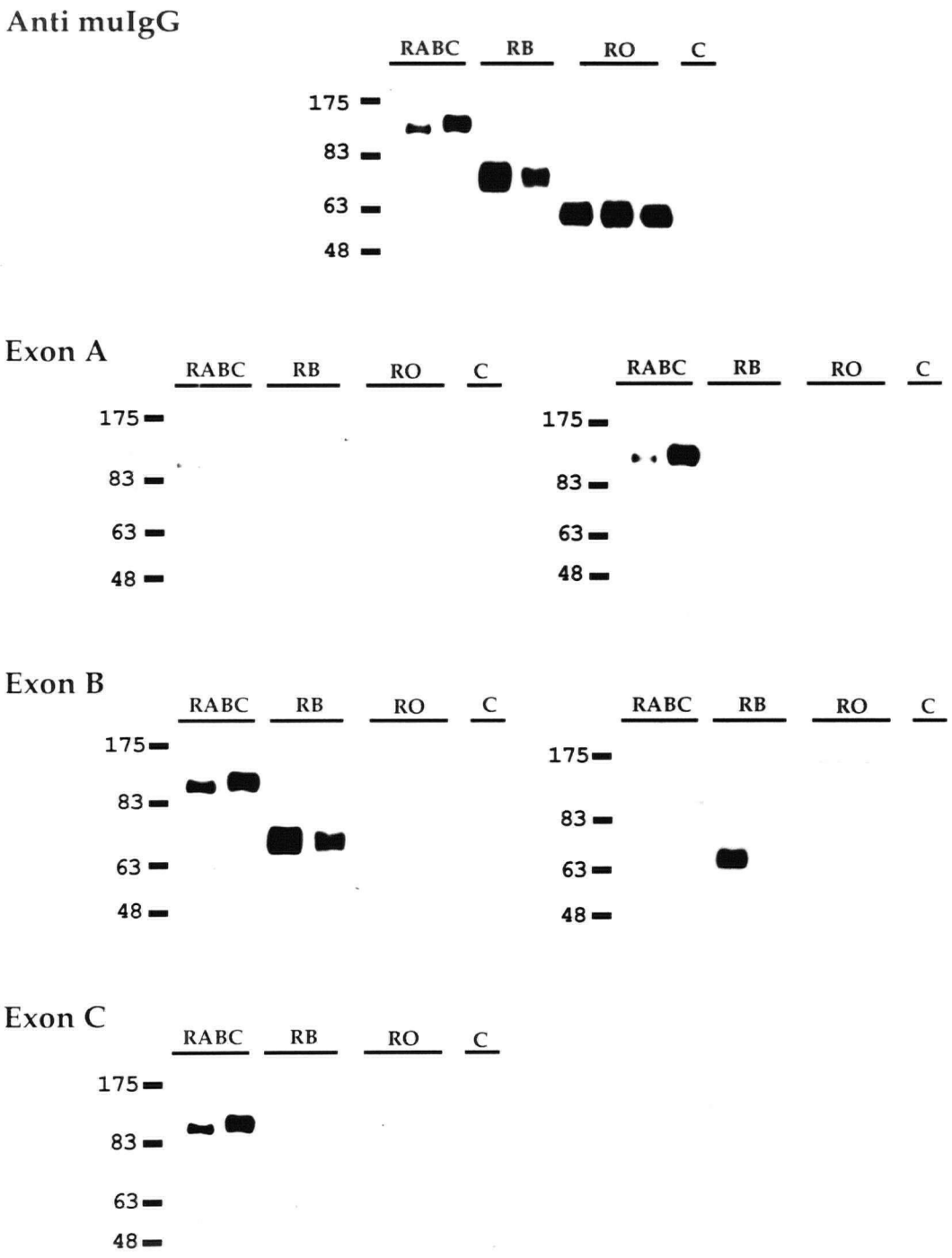


Figure 12: Reactivity of CD45-Immunoglobulin Fusion Proteins Expressed in Cos 7 cells with Anti-CD45 Exon-specific Antibodies.

Precipitation of 100 µl of cell culture supernatant with 20 µl of a 10% solution of protein A producing Pansorbin cells followed by 7.5% SDS-PAGE and western blotting with the antibody indicated. From left to right, top to bottom: Anti muIgG, GAMIgG-HRP; Exon A, 14.8 and RA3 6B2; Exon B, MB 4B4 and 23 G2; Exon C, DNL1.9. C, negative control.

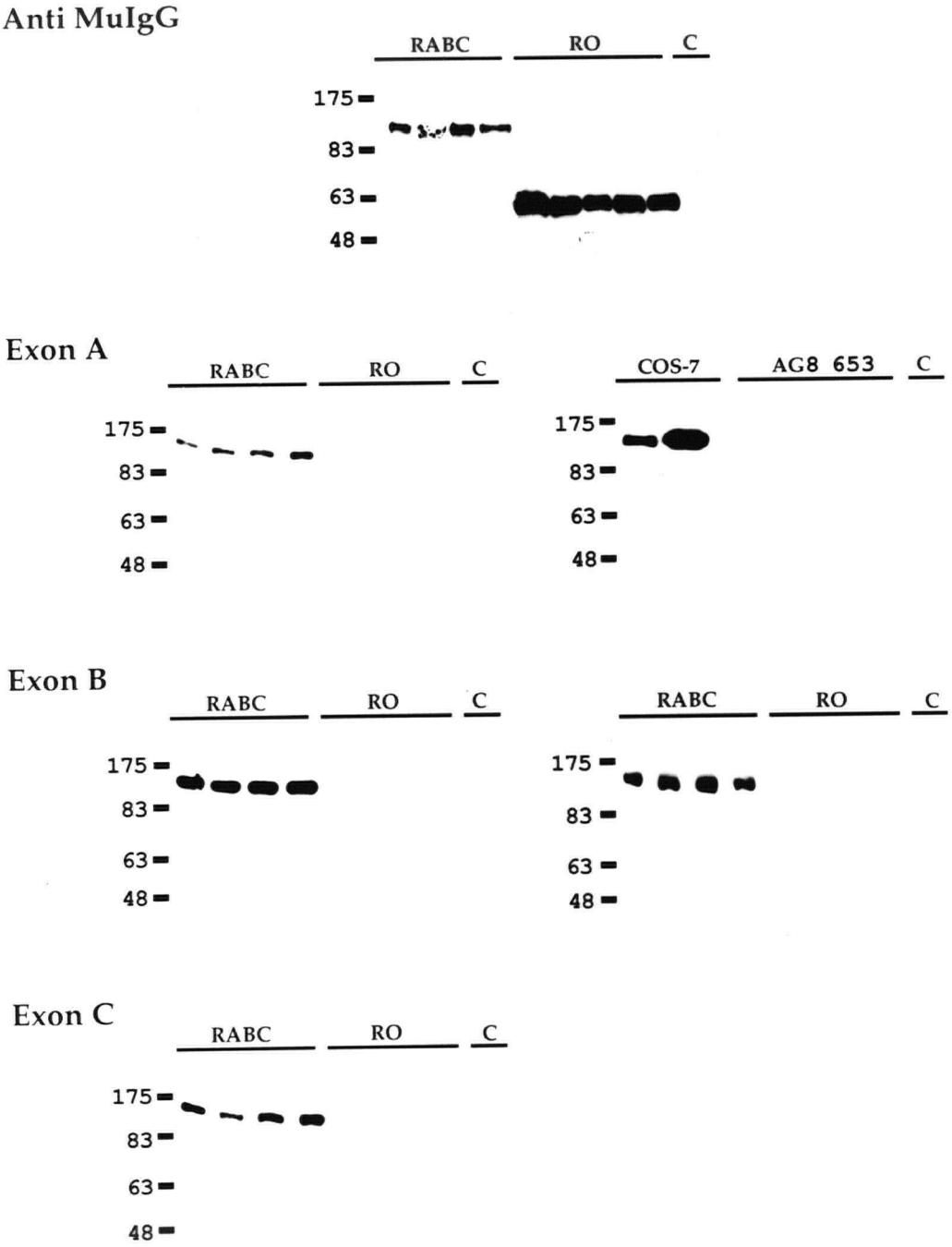


Figure 13: Reactivity of CD45-Immunoglobulin Fusion Proteins Expressed in X63-Ag8.653 cells with Anti-CD45 Exon-specific Antibodies.

Precipitation of 100 µl of cell culture supernatant with 20 µl of a 10% solution of protein A producing Pansorbin cells followed by 7.5% SDS-PAGE and western blotting with the antibody indicated. From left to right, top to bottom: Anti muIgG, GAMIgG-HRP; Exon A, 14.8 and RA3 6B2 (CD45RABC:MuIgG only); Exon B, MB 4B4 and 23 G2; Exon C, DNL1.9. C, negative control.

proteins expressed by X63-Ag8.653 cells reacted with exon A-specific 14.8, exon B-specific MB 4B4 and 23 G2, and exon C-specific DNL1.9 as expected depending on the presence of the appropriate alternatively spliced exon (Figure 12 versus Figure 13). In contrast to CD45RABC:MuIgG expressed by Cos 7 cells, CD45RABC:MuIgG expressed by X63-Ag8.653 cells did not react with B220 isoform-specific RA3-6B2.

Figure 14 shows antibody reactivity of CD45:MuIgG expressed transiently by T28 cells. Results were similar to that obtained by using fusion proteins expressed by X63-Ag8.653 cells (Figure 13 versus Figure 14). Each of two independent clones for CD45RABC, CD45RB, and CD45R0 reacted with anti-muIgG and the exon-specific antibodies previously outlined as expected. In agreement with the results obtained using X63-Ag8.653 cells but in contrast to results obtained using Cos 7 cells, fusion proteins secreted by T28 cells did not react with the B220 isoform-specific antibody RA3 6B2.

4.0.2 Analysis of N-linked Glycosylation

The presence of N-linked carbohydrate was detected and analyzed by digestion with PNGase F (Figure 15). Treatment of protein A-precipitated CD45RABC:MuIgG and CD45R0:MuIgG with PNGase F resulted in a slight decrease in apparent molecular weight of approximately 5 kDa for both isoforms of CD45. As a positive control, endogenous CD45 expressed by T28 cells was precipitated with the I 3/2 antibody conjugated to sepharose beads, and then digested with PNGase F, resulting in a decrease in apparent molecular weight as visualized by SDS-PAGE and western blotting with CD45 exon B-specific MB 4B4.

CD45:MuIgG fusion proteins can be cut into two pieces at the engineered thrombin cleavage site between CD45 and murine IgG (see Figure 5). This treatment allows for determination of the the apparent molecular weight of each portion of the fusion protein. The results of thrombin cleavage are shown in Figure 16.

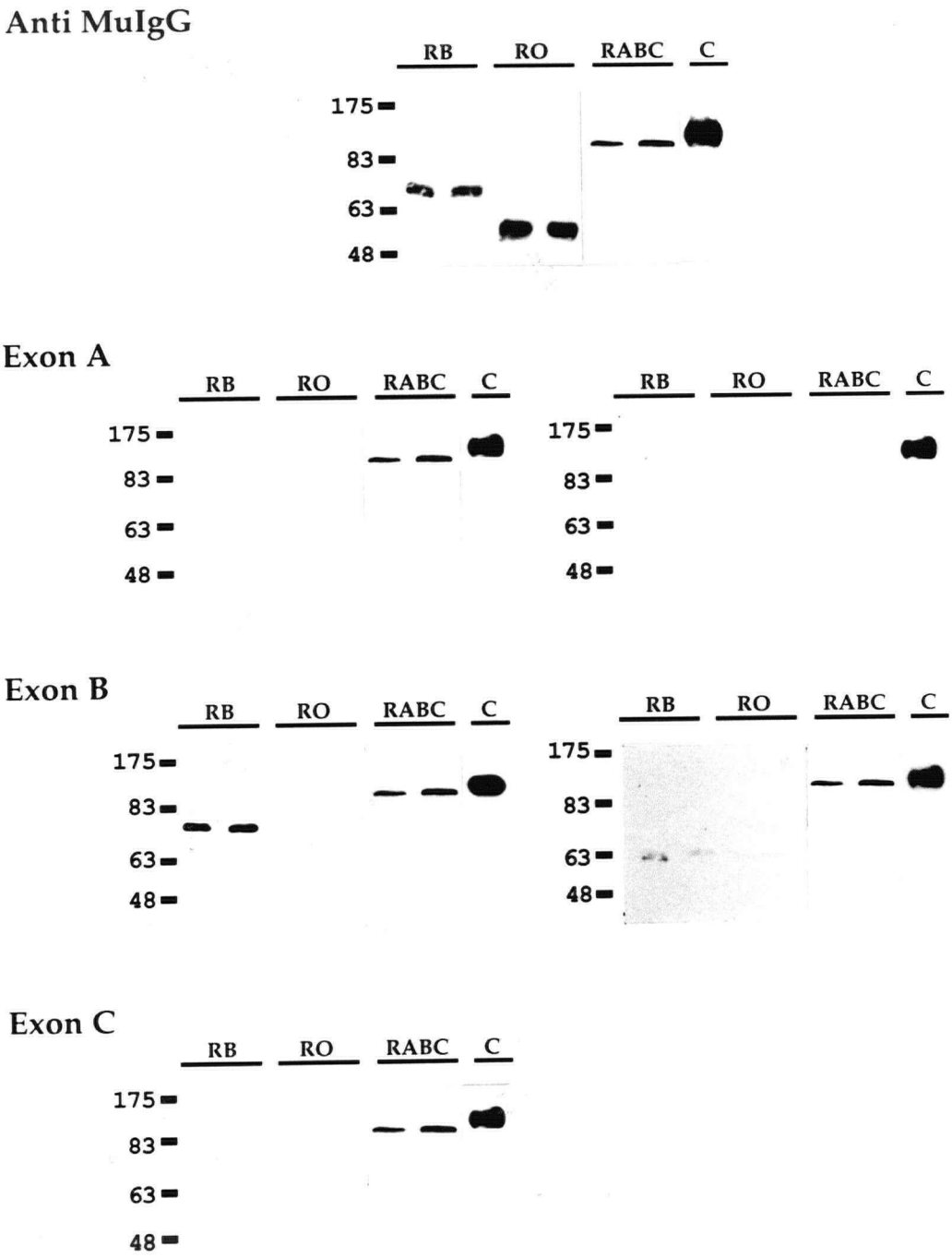


Figure 14: Reactivity of CD45-Immunoglobulin Fusion Proteins Expressed in T28 cells with Anti-CD45 Exon-specific Antibodies.

Precipitation of 100 µl of cell culture supernatant with 20 µl of a 10% solution of protein A producing Pansorbin cells followed by 7.5% SDS-PAGE and western blotting with the antibody indicated. From left to right, top to bottom: Anti muIgG, GAMIgG-HRP; Exon A, 14.8 and RA3 6B2; Exon B, MB 4B4 and 23 G2; Exon C, DNL1.9. C, positive control, CD45RABC:MuIgG expressed by Cos 7 cells precipitated in the same manner.

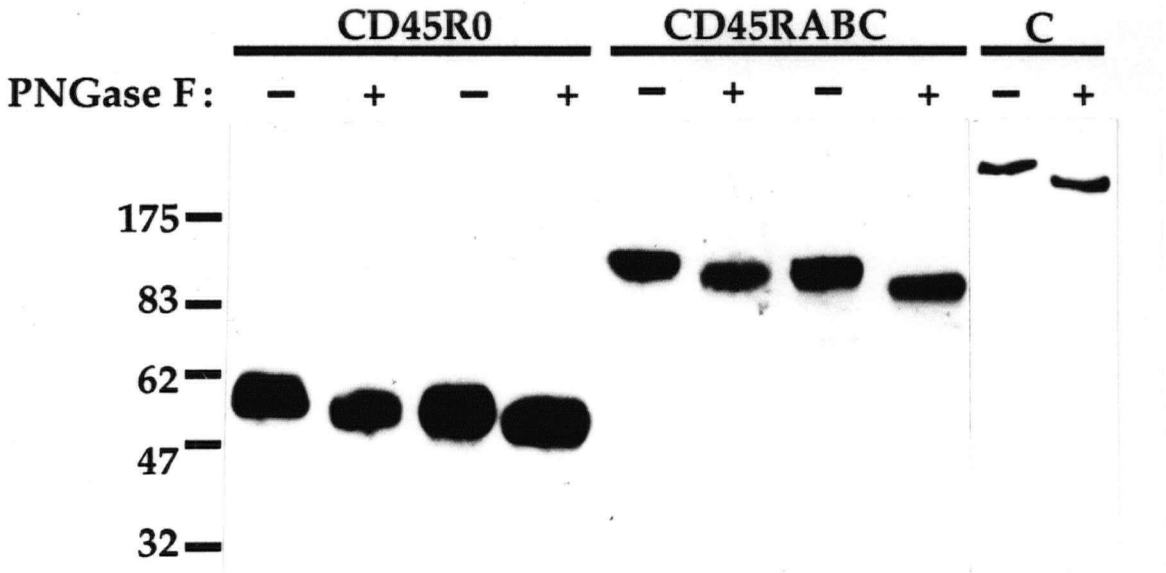


Figure 15: PNGase F Digestion of CD45-Immunoglobulin Fusion Proteins .

100-600 µl of cell culture supernatant was precipitated with 20 µl of a 10% solution of protein A producing Pansorbin cells followed by treatment with 1000 Units of PNGase F (New England Biolabs) for one hour at 37°C. The entire sample was subjected to 7.5% SDS-PAGE and western blotting with 1/5000 GAMIgG-HRP. +, treated samples; -, untreated samples. From left to right: isoform-specific fusion proteins secreted by Cos 7 cells, isoform-specific fusion proteins secreted by X63-Ag8.653 cells. C, PNGase F treated CD45 precipitated from 3 X 10^6 T28 cells with 20 µl of I 3/2 conjugated sepharose beads.

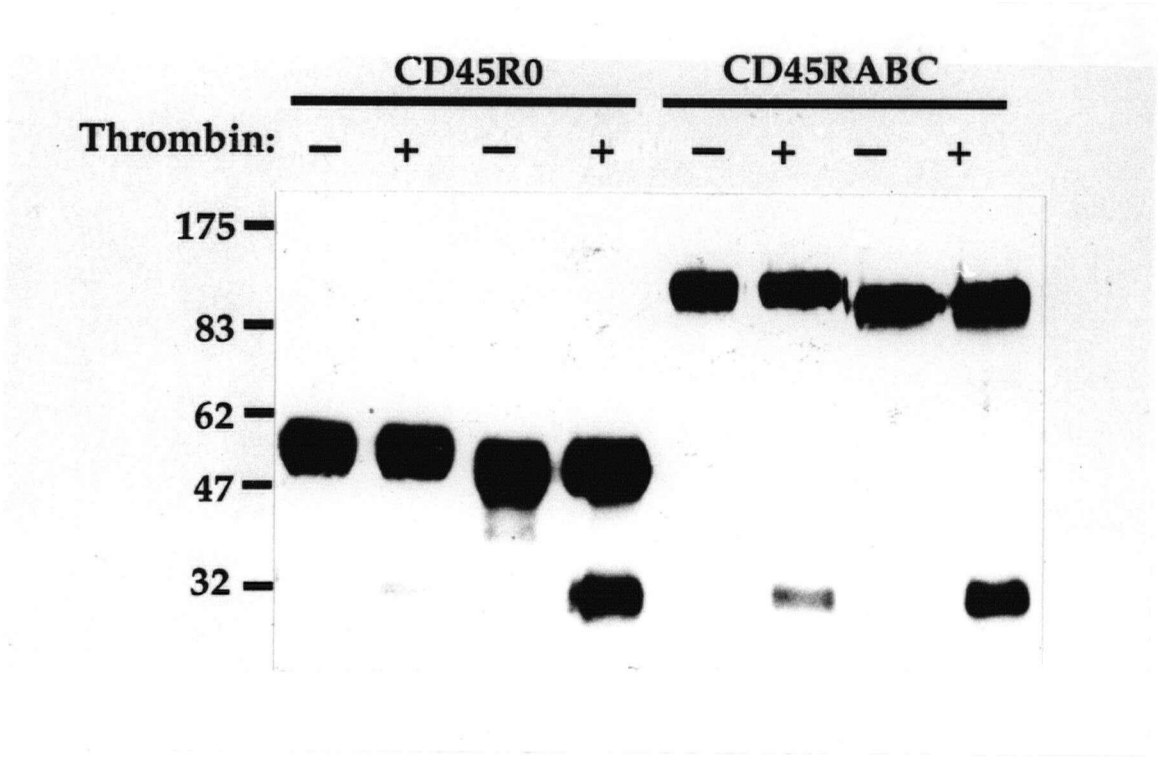


Figure 16: Thrombin Cleavage of CD45-Immunoglobulin Fusion Proteins.

100-600 µl of cell culture supernatants was precipitated with 20 µl of a 10% solution of protein A producing Pansorbin cells followed by treatment with 12.5 cleavage units of thrombin (Sigma) for two hours at room temperature. The entire sample was subjected to 7.5% SDS-PAGE and western blotting with 1/5000 GAMIgG-HRP. +, treated samples; -, untreated samples. From left to right: isoform-specific fusion proteins secreted by Cos 7 cells, isoform-specific fusion proteins secreted by X63-Ag8.653 cells.

Digestion of protein A-precipitated CD45RABC:MuIgG and CD45R0:MuIgG with thrombin resulted in the appearance of a 34 kDa band upon western blot analysis with anti-muIgG. As two bands are observed in the lanes treated with thrombin, one at the apparent molecular weight previously observed for the isoform-specific fusion protein and the second band at 34 kDa, thrombin cleavage was not 100% complete. Removal of the immunoglobulin portion may be necessary during ligand identification. For example, macrophages express Fc receptors which can non-specificially bind to the Ig portion of fusion proteins, thus allowing cellular interactions to occur that are not specific to CD45.

4.0.3 Analysis of O-linked Glycosylation

The addition of O-linked sugars to CD45:MuIgG was analyzed by digestion with O-glycosidase (Figure 17). Prior to treatment with *Diplococcus pneumoniae* O-glycosidase, terminal sialic acid residues were removed by digestion with *Vibrio cholerae* neuraminidase, thus allowing for optimal cleavage of O-linked sugars by O-glycosidase. Upon digestion with O-glycosidase, a decrease in apparent molecular weight of 10-12 kDa was observed for CD45R0:MuIgG expressed by both Cos 7 and X63-Ag8.653 cells, as visualized by precipitation with protein A and western blotting with anti-muIgG. The predicted molecular weight of unglycosylated CD45R0:MuIgG is 34 kDa. In X63-Ag8.653 cells, the observed 50 kDa fusion protein is comprised of 5 kDa N-linked carbohydrate (as determined by PNGase F digestion) and 11 kDa O-linked carbohydrate (as determined by O-glycosidase digestion) presumably attached to the predicted 34 kDa protein backbone. The predicted molecular weight of unglycosylated CD45RABC:MuIgG is 50 kDa. Upon comparing untreated and O-glycosidase treated samples, a decrease in apparent molecular weight of 30 kDa was observed for CD45RABC:MuIgG in X63-Ag8.653 cells. A smaller decrease in apparent molecular weight of 14 kDa was

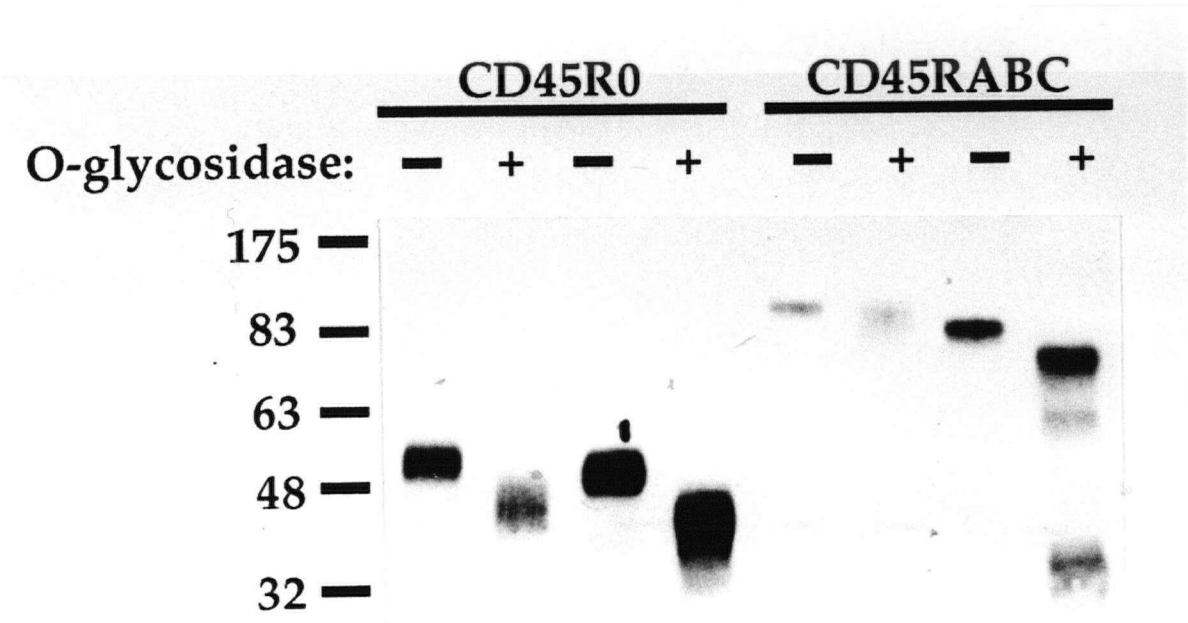


Figure 17: O-Glycosidase Treatment of CD45-Immunoglobulin Fusion Proteins.

50-200 µl of cell culture supernatant was precipitated with 20 µl of a 10% solution of Pansorbin cells. The sample was digested with 1 mUnit of neuraminidase for 4 hours at 37°C followed by digestion with 1 mUnit of O-glycosidase for 20 hours at 37°C, subjected to 7.5% SDS-PAGE and western blotting with 1/5000 GAMIgG-HRP. +, treated samples; -, untreated samples. Leftmost two lanes, isoform-specific fusion proteins secreted by Cos 7 cells; rightmost 2 lanes, isoform-specific fusion proteins secreted by X63-Ag8.653 cells.

observed for CD45RABC:MuIgG secreted by Cos 7 cells upon O-glycosidase digestion.

4.0.4 Characterization of Carbohydrate residues by Lectin Binding

The identity of specific carbohydrate residues on CD45RABC:MuIgG and CD45R0:MuIgG expressed by both Cos 7 and X63-Ag8.653 cells was determined by precipitation of culture supernatant with protein A followed by blotting membranes with various biotinylated sugar residue-specific lectins and detection with streptavidin-HRP (Figure 18). A summary of observed lectin reactivity of CD45:MuIgG is compiled in Table VII. Two isoforms of CD45 were analyzed: the highest molecular weight form containing alternatively spliced exons A, B, and C, and the lowest molecular weight isoform that does not contain the alternatively spliced exons. As exons A, B, and C contain multiple sites for addition of O-linked sugars, the two isoforms indicated are predicted to have differing lectin reactivity. In addition, lectin reactivity may be different between fusion proteins expressed by different cell lines. If ligand binding is dependent on specific carbohydrate residues on CD45, differing lectin reactivity could provide insight into the identity of an isoform-specific ligand. The strong reaction of both fusion proteins with anti-muIgG serves as a control ensuring that relatively equal amounts of fusion protein are present for subsequent lectin analysis.

Wheat Germ Agglutinin (WGA) reacted with CD45RABC:MuIgG expressed by Cos 7 cells but not with the same fusion protein expressed by X63-Ag8.653 cells. *Vicia villosa* (VVA) lectin did not react with either fusion protein expressed by either cell line. *Ricinus communis* 120 (RCA) reacted strongly with CD45RABC:MuIgG expressed by both Cos 7 and X63-Ag8.653 cells. In addition, CD45R0:MuIgG expressed by X63-Ag8.653 cells reacted moderately with RCA. Concanavilin A (Con A) reacted faintly with CD45RABC:MuIgG expressed by X63-Ag8.653 cells but did not react with other fusion proteins secreted by Cos 7 cells.

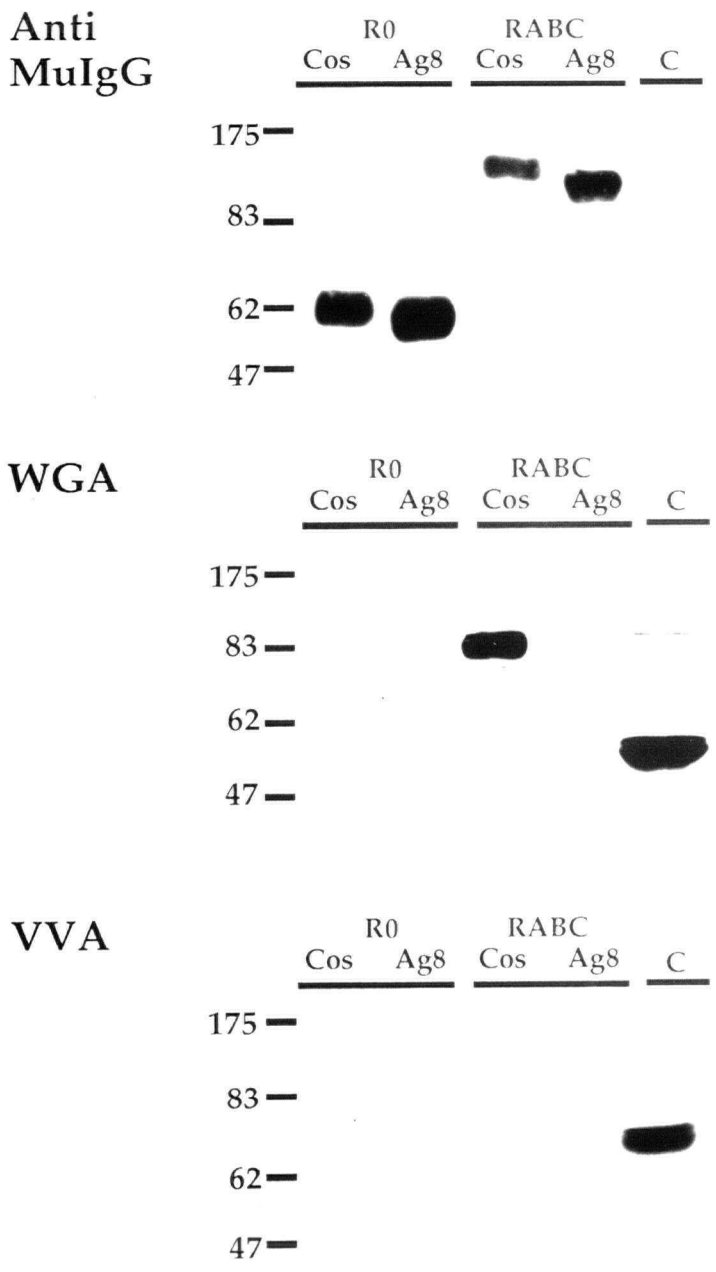


Figure 18: Reactivity of CD45-Immunoglobulin Fusion Proteins with Various Lectins.

CD45:MuIgG fusion proteins were precipitated from 100-500 µl of cell culture supernatant using 20 µl of a 10% solution of protein A producing Pansorbin cells followed by 7.5% SDS-PAGE, lectin blotting, and detection with Streptavidin-HRP. Page 1, top to bottom: Anti MuIgG, 1/5000 GAMIgG-HRP; 0.5 µg WGA; 0.5 µg VVA. Page 2, top to bottom: 0.5 µg RCA, 0.5 µg Concanavalin A, 3 µg SNA, 0.5 µg MAA. C, positive control - 3 X 10^6 T28 cells lysed in 20 µl 1% Triton/TNE.

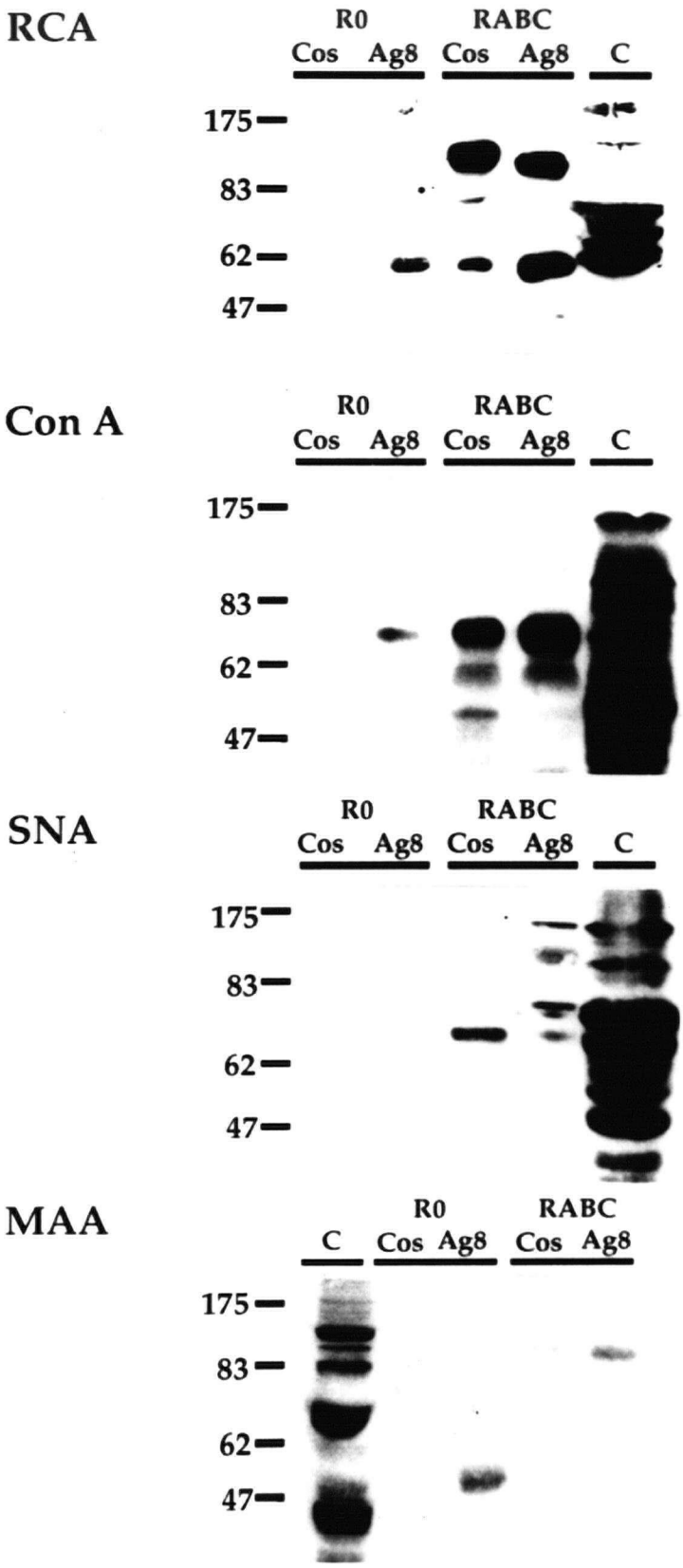

RCA
R0
RABC
Cos
Ag8
Cos
Ag8
C
175
83
62
47
Con A
R0
RABC
Cos
Ag8
Cos
Ag8
C
175
83
62
47
SNA
R0
RABC
Cos
Ag8
Cos
Ag8
C
175
83
62
47
MAA
C
R0
Cos
Ag8
RABC
Cos
Ag8
175
83
62
47

Table VII

Lectin	Specificity	Cos 7		X63-Ag8.653		C
		RO	RABC	RO	RABC	
PNA	β gal(1,3)galNAc (O linked)	-	+/-	++	++	++++
RCA	term β gal(1,4)glcNAc (N linked)	-	+++	+	++	++++
VVA	term α galNAc	-	-	-	-	++++
Con A	α man, α glc	-	-	-	+/-	++++
WGA	glcNAc α neuNAc	-	+++	-	-	++++
SNA	α(2,6) sialic acid (N linked, O linked)	-	-	-	+	++++
MAA	α(2,3) sialic acid (N linked)	-	+/-	++	+	++++

Table VII: Reactivity of CD45-Immunoglobulin Isoform Fusion Proteins with Various Lectins.

CD45:MuIgG fusion proteins were precipitated from 100-500 μl of culture supernatant using 20 μl of a 10% solution of protein A producing Pansorbin cells. The entire sample was subjected to 7.5% SDS-PAGE and transfer to Immobilon P membrane. The lectins are as follows: RCA, *Ricinus communis* 120; PNA, *Arachis hypogaea* (Peanut agglutinin); VVA, *Vicia villosa*; Con A, Concanavalin A; WGA, *Triticum vulgaris* (Wheat germ agglutinin); SNA, *Sambucus nigra*; MAA, *Maackia amurensis*.. Signal strength is as follows: C,++++, positive control; +++, very strong reaction; ++, strong reaction; +, moderate reaction; +/-, faint reaction; -, no reaction. Lectin specificity reflects carbohydrate residues commonly found on N-linked or O-linked chains as indicated.

Incubation with *Sambucus nigra* (SNA) resulted in a moderate reaction with CD45RABC:MuIgG expressed by X63-Ag8.653 cells although SNA did not react with CD45R0:MuIgG expressed by X63-Ag8.653 or either fusion protein expressed by Cos 7 cells. Finally, *Maackia amurensis* (MAA) reacted strongly with CD45R0:MuIgG secreted by X63-Ag8.653 cells, moderately with CD45RABC:MuIgG secreted by X63-Ag8.653 cells, faintly with CD45RABC:MuIgG secreted by Cos 7 cells and not at all with CD45R0:MuIgG secreted by Cos 7 cells. These results indicate that the same isoform of CD45 can be glycosylated differently in different cell types, possibly due to cell-specific expression of certain glycotransferases.

4.0.5 Determination of Peanut Lectin Reactivity in the Absence of Sialic Acid

The reactivity of *Arachis hypogaea* Peanut Lectin (PNA) with CD45:MuIgG in the presence and absence of sialic acid was determined (Figure 19). The presence of sialic acid on the end of a carbohydrate chain prevents PNA from binding as this lectin binds to galactose residues beneath sialic acid on O-linked chains. Removal of sialic acid will therefore increase PNA reactivity, thus providing a method to quantitate the relative amount of sialic acid present. Panel A of Figure 19 shows reactivity of CD45R0:MuIgG and CD45RABC:MuIgG secreted by both Cos 7 and X63-Ag8.653 cells with anti-muIgG. This experiment demonstrated that equal amounts of fusion protein were present for subsequent experiments. The reactivity of CD45:MuIgG with PNA lectin is shown in Panel B of Figure 19. This experiment serves to show the initial level of PNA reactivity of fusion proteins before the removal of sialic acid by neuraminidase (*Vibrio cholerae*) digestion. Incubation of CD45R0:MuIgG expressed by either cell line with neuraminidase did not affect PNA reactivity (Figure 19, Panel C). In contrast, digestion of CD45RABC:MuIgG secreted by Cos 7 cells with neuraminidase significantly increased PNA reactivity, while similar incubation of CD45RABC:MuIgG expressed by X63-Ag8.653 cells increased

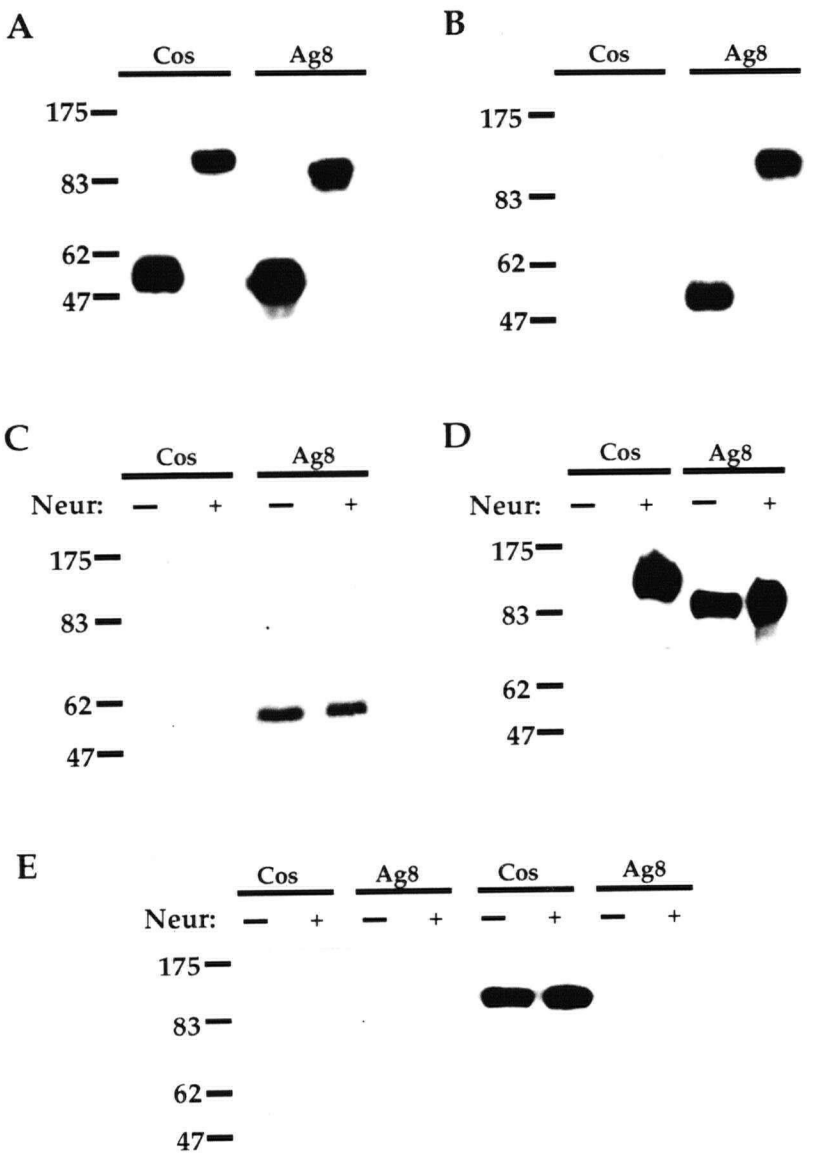


Figure 19: Peanut Agglutinin and RA3 6B2 Reactivity of Neuraminidase Treated CD45-Immunoglobulin Fusion Proteins.

CD45:MuIgG fusion proteins were precipitated from 100-500 µl of cell culture supernatant using 20 µl of a 10% solution of protein A producing Pansorbin cells followed by treatment with 1 mUnit of Neuraminidase for one hour at 37˚C and electrophoresis on 7.5% SDS-PAGE. A, CD45R0:MuIgG and CD45RABC:MuIgG expressed by Cos 7 and X63-Ag8.653 cells blotted with 1/5000 GAMIgG-HRP; B, 0.5 µg *Arachis hypogaea* (Peanut agglutinin); C, PNA reactivity of neuraminidase treated CD45R0:MuIgG; D, PNA reactivity of neuraminidase treated CD45RABC:MuIgG; E, RA3 6B2 reactivity of neuraminidase treated CD45R0:MuIgG and CD45RABC:MuIgG.

PNA reactivity, but to a much lesser extent (Figure 19, Panel D). Neuraminidase treatment of CD45:MuIgG did not affect subsequent RA3 6B2 reactivity of fusion proteins secreted by either cell line (Figure 19, Panel E). As sialic acid residues have been shown to be instrumental in the binding of CD22 to endogenous CD45, it is possible that sialic acid residues on CD45:Ig fusion proteins may function in a similar manner.

4.0.6 Apparent Molecular Weight in Non-reducing SDS-PAGE Conditions

The apparent molecular weight of CD45:MuIgG was determined in both reducing and non-reducing SDS-PAGE conditions (Figure 20). In the presence of the reducing agent, DTT, protein A-precipitated fusion proteins migrated to the apparent molecular weights previously described (Figure 8, Figure 11, Table III). However, in the absence of DTT, fusion proteins expressed by both Cos 7 and X63-Ag8.653 cells were observed to have significantly higher apparent molecular weights of 120 kDa for CD45R0:MuIgG and approximately 240 kDa for CD45RABC:MuIgG, thus indicating dimer formation. In addition, a band at approximately 190 kDa was observed for CD45R0:MuIgG secreted by Cos 7 cells, indicated the possible formation of a multimer.

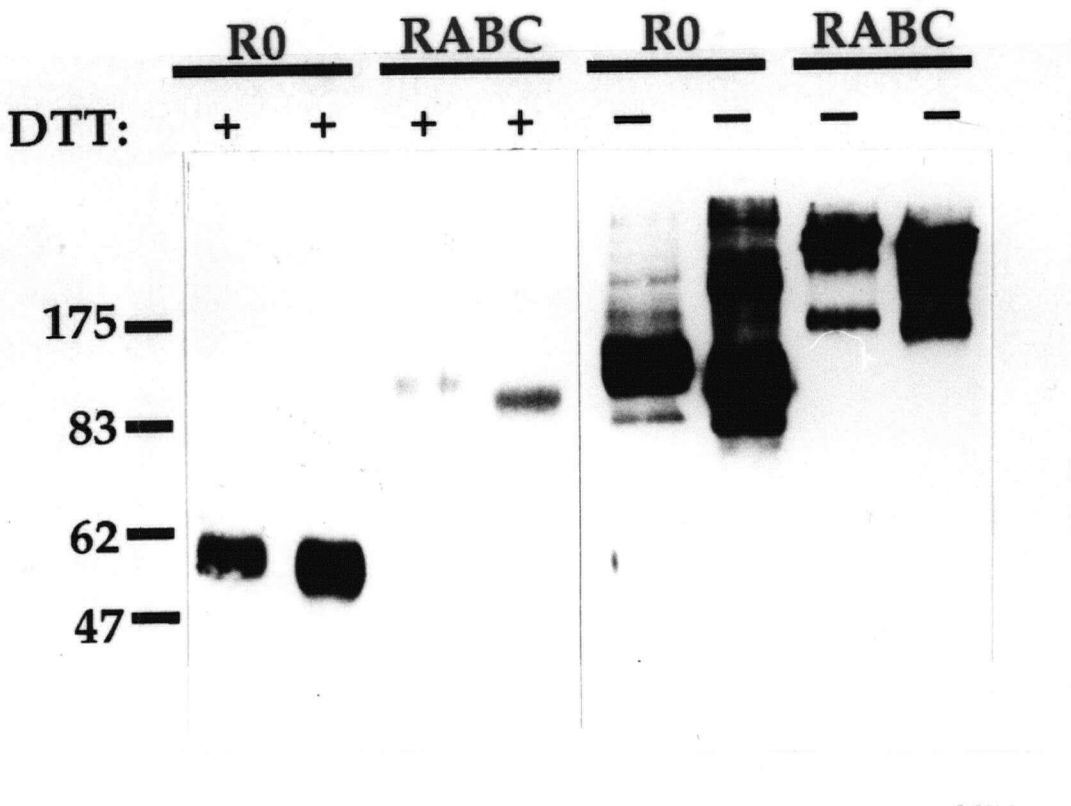


Figure 20: SDS-PAGE Analysis of CD45-Immunoglobulin Fusion Proteins in Reducing and Non-reducing Conditions.

100-500 µl of cell culture supernatant was precipitated with 20 µl of a 10% solution of protein A producing Pansorbin cells. Samples were boiled in 1 X SDS-PAGE sample buffer in the presence or absence of 0.1 M Dithiothreitol (DTT) followed by electrophoresis on 7.5% SDS-PAGE and western blotting with 1/5000 GAMIgG-HRP. Leftmost two lanes, isoform-specific fusion proteins secreted by Cos 7 cells; rightmost 2 lanes, isoform-specific fusion proteins secreted by X63-Ag8.653 cells.

DISCUSSION

Creation of murine CD45-Immunoglobulin Fusion Constructs

At the start of this work, previous attempts at expression of human CD45-immunoglobulin constructs with the native CD45 signal sequence were unsuccessful. To try to improve expression levels, two things were changed: the signal sequence and the expression vector. The Onco M signal sequence has been used successfully by other groups to mediate the secretion of human IgG fusion proteins of the B lymphocyte activation antigen B7, its counter receptor CD28, on T lymphocytes, CD5 [39] as well as CTLA-4 [54]. The inclusion of the Onco M signal sequence in place of the native CD45 signal sequence increased the expression of human CD45-immunoglobulin fusion proteins by over 10 fold. As a result, the Onco M signal sequence was included in fusion constructs consisting of different isoforms of murine CD45 linked to the hinge, CH2 and CH3 regions of the murine IgG_{2a} heavy chain.

Polymerase Chain Reaction is an extremely useful method for the exponential amplification of small fragments of DNA. By using oligonucleotide primers complementary to the ends of the desired sequence, a specific segment of DNA can be targeted and amplified for further analysis. In this body of work, the creation of murine CD45 isoform-specific inserts by PCR for subcloning into the modified Ig vector, 1 ng of plasmid DNA template was amplified to approximately 2 μg of final product - an efficient increase of over 2000 fold. The presence of non-specific bands upon agarose gel electrophoresis of PCR products was due to binding of oligonucleotide primers to non-complementary sequences, resulting in the extension and subsequent amplification of an alternate portion of the DNA strand. Annealing of oligonucleotide primers is optimal at the melting temperature (T_m) of the primer. T_m can be calculated for oligonucleotide primers shorter than 18

nucleotides by allowing 2°C for each adenine or thymine and 4°C for each cytosine or guanine. For longer primers, an equation based on guanine/cytosine content and ionic strength can be used to get an more accurate value [55]. As primer annealing does not occur at temperatures above the calculated T_m, hybridization reactions should be carried out under stringent conditions - typically 5-10°C below the calculated T_m. To eliminate non-specific bands, the annealing temperature should be increased to the highest temperature possible without going above the calculated T_m.

The occurrence of smearing and apparent degradation of some isoform-specific PCR products was due to two factors. Firstly, the 30 second extension time at 72°C using Vent DNA polymerase (New England Biolabs) may have been too long. To minimize smearing, manufacturers instructions suggest using an extension time correlating to the expected length of the final product: 1 minute for every 1000 base pairs of DNA. For CD45RA and CD45RB, the predicted size of the final products was 280 bp and 300 bp respectively. Therefore, the optimal extension time for CD45RA was 17 seconds and for CD45RB, 18 seconds. Alternatively, the smear extending down from the agarose gel well commonly indicates that a particular reagent or condition is in excess. Therefore, using less enzyme, primer, or template may have reduced smearing.

Ligation of muCD45 isoform-specific inserts into the modified Ig vector containing the Onco M signal sequence required the correct restriction enzyme-generated overlapping ends. Digestion of PCR inserts with Sph 1 was inefficient for two reasons. Firstly, the half life of activity for Sph 1 is approximately one hour. Secondly, Sph 1 digests at the end of DNA strands with a very low efficiency: 25% in 20 hours when there are 8 nucleotides (nt) 5' to the cleavage site, 10% in 2 hours or 50% in 20 hours when there are 9 nt 5' to the cleavage site. Taken together, these statistics indicated that Sph 1 was not an efficient enzyme for creation of a restriction enzyme site at the end of a PCR fragment. Blunt end ligation, end to end, of PCR

products allowed for creation of multimers that could then be more efficiently digested with Sph 1 to create the required subcloning site.

Expression of murine CD45-Immunoglobulin Fusion Proteins

Cos 7 cells, a non-hematopoietic monkey kidney cell line, were used as an initial expression system to determine if murine CD45 isoform-specific fusion proteins could be secreted into the culture supernatant at levels high enough to be 1) detected by precipitation with protein A and western blot analysis and 2) purified in larger amounts by protein A affinity chromatography. If muCD45:MuIgG fusion proteins were expressed by Cos 7 cells, it would be reasonable to hypothesize that expression in more functionally relevant myeloid and lymphoid cell lines would also be successful.

Transient expression of five isoforms of CD45 linked to murine heavy chain constant regions was obtained in Cos 7 cells by the DEAE-dextran method. In addition, stable expression in Cos 7 cells of three isoform-specific constructs was obtained by calcium phosphate transfection. Both DEAE-dextran and calcium phosphate transfection are highly efficient and widely used methods for the introduction of plasmid DNA into eucaryotic cells [55]. Although the exact mechanism remains obscure, the DNA is thought to enter the cell by endocytosis followed by transport to the nucleus. While DEAE-dextran transfection is used only for transient expression, the calcium phosphate method can be used for both transient expression and stable integration of DNA into the eucaryotic genome.

Although repeated attempts were made, transient and stable expression of one isoform of muCD45, CD45RC, was not observed. While all other isoform constructs in pBluescript were 100% confirmed by DNA sequencing prior to subcloning into the mammalian expression vector, pBCMGSneo, 8/300 nt of CD45RC could not be confirmed due to compression of guanine/cytosine rich

sequences resulting in unreadable results with respect to the nitrogenous base present at that position of the DNA sequence. Hence, the DNA sequence of CD45RC was only 97% confirmed. It is plausible that one or more of the unconfirmed nucleotides was incorrect, resulting in the substitution of a different amino acid residue that then acted to prevent subsequent expression of the fusion protein. In addition, it is possible that the unconfirmed sequence contained the incorrect number of nucleotides, resulting in the downstream protein being out of frame and subsequently being translated into an entirely different protein that could not be detected by the methods employed.

The apparent molecular weight of CD45:MuIgG fusion proteins secreted by Cos 7 cells as determined by precipitation with protein A and western blotting with anti-muIgG was significantly higher than the predicted molecular weight of unglycosylated protein (Table III). In Cos 7 cells, the difference between predicted and apparent molecular weight is 21 kDa for CD45R0:MuIgG and 75 kDa for CD45RABC:MuIgG. As each alternatively spliced exon consists of approximately 50 amino acids, the addition of all three exons would increase the apparent molecular weight by 18 kDa. Thus, the remaining 57 kDa observed for CD45RABC:MuIgG can be explained by the fact that exons A, B, and C contain multiple sites for O-linked carbohydrate addition, which upon addition, would result in a further increase in apparent molecular weight.

The appearance of lower molecular weight bands in lysates of Cos 7 cells can be readily explained. The observed bands react with the anti-muIgG antibody, suggesting they contain the heavy chain constant regions of murine IgG. Hence, the lower molecular weight proteins may be CD45:MuIgG fusion proteins that are not yet fully processed by addition of carbohydrate in the endoplasmic reticulum and golgi apparatus. The lack of complete glycosylation accounted for the lower apparent molecular weight and inability to be secreted into the culture supernatant. Although the lower molecular weight proteins did not react with anti-CD45 exon A-

specific 14.8 and exon B-specific MB 4B4 and 23 G2, purified exon C-specific DNL1.9 did react with proteins in the lysates, implying that exon A and B-specific antibodies may recognize a combination of protein sequence and carbohydrate residues whereas DNL1.9 may recognize protein sequence only. Related evidence from isoforms of human CD45 expressed in *Escherichia coli* as unglycosylated glutathione-S-transferase (GST) fusion proteins also suggested that antigenic determinants encoded by alternatively spliced exons of CD45 are determined by the polypeptide sequence but influenced by glycosylation[56]. For example, in this study, the monoclonal antibody MRC OX22, which recognizes the product of exon C in the rat, was shown to have significantly higher binding affinity to glycosylated CD45 from spleen than to unglycosylated CD45-GST fusion proteins produced in *E. coli.*

Expression of CD45:MuIgG by Cos 7 cells incubated in DMEM/10% FCS resulted in the appearance of three anti-IgG antibody unreactive protein bands at 55 kDa, 65 kDa and > 175 kDa upon precipitation with protein A and coomassie brilliant blue staining. The contaminating proteins were components of fetal calf serum, as incubation of CD45R0:MuIgG and CD45RABC:MuIgG in serum free hybridoma media resulted in fusion proteins free of contaminating bands at the previously observed molecular weights. Scanning densitometry revealed that approximately 1.5-2.0 μg of CD45RABC:MuIgG and 3.0-4.0 μg CD45R0:MuIgG was secreted per ml of serum free media (1×10^6 cells in 10 ml media) over a three day period. The fusion protein containing the smallest molecular weight form of CD45, CD45R0, was secreted at a higher concentration possibly due to the decreased requirement for addition of carbohydrate residues during processing. As CD45R0:MuIgG lacks alternatively spliced exons A, B, and C, CD45R0:MuIgG fusion proteins contain only 18 potential sites for O-linked sugar addition and 1 site (on Ig portion) for N-linked sugar addition. Therefore, the time required for processing and secretion of CD45R0:MuIgG would be less than that required for CD45RABC:MuIgG, which contains 57 potential O-linked glycosylation sites and 3

potential N-linked glycosylation sites. The yield of 1.5-4.0 μg/ml is comparable to that observed by other groups performing similar experiments: B7Ig and CTLA-4Ig fusion proteins (both containing the Onco M signal sequence) were secreted by 1 X 10^6 Cos 7 cells at 1.5-4.5 μg/ml over a three day period [39, 54] whereas CD44Ig accumulated in Cos 7 cell supernatants at 0.5 μg/ml 7 days post-transfection [53].

Once it had been determined that CD45:MuIgG could be expressed at reasonable levels by Cos 7 cells, the focus of subsequent experiments was to express fusion proteins in the myeloma cell line, X63-Ag8.653, and the T lymphoma cell line, T28. As observed with Cos 7 cells, fusion proteins secreted by X63-Ag8.653 and T28 cells had apparent molecular weights significantly higher than the predicted protein size, suggesting extensive post-translational modifications via carbohydrate addition. The apparent molecular weight of CD45RABC:MuIgG, CD45RB:MuIgG, and CD45R0:MuIgG was generally 5-10 kDa lower than the equivalent fusion protein expressed in Cos 7 cells (Table III). One explanation for this result could be increased addition of carbohydrate residues by Cos 7 cells when compared to the sugars added by X63-Ag8.653 and T28 cells, resulting in the formation of a different glycosylation pattern. In addition, this result suggests that expression of the same protein by different cell lines can result in proteins with differing carbohydrate patterns which then may, in turn, affect subsequent ligand interactions. Therefore, the importance of expressing CD45:MuIgG fusion proteins in more than one cell line becomes apparent in order to identify all potential ligand interactions due to the presence or absence of a particular carbohydrate residue(s). The yield of CD45:MuIgG obtained in X63-Ag8.653 cells was not significantly different from that observed in Cos 7 cells and, as previously described, is in the range of concentration observed by other groups performing similar experiments.

Transient expression of CD45:MuIgG was observed in T28 cells. The apparent molecular weights of fusion proteins were comparable to that obtained by expression in X63-Ag8.653 cells (Table III). Unfortunately, the level of fusion protein

expression by T28 cells decreased steadily over a period of three days, eventually leading to undetectable levels by precipitation with protein A and subsequent western blotting with anti-muIgG. The decrease in fusion protein expression may have occurred because fusion protein-secreting clones were contaminated with non-fusion protein-secreting cells. As untransfected T28 cells grow faster than transfected cells, the non-expressing cells eventually became the dominant cell type in the population. Possible ways to prevent this problem in future experiments may be to further increase the already high concentration of active G418 used for selection, thus allowing for complete destruction of cells that do not carry the gene for neomycin resistance. Alternatively, increasing the voltage and/or capacitance used for electroportation may result in a cell population in which neomycin sensitive cells are more stressed, thus more easily killed. As T28 cells appear to have a higher growth rate and faster recovery from electric shock than other T cell lines, electroportation of half the number of cells per sample (5×10^6 cells rather than 1×10^7) followed by selection of neomycin resistance by addition of G418 at 24 hours rather than 48 hours, may increase the percentage of fusion protein-secreting cells in the population and prevent neomycin sensitive cells from further propagating.

Characterization of Expressed CD45-Immunoglobulin Fusion Proteins

Although an isoform-specific ligand for CD45 has not yet been identified, one theory postulates that specific carbohydrate residues on CD45 mediate the interaction(s) with a putative ligand(s). Therefore, if carbohydrate residues are important, it is critical that CD45:MuIgG fusion proteins be correctly glycosylated so that they express the necessary sugars required for ligand binding. As endogenous CD45 is expressed on the surface of all nucleated hematopoietic cells, the glycosylation pattern of fusion proteins expressed by lymphoid cells would probably

be closer to that of endogenous CD45 than that of fusion proteins expressed by non-hematopoietic Cos 7 cells.

The reactivity of secreted CD45:MuIgG fusion proteins with various murine CD45-specific antibodies was analyzed. All fusion proteins, expressed transiently or as stable clones by all three cell lines, reacted with an antibody recognizing the murine IgG heavy chain, suggesting that all fusion proteins contain the predicted heavy chain regions. At the transient expression level in Cos 7 cells, all isoform-specific fusion proteins reacted with anti-CD45 exon-specific antibodies as predicted with the exception of RA3 6B2, a B220 isoform-specific antibody observed to react not only with CD45RABC:MuIgG, but also with CD45RA:MuIgG (Figure 7, Panel B). This suggests that although RA3 6B2 can bind to the largest isoform of CD45 containing all three alternatively spliced exons, its reactivity is dependent on the presence of exon A. This is the first demonstration that RA3 6B2 reactivity is dependent on the expression of exon A. In addition, as RA3 6B2 did not react with lower molecular weight proteins in lysates of Cos 7 cells, the formation of the RA3 6B2 epitope may possibly depend on carbohydrate modifications of exon A. Interestingly, RA3 6B2 reacted with stable clones in Cos 7 cells secreting CD45RABC:MuIgG but did not react with stable CD45RABC:MuIgG clones in X63-Ag8.653 cells or transient clones in T28 cells. The difference in the binding of the B220-isoform-specific antibody may be due to differences in carbohydrate addition between Cos 7 versus X63-Ag8.653 and T28 cells. It is plausible that differential carbohydrate modification of fusion proteins expressed by different cell lines may contribute to the presence or absence of different epitopes for antibody binding. In addition, the fact that fusion proteins expressed by Cos 7 cells had a slightly higher apparent molecular weight than those secreted by X63-Ag8.653 or T28 cells, may correlate with additional glycosylation and the appearance of RA3 6B2 reactivity.

Analysis of apparent molecular weight suggests that CD45:MuIgG fusion proteins were extensively glycosylated. Hence, it was important to characterize the

carbohydrate residues expressed by the fusion proteins secreted by different cell lines. Fusion proteins containing CD45RABC are more highly modified, presumably by addition of carbohydrate, than those containing CD45R0. Removal of asparagine (N) -linked glycan chains by PNGase F digestion followed by SDS-PAGE and western blot analysis with anti-muIgG (Figure 15) revealed a small reduction in apparent molecular weight of approximately 5 kDa. Interestingly, approximately equal decreases in apparent molecular weight upon PNGase F digestion for both CD45R0 and CD45RABC Ig fusion proteins suggests that the N-linked sugars present must be located on a common portion of the fusion proteins. This common region could be one of two parts of the fusion protein: exons 3, 7, and 8 of CD45, or the heavy chain regions of muIgG_{2a}. As exons 3, 7, and 8 of CD45 lack any potential N-linked glycosylation sites conforming to the required sequence motif - Asn-X-Ser/Thr where X is any amino acid except proline [57] - N-linked glycosylation will not occur in this region. Therefore, N-linked sugars present are likely located on the murine IgG portion of the fusion protein. Indeed, there is a conserved site between all isotypes of IgG molecules (Asn 297) for potential N-linked sugar addition conforming to the required motif [58]. The murine IgG portion was shown to be 34 kDa by thrombin cleavage (unglycosylated protein predicted to be 28 kDa). The size of the IgG portion did not vary between isoforms of CD45 suggesting that the observed differences in apparent molecular weight between fusion proteins containing different isoforms of CD45 were due solely to differential post-translational modifications of CD45. In addition, the size of the IgG portion was the same in fusion proteins expressed by Cos 7 cells and X63-Ag8.653 cells, suggesting that the IgG region was similarly processed by these two cell lines prior to secretion. As it has been determined that there is 5 kDa of N-linked glycosylation (out of a total of 16-21 kDa for CD45R0 and 60-75 kDa for CD45RABC in X63-Ag8.653 cells and Cos 7 cells respectively), the majority of apparent carbohydrate must be O-linked to serine or threonine. Digestion of CD45R0:MuIgG expressed in both Cos 7 cells and

X63-Ag8.653 cells with O-glycosidase revealed a decrease in apparent molecular weight of approximately 10-12 kDa. The predicted molecular weight of CD45R0:MuIgG is 34 kDa, so the addition of 5 kDa of N-linked carbohydrate and 11 kDa of O-linked carbohydrate by X63-Ag8.653 cells results in the apparent molecular weight observed - 50 kDa- by SDS-PAGE analysis and western blotting. On the other hand, digestion of CD45RABC:MuIgG expressed by both Cos 7 and X63-Ag8.653 cells did not appear to be complete. A decrease in apparent molecular weight of 30 kDa for CD45RABC:MuIgG in X63-Ag8.653 cells and 14 kDa for CD45RABC:MuIgG in Cos 7 cells was observed upon O-glycosidase digestion. These numbers do not account for the total amount of O-linked glycosylation expected. However, it is possible that O-glycosidase digestion of CD45RABC:MuIgG was not complete due to the presence of terminal sialic acid resides, which act to prevent cleavage by O-glycosidase.

It has been determined that fusion proteins expressed by different cell lines have slightly differing apparent molecular weights and antibody reactivity. In addition, it has been suggested that fusion proteins expressed by different cell lines may have differential carbohydrate modifications. Therefore, in order to determine the identity of specific carbohydrate residues, each fusion protein was tested for reactivity with various sugar residue-specific lectins (Figure 18). The results are summarized in Table VII. In general, it was determined that fusion proteins expressed by different cell lines but expressing the same isoform of CD45 had different lectin binding properties. In some cases, lectin reactivity was different between CD45RABC:MuIgG and CD45R0:MuIgG expressed by the same cell line.

Lack of binding to *Vicia villosa* (VVA) lectin and Concanavalin A (Con A) revealed the absence of α-linked N-acetygalactosamine, mannose, and glucose on all fusion proteins. The lack of mannose can be explained by the fact that high mannose structures are commonly associated with N-linked sugar chains, which were shown by PNGase F digestion to contribute to just 6% of total post-translational modifications of CD45RABC:MuIgG. In addition, two glucose residues

are often removed from precursor N-linked chains to allow for association with calnexin, a protein found in the endoplasmic reticulum (ER) that functions to prevent incompletely folded or misfolded glycoproteins from further processing [59]. Calnexin binds to monoglucosylated, incorrectly folded glycoproteins in the ER, thus preventing further transport to the cell surface. Removal of the final glucose residue from correctly folded glycoproteins is required for release of calnexin and subsequent downstream processing.

Ricinus communis 120 (RCA) is a group II galactose-specific phytohemagglutinin that commonly binds to carbohydrate chains containing terminal N-linked galactose linked β-1,4 to N-acetyglucosamine [60]. Peanut agglutinin (PNA) is a group I lectin that commonly binds carbohydrate chains containing O-linked galactose linked β-1,3 to N-acetylgalactosamine. RCA reactivity of CD45:MuIgG fusion proteins expressed by Cos 7 cells was higher than PNA reactivity, suggesting that the majority of N-linked chains end in galactose whereas the majority of O-linked chains have an additional sugar present that acts to mask galactose-specific PNA reactivity. O-linked carbohydrate chains commonly have N-acetylneuraminic acid or sialic acid as the ultimate residue. Lectins that have been previously shown to recognize α-2,6 and α-2,3 N-linked sialic acid, SNA and MAA, [50, 51] did not react significantly with fusion protein expressed by Cos 7 cells, confirming previous conclusions from RCA and PNA data that suggest that the majority of N-linked chains on CD45:MuIgG fusion proteins expressed by Cos 7 cells end in a galactose residue. Unfortunately, a lectin that binds with high affinity to O-linked sialic acid could not be found in order to further confirm the outlined conclusions.

Sialic acids often act as to mask antigenic sites. For example, the surfaces of trophoblast cells are rich in sialic acids which are thought to serve as an immunobarrier between mother and embryo [14]. Partial loss of this barrier has been proposed as one of the causes of autoimmune disease. In a similar manner,

the presence of terminal sialic acid residues on CD45:MuIgG fusion proteins acts to mask reactivity of galactose-specific peanut agglutinin (PNA). Therefore, removal of sialic acid by digestion with neuraminidase (sialidase) will expose galactose residues that will then react with PNA. Digestion of CD45R0:MuIgG expressed in Cos 7 or X63-Ag8.653 cells with neuraminidase did not result in an increase in subsequent PNA reactivity, suggesting that very little sialic acid was added to CD45R0 during processing and secretion (Figure 19). On the other hand, digestion of CD45RABC:MuIgG expressed by Cos 7 cells with neuraminidase resulted in a significant increase in subsequent PNA reactivity while digestion of CD45RABC:MuIgG expressed by X63-Ag8.653 cells also resulted in an increase in PNA reactivity, but to a lesser extent than that observed with Cos 7 expressed fusion proteins. This data suggests that although sialic acid appears to be added to CD45RABC:MuIgG, a much larger amount is added by Cos 7 cells than X63-Ag8.653 cells. This data correlates with the previous observation that fusion proteins expressed by Cos 7 cells have a slightly higher apparent molecular weight. The increased addition of sialic acid can account for the observed increase in apparent molecular weight of fusion proteins expressed by Cos 7 cells.

The next logical question to consider was whether or not the increased addition of sialic acid to fusion proteins expressed by Cos 7 cells accounts for the observed reactivity of B220 isoform-specific antibody RA3 6B2. Digestion of CD45RABC:MuIgG with *Vibrio cholerae* neuraminidase did not affect RA3 6B2 reactivity of fusion proteins expressed by either Cos 7 or X63-Ag8.653 cells (Figure 19). Hence, the binding of this antibody does not appear to depend on the presence of sialic acid. *Vibrio cholerae* neuraminidase is known to cleave α-2,6, α-2,3, and α-2,8 sialic acid residues so this enzyme should remove most residues from CD45. However, it is possible that some sites may be more accessible than others and thus it would be better in future experiments to metabolically label sialic acids and show that they are all removed by this treatment. However, it is clear from lectin analysis

that fusion proteins expressed by different cell lines have differential carbohydrate addition. It is likely that RA3 6B2 reactivity depends on the formation of an epitope containing sugars other than sialic acid. In agreement with RA3 6B2 reactivity, CD45RABC:MuIgG expressed by Cos 7 cells reacted with wheat germ agglutinin (WGA) whereas the equivalent fusion protein expressed by X63-Ag8.653 cells did not bind to WGA. Although the binding properties of WGA are complex, it is generally thought to bind to N-acetylglucosamine, poly-N-acetyl-lactosamine, and sialic acid residues[61]. Interestingly, carbohydrate structures on endogenous CD45 are thought to be rich in poly-N-acetyl-lactosamine [62] and N-acetylglucosamine. Whether the reactivity of RA3 6B2 correlates with the presence of N-acetylglucosamine and/or poly-N-acetyl-lactosamine on fusion proteins remains to be fully elucidated.

Fusion proteins expressed by both Cos 7 and X63-Ag8.653 cells were observed to form dimers in non-reducing SDS-PAGE conditions. In the absence of dithiothreitol (DTT), a reducing agent known to break disulfide bonds between cysteine residues, CD45:MuIgG fusion proteins migrated to an apparent molecular weight of approximately two fold of that observed in the presence of DTT - 50-55 kDa in reducing conditions versus 120 kDa in non-reducing conditions for CD45R0:MuIgG whereas CD45RABC:MuIgG migrated to 110-125 kDa in reducing conditions versus approximately 240 kDa in non-reducing conditions. In X63-Ag8.653 cells, CD45R0:MuIgG was also expressed in a form with an apparent molecular weight of approximately 190 kDa, which is consistent with the formation of a multimer.

The expression and characterization of isoform-specific CD45:MuIgG fusion proteins revealed the following observations. Firstly, the levels of CD45:MuIgG fusion protein expression was in the same range between Cos 7 and X63-Ag8.653 cells. The fusion protein containing the smallest isoform of CD45, CD45R0, was expressed approximately 2 fold more than the fusion protein containing the largest isoform of CD45, CD45RABC, which contains alternatively spliced exons A, B, and

C. The apparent molecular weight of CD45:MuIgG expressed by Cos 7 cells was 5-10 kDa higher than the equivalent fusion protein secreted by X63-Ag8.653 or T28 cells. All isoform-specific fusion proteins reacted with anti-CD45 exon-specific antibodies as predicted with the exception of RA3 6B2, which was observed to react with both transiently expressed CD45RABC:MuIgG and CD45RA:MuIgG. In addition, RA3 6B2 reacted with stable fusion proteins secreted by Cos 7 cells whereas this antibody did not react with fusion proteins secreted by X63-Ag8.653 or T28 cells. Isoform-specific fusion proteins are extensively O-glycosylated on serine and threonine residues. PNGase F digestion revealed that although minimal N-linked glycosylation was present, N-linked glycans that were present were most likely located on the murine IgG portion of the fusion protein, determined to be 34 kDa by thrombin cleavage. Lectin analysis of fusion proteins expressed by Cos 7 cells revealed that the majority of N-linked carbohydrate chains terminated in a galactose residue, whereas the majority of O-linked chains had been further modified by the addition of sialic acids. Removal of sialic acid by digestion with neuraminidase resulted in a significant increase in subsequent PNA reactivity of CD45RABC:MuIgG from Cos 7 cells whereas only a slight increase in PNA reactivity was observed for the equivalent fusion protein expressed by X63-Ag8.653 cells, suggesting that a larger amount of sialic acid was added to fusion proteins expressed by Cos 7 cells. The increased addition of sialic acid may account for the slightly higher apparent molecular weight but does not account for the differences observed in B220-specific RA3 6B2 reactivity.

The work presented in this thesis has established the feasibility of generating isoform-specific CD45-immunoglobulin fusion proteins that can now be used in the search for an isoform-specific ligand for CD45. A CD45-immunoglobulin fusion construct has been developed which will allow for secretion of sufficient amounts of fusion protein for purification and subsequent use in ligand identification. It has been established that CD45-immunoglobulin fusion proteins can be easily purified in one step by precipitation of fusion proteins with protein-A-sepharose from cells

grown in serum free hybridoma media. In addition, it has been determined that the same fusion protein can be differentially glycosylated by expression in different cell lines. Moreover, the presence of carbohydrate residues on the protein backbone was shown to affect antibody reactivity, and therefore, could affect subsequent ligand interactions. Given the observations presented, it is now possible to produce large amounts of purified fusion protein from cells and use this fusion protein as a diagnostic tool in immunoadherence assays to identify isoform-specific ligand interactions of CD45. In particular, it would be interesting to determine if CD45-immunoglobulin fusion proteins expressed by different cell lines bind to the B cell specific molecule, CD22. If specific binding is observed, it then must be determined whether or not the observed interaction is dependent on the expression of certain carbohydrate residues on CD45, specifically α-2,6 linked sialic acid residues. In addition, it would be useful to determine if CD45-immunoglobulin fusion proteins bind to thymocytes expressing galectin-1, and if so, to determine if that binding was inhibited by anti-CD45 exon-specific antibodies. Further characterization experiments need to be done in order to determine if the carbohydrate residues on CD45:Ig fusion proteins compares to the carbohydrate on CD45 expressed on the surface of lymphocytes. The results of future experiments using isoform-specific CD45-immunoglobulin fusion proteins could aid in the further understanding of the isoform-specific ligand interactions of murine CD45.

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