THE DEVELOPMENTAL EXPRESSION OF CD45 GLYCOPROTEIN IN MURINE T LYMPHOCYTES

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ABSTRACT

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The CD45 family of lymphoid and myeloid cell surface glycoproteins exhibits molecular weight heterogeneity arising in part from alternate exon use among exons 4, 5, and 6. I developed a new method combining reverse transcription and polymerase chain reaction (RT-PCR) using only 3,000-30,000 cells to study the exact alternate exon use in the murine lymphoid cells. Based on the results from size measurement, Southern hybridization, and direct sequencing of the gel-purified fragment, I concluded that exon 7 and exon 8 could also be alternately used. My data indicated that, under the experimental conditions, smaller isoforms of CD45 cDNA were amplified more efficiently than larger ones at large number of PCR cycles. However, each individual cDNA isoform, regardless of the length, was amplified at approximately the same rate at least before the end of the 25th cycle. Therefore, RT-PCR has been limited to 24 cycles in examining the CD45 isoform expression in murine T cells. My results showed that Stage I thymocytes ($CD4^-CD8^-$) expressed only trace amounts of minus-one [Ex(-1)] and minus-two exon [Ex(-2)] isoforms, with no other isoforms detected. A Ex(-2) isoform was also detected in all thymocytes and T cells analyzed but only in trace amounts. Stage II thymocytes ($CD4^+CD8^+$) expressed high and approximately equal amounts of Ex(-1) and zero alternate exon [Ex(0)] isoforms, with minor quantities of one exon [Ex(1)] and two exon [Ex(2)] isoforms. Among Stage III thymocytes, both $CD4^+CD8^-$ and $CD4^-CD8^+$ cells expressed significant quantities of only the

Ex(-1) and Ex(0) isoforms. Comparison of CD45 alternate exon use in resting $CD4^+$ and $CD8^+$ lymph node T cells revealed evidence of divergent exon use. Examination of allogeneically activated T cells revealed that the $CD4^+$ BC-3 helper T cell clone expressed less of the Ex(1) isoform, while the $CD8^+$ 8.2.2 CTL clone increased its production of higher alternate exon isoforms, including Ex(2) and Ex(3) isoforms. Analysis of isoform expression among thymocytes and T cells suggests that shuffling of CD45 alternate exons occurs in an organized and predictable sequence during the process of T cell maturation and activation.

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INTRODUCTION

The overall objective of the experiments described in this dissertation was to identify the CD45 isoforms expressed in murine lymphoid cells at the mRNA level. The main focus of the research was on the characterization of CD45 expression in T cells during development and activation. CD45, also known as Ly-5, T200, B220, and LCA, is an alloantigen expressed on all hemopoietic cells except erythrocytes. Since its first discovery by Dr. I. Trowbridge in 1978, CD45 has been of interest as a differentiation antigen and has recently been implicated in the regulation of the immune response. A few examples include B cell proliferation, cytolytic T cell lysis, target cell binding and lysis by NK effector cells. Recent data showed that CD45 was a substrate for protein kinase C, suggesting an involvement in transmembrane signal transduction. Most recently CD45 was found to have protein tyrosine phosphatase activity in its cytoplasmic domain. It is possible that this phosphatase activity may strip the phosphate groups from kinases themselves or directly dephosphorylate the substrates for tyrosine kinase. This finding, combined with the observation that cells with higher phosphatase activity were more recalcitrant to transformation, suggested that CD45 may function as a tumor suppressor gene.

CD45 consists of a family of high molecular weight transmembrane glycoproteins with apparent molecular weight normally ranging from 180 kDa to 220 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis

(SDS-PAGE). CD45 isoform of extraordinarily high molecular weights (about 260 kDa) have been reported in intraepithelial lymphocytes (IEL). It has been shown that there is only one copy of CD45 gene in the mouse genome and the gene spans a length of about 130 kb, consisting of 34 exons. The molecular weight heterogeneity observed by SDS-PAGE is due to both differential glycosylation and alternate exon use among exons 4, 5, and 6. Evidence for alternate exon use comes from sequencing cDNA clones isolated from thymocytes and pre-B cells. It is believed that each of the three exons can be used independently of each other, which generates the possibility of eight combinations of the three exons. Exon 1a and 1b are also alternately used but in a mutually exclusive way. Since 1a and 1b are in the 5′ untranslated region, the selective use of either exon does not affect the molecular weight of CD45 glycoprotein. So far, there have been no reports of the existence of other alternate exons.

The expression of CD45 has been found to be cell lineage-specific and dependent on the state of maturation and activation. It was reported that thymocytes expressed predominantly the smallest CD45 mRNA (4.8 kb), pre-B cells the largest (5.5 kb), and lymph node T cells the intermediate sizes. The 4.8 kb mRNA was considered to be the isoform without any of the three alternate exons, and the largest one to be the isoform containing all the three exons. There have been no conclusive data showing exon use in T cells expressing the intermediate sizes of mRNA.

By using either exon-specific probes in Northern hybridization or labeling the cells with exon-specific monoclonal antibodies, it was possible to determine whether a certain exon was present in the isoform but it was impossible to determine how many CD45 isoforms were present or whether a specific exon was used in each isoform, alone or in conjunction

with the other two exons. In addition, these methods were not sensitive enough to detect minor isoforms of CD45 which may have important functional roles. Therefore we attempted to establish a methodology which could not only detect the major and minor isoforms of CD45 mRNAs in cells but also the exact exon use in each of the isoforms.

In chapter two, a methodology is described which combines reverse transcription, polymerase chain reaction (RT-PCR) and restriction enzyme digestion to precisely define alternate exon use of CD45 mRNA. PCR is an extremely powerful method for the production of large quantities of DNA for various kinds of analysis, but has not been used widely until the isolation of thermally stable Taq polymerase from *Thermus aquaticus*. The procedure used consisted of multiple runs of two DNA primer extensions (extending toward each other on separate strands) in the presence of a large excess of primers. By raising the temperature to 94°C, the double-stranded template DNA was denatured into two single-stranded DNAs. The primers were then allowed to anneal when the temperature was dropped to the annealing temperature. Finally the primer was extended to the end of its template at 72°C, which was about the optimal temperature of Taq polymerase. The temperature change from denaturing to annealing, and then to extension consisted of one cycle of PCR. PCR normally was conducted for 20-35 cycles depending on the purpose of the experiment. All of the extended products or the amplified products from the previous cycle are denatured and serve as potential templates for the following cycles. This allows PCR to increase the quantity of the target DNA exponentially for the first 15-25 cycles depending on the starting template concentration.

The basic concept behind the protocol was to use RT-PCR to analyze the CD45 mRNA isoforms by first reversely transcribing RNA into cDNA which

was then used as template in the subsequent PCR. After PCR, the double-stranded DNA products were digested by restriction enzymes to confirm the presence of the alternate exons in each individual isoform of interest. PCR primers flanking the alternate exon region were designed in such a way that the sizes of PCR products were between 620 bp and 190 bp representing, respectively, the mRNA containing all three exons (three exon form) and none of the three exons (zero exon form). Since each of the exons is about 140 bp in length, PCR products containing one of the three exons would be around 330 bp in length (one exon form), while the PCR products around 470 bp in length would contain two of the three exons (two exon form). Therefore, by determining the sizes of the PCR products by electrophoresis, the number of exons in each isoform was readily determined. NciI and BanI digestion of the PCR products provided conclusive information about which exons were present in particular CD45 mRNA isoforms.

By use of RT-PCR, we have confirmed 1) that one cell line can express more than one CD45 mRNA isoform; 2) that pre-B cell lines expressed predominantly the largest isoform together with smaller amounts of some intermediate forms; and 3) that T cells expressed the smallest and the intermediate mRNAs as the major isoforms.

In chapter three, the nature of two unidentified bands observed in the initial studies was determined by directly sequencing the isolated PCR products. The results indicated that the unknown amplified products were new CD45 isoforms. One of the newly identified isoforms did not have any of the exons between exon 3 and 8 (minus one form), thereby showing that exon 7 is another alternate exon. Evidence from Southern hybridization with exon specific probes suggested that exon 8 was also alternately used

giving rise to a CD45 isoform missing exons between exon 3 and 9 (minus two form).

In addition, the potential of using PCR to compare relative amounts of mRNA by using ^{32}-P end-labeled primer was explored. It was found that the PCR procedure amplified shorter templates more efficiently than larger templates. Under the experimental conditions, between cycle 10 and 25, the one exon form, the zero exon form, and the minus one form were amplified exponentially at about the same rate. However, after the 25th cycle the increase of the one exon form reaches saturation faster than the shorter forms. Based on this study, the method was modified to terminate the PCR amplifications at cycle 24, so that the intensity of each isoform at the end of PCR better represented the relative quantity of the mRNA isoforms present at the outset of the experiment.

The reverse transcription-polymerase chain reaction method has several advantages. First, successful amplification requires very few cells, only 300-30,000, which makes possible analysis of rare T cell subpopulations. Second, the method is effective with fresh or frozen cells, thus enabling the analysis of samples collected over a long period of time. Third, RT-PCR saves time and reagents since it does not require prior RNA isolation. Finally, RT-PCR is sensitive enough to detect the presence of minor isoforms in complex mixtures.

Our analysis of CD45 expression during thymocyte and T cell development indicated that, except for double negative thymocytes, the zero and the minus one exon isoforms were among the most abundant mRNA isoforms present. The putative minus two exon isoform was also detectable, in trace amounts, in all thymocytes and T cells analyzed. A comparison of other subsets of T cells indicated that double negative

thymocytes had low expression of CD45 in general and these cells only expressed the minus one and minus two exon isoform. On the other hand double positive thymocytes expressed comparably higher levels of CD45, which consisted mainly of zero and minus one exon isoforms. In general, mature T cells, except the $CD4^+$ T cell clone, exhibited increased expression of higher exon isoforms as they moved to higher differentiation and activation states. After the digestion with NciI, HinfI, and BanI, the one exon isoforms observed in both $CD4^+$ and $CD8^+$ cells were found to contain mainly exon five with very little exon 6 and exon 4.

LITERATURE REVIEW

Discovery and distribution. The existence of the CD45 glycoprotein family (also known as LCA, Ly5, T200, and B220) was first described in 1975 by Trowbridge et al. (1). These glycoproteins were found on the surface of mouse T and B cells, as well as on mouse T lymphoma cells with an apparent molecular weight around 200 kDa as determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)(1). CD45R (restricted) has been used to indicate monoclonal antibodies recognizing unique epitopes of CD45 whose expression was found to be restricted to certain isoforms (2, 3). Initially, the expression of CD45 was thought to be lymphocyte-specific (4), but later it was demonstrated that CD45 was expressed on all hemopoietic cells except erythrocytes (5-7).

Based on antibody binding, it was estimated that thymocytes and spleen cells had about 50,000-100,000 CD45 molecules per cell surface, covering about 10% of the cell membrane (8). CD45 together with Thy-1 glycoprotein constitute the major proteins on the thymocyte plasma membrane (8). Compared to splenocytes, bone marrow cells have lower amounts of CD45, while the T cell lymphoma cell line, BW5147, expresses about four times more CD45 than splenic lymphocytes (8). In general, the expression of CD45 on macrophages and granulocytes is lower than that found in lymphocytes (9).

CD45 expression was found to be enhanced by IL-2, interferon, or by certain degranulating agents (10, 11). Recently, an intracellular pool (in the tertiary granules) has been reported to contain at least 50% of the total CD45 molecules in human neutrophils (10). Upon stimulation with a degranulation-inducer, much of the intracellular CD45 relocalized to plasma membranes. It is not clear whether other cell types also have a

similar intracellular pool. Phorbol myristyl-13-acetate (PMA) was found to cause the reexpression of CD45 in a CD45 negative mutant derived from a human T cell leukaemia line (12).

Heterogeneity of CD45 glycoproteins. CD45 is a molecularly heterogenous family of transmembrane glycoproteins that have been found in all the mammalian systems studied so far, including mouse, human, and rat (13-18). The apparent molecular weights observed by SDS-PAGE analysis normally ranged from 175 kDa to 240 kDa depending on the investigator. The molecular weight heterogeneity has been proposed to be due to variable N-terminal peptide sequences coded by three alternate exons, namely exons 4, 5 and 6 (16-18); and to large differences in carbohydrate content (14, 16-18). Recently, a large isoform of CD45 (260 kd) was reported on intra-epithelial lymphocytes (IEL) from the mouse (19, 20). Since other alternate exons have not been reported, the molecular weight increase over the normal range was believed to be due to extra glycosylation (20). Experiments from many different laboratories suggested that one cell type can express more than one CD45 isoform, although some of the isoforms may be present in barely detectable levels (13-18, 21). Normally, B cells and thymocytes expressed the highest and lowest forms respectively, while T cells, macrophages, and granulocytes expressed multiple forms of intermediate size (5, 8, 22). In addition, each cell lineage may express CD45 differently at different developmental stages (23-25).

Genomic structure. Mouse CD45 cDNA from a T cell clone, Cl.Lyl-Tl, was cloned and sequenced by Shen and Saga (13). Based upon their data from Southern hybridization, as well as from restriction mapping of overlapping genomic clones, they concluded that there was only one copy of the CD45 gene in the mouse genome. They further proposed that CD45

isoforms were generated by differential processing of transcripts rather than by gene rearrangement (13). The hypothesis was later confirmed by sequencing cDNA clones (16-18).

At least three alleles of CD45 have been identified by observation of restriction fragment length polymorphisms and by serological phenotyping (26) and have been designated Ly-5^a, Ly-5^b and Ly-5^c (27, 28). Ly-5^b appeared as the most common genotype (27) and was found in Balb/c, C57BL/6J, DBA/2 and in many other common strains (27). On the other hand, Ly-5^c was the least common and was only found in mice of ST/bJ strain (27). The nomenclature for the Ly-5 alleles has recently been revised (29).

The mouse and human CD45 gene locus have both been mapped to chromosome 1 (14, 27). The mouse gene spanned about 130 kb including 34 exons, 1a or 1b, and 32 additional exons (16, 17, 28; Figure 1). The mature mRNA was about 5.1 kb coding for 1,268 amino acids (30). Exons 3-15 were found to encode amino acid residues 1-538 of the extracellular domain, exon 16 encoded residues 539-574, encompassing the transmembrane domain, and exon 17-33 encoded residues 575-1268 of the cytoplasmic domain (30). The exons were found to vary in size, but most were between 50 and 200 base pairs in length (28, 30). The largest exon, 33, was about 1.1 kb in length and contained a termination codon plus the 3' untranslated region (30). The splice junctions of all exons followed the AG/GT rule (16, 28). The sizes of the introns were widely different, from the shortest of 81 base pairs between exons 2 and 3, to about 50 kb in length between exons 3 and 4 (28).

Based upon primer extension studies, the first two exons, namely 1a and 1b, were shown to be expressed in a mutually exclusive way (16), and were presumably from two different transcription initiation sites (16).

Figure 1. Genomic structure of mouse CD45 gene. Exons are represented by the vertical lines. Exons 1a to 15 are in the extracellular domain, while exons 17 to 33 in the cytoplasmic domain. TM: transmembrane region.

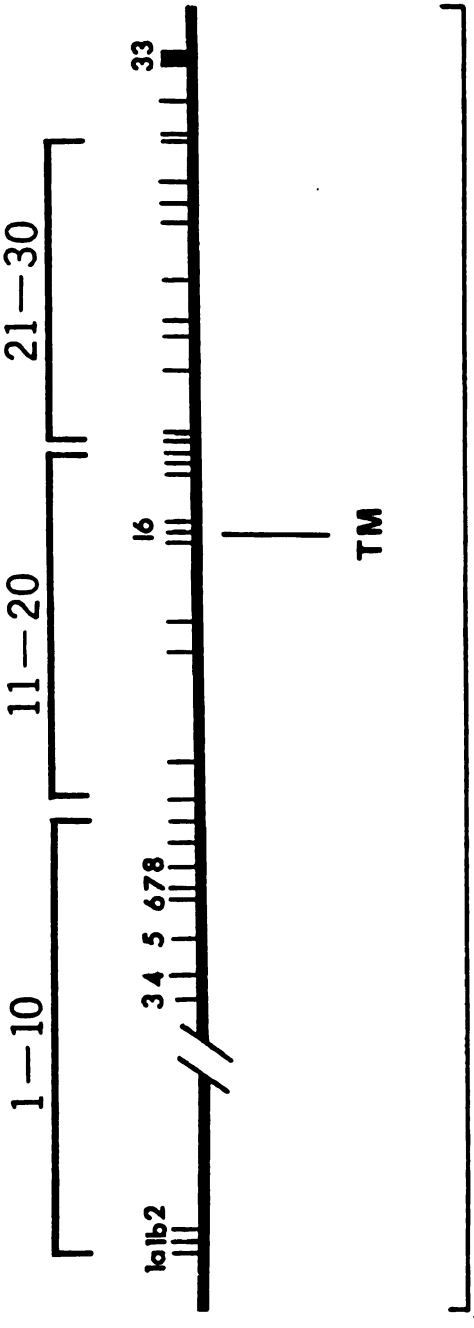

Figure 1.

In human, exons 1a and 1b were used differentially but not in a cell type-specific manner (16). According to the accumulated data, 1b seemed to be more frequently selected (16). However, it was reported that in mice the 1a containing species of mRNA was much more abundant in T cells than in B cells (28). Since each of these two exons encodes the 5′ untranslated region, the use of either exon does not appear to affect the peptide sequence. The significance of the alternative use of exons 1a and 1b is still unknown. Exon 2 encodes part of the 5′ untranslated region and most of the leader sequence (23 amino acid residues). Exon 3 includes the last 2 nucleotides for the last amino acid in the leader sequence and encodes the first seven amino acids of the mature protein, a sequence that is found to be common to all CD45 isoforms discovered so far.

The main region for alternative splicing was found from exon 4 to exon 6 (17, 28). In the murine system these exons were 129, 147, and 141 base pairs or 43, 49, and 47 amino acid residues in length, respectively (28). The cDNA clone from a pre-B cell line (70Z/3.12), which expressed mainly a 220 kd isoform, contained all the exons except 1a (due to the presence of the mutually exclusive exon 1b), whereas the cDNA from thymocytes expressed the 180 kd form lacking exons 4, 5 and 6 (16, 17). It was further established that each of the three exons was independently used, alone or in combination. Potentially each cell type could contain up to eight distinct isoforms (28). The use of the three exons not only changed the peptide sequence but also the extent and pattern of glycosylation, which in turn determined the molecular weight of the CD45 isoforms on SDS-PAGE. It is now believed that the expression of CD45 isoforms is controlled in a cell type-specific way (1, 4, 8, 16, 28) and that any one type of cells can express more than one isoform. So far, six

of the isoforms have been identified (14, 16, 17).

Although it is possible that there are still some alternate exons to be discovered for CD45, there has been no evidence for the existence of other alternate exons other than 1a, 1b, 4, 5, and 6. Therefore epitopes on the peptide sequences encoded by exon 3 and by exons 7 to 32 are believed to be present on all CD45 isoforms, and have been termed common epitopes (31, 32). By contrast, epitopes that were found on the protein sequences coded by the alternate exons were not common to all the isoforms, and thus were referred to as restricted epitopes (CD45R). In addition, the restricted epitopes were not necessarily due to differences in the peptide sequence but were also believed to be due to differential glycosylation.

Mechanism of splicing. Although the mechanism controlling cell lineage-specific CD45 splicing is still unknown, it is believed that selective use of the transcription initiation sites does not determine splicing patterns (33). Consequently, the existence of both trans-acting and cis-acting factors was proposed to account for the regulation of the splicing of CD45 RNA. Some knowledge of the regulatory elements for splicing has been acquired from studies of a human-mouse chimeric system (34, 35). In order to study the factors involved in tissue-specific splicing, Streuli et al. transfected the murine pre-B cell line 300-19 and thymocyte cell line EL-4 with mini-gene constructs that contained only part of the human CD45 sequence, including several of the alternate exons (exon 4 and/or exon 6) and the SV40 promoter. The transcripts were then analyzed by primer extension to determine whether pre-B cells retained the alternate exons in their mature mRNA, and whether the thymocyte isoform excluded all of the alternate exons. The results indicated that the same

primary transcripts were discriminately processed in the pre-B cell line and thymocyte line as expected (36). This finding suggested that; (a) the phenomenon of alternate splicing for CD45 mRNA probably was not the consequence of different pre-mRNA structures resulting from alternative promoter usage or alternative poly(A) site usage; (b) there was some trans-acting factor that regulated the specificity of the CD45 pre-mRNA splicing; and (c) whatever the trans-acting factor was, it must have been well conserved between human and mouse, in that the trans-acting factor from the murine system seemed to be functional on the human DNA sequence and this factor worked with both exon 4 and exon 6 (36). It was also concluded that exon 5 was used in a poorly regulated way and thus its use might not be tissue-specific (36).

Transfection of CD45 into the nonhemopoietic cells, NIH-3T3, HeLa, and L cells indicated that the splicing pattern was essentially the same as that in thymocytes (36). This suggested that thymocytes and non-hemopoietic cells have the same or functionally identical trans-acting factor, if present. It was hypothesized that there was no such trans-acting factor in the thymocytes and nonhemopoietic cells and the primary transcript was thus processed to remove all the alternate exons. This hypothesis has been supported by the findings from Saga's group (37). They examined the splicing pattern of mouse CD45 in hybrid cells made by fusing T cells and B cells and found that all the hybrids used the alternate exons in a B cell-specific pattern. Thus, they concluded that all the information required for faithful splice-site selection according to cell type was contained within the resulting pre-mRNA, and the splicing pattern manifested by nonexpressor cells or thymocytes may represent a default, nonregulated type (37). So far, no trans-acting factors have

been identified.

There are several models proposed to explain the alternate splicing phenomenon observed for CD45 (36), but none of these has been found to be entirely satisfactory. The results obtained in studies of cis-acting elements have been more conclusive. By use of various kinds of deletion mutants, it has been demonstrated that the critical cis-elements for specific splicing are inside the alternate exons themselves and in their immediate flanking intron regions (from 10 to 200 bp) (36). In addition, the absence of neighboring constant exons, Ex-3 and Ex-7, did not seem to affect alternate splicing (36).

By use of linker scanning, it has been found that for Ex-4 to be alternatively used, at least three different cis-elements within Ex-4 were crucial. In other words, if certain sequences were modified without significantly changing the length, the splicing of the mutated transcripts were no longer tissue-specific. The critical sequences were between nucleotide positions 8 and 17, 40 and 91, and 127 and 137 in Ex-4 (36). For Ex-6 the critical sequences lie between nucleotide positions 16 and 144 (34). However, Saga et al. reported that some of the intron sequences flanking both sides of exon 6 were also important in deciding the exclusion of that exon in mouse T cells (37). Although it has not been confirmed, it is possible that Ex-5 has crucial sequences for tissue-specific splicing. Although cis-elements have been identified, the mechanism by which they affect specific splicing is still obscure. It is possible that they consist of binding sites for one or more trans-acting factors, or perhaps they are important for secondary structure formation (36).

Structure of CD45 glycoprotein. The complete structure of CD45

glycoproteins of mouse, human, and rat have been elucidated by analysis of cDNA clones (13-18, 38). The protein was found to consist of 1118 to 1281 amino acids depending on the species and alternative mRNA splicing. The mature murine CD45 glycoprotein was be divided into three distinct domains: the N-terminal, extracellular domain (402-541 amino acids residues depending upon the variable exon usage), the transmembrane domain (22 amino acids residues), and the C-terminal, cytoplasmic domain (705 amino acids residues) (Figure 2). Electron microscopic examination of the rat CD45 molecule indicated that it had the shape of a comma (39). The extracellular domain appeared to be the elongated (tail) portion and the cytoplasmic domain the globular (head) portion.

Based on protein sequence analysis and biochemical studies, the extracellular domain was further divided into at least two different areas: an O-linked, glycan-rich area and an N-linked, glycan-rich, cysteine-rich area. Peptide sequence coded by exons 3-8 was rich in serine and threonine (around 34%) but contained no cysteine, which is characteristic of O-linked carbohydrate sites (40). Therefore this area contained potential sites for O-linked glycosylation, and the use of the alternate exons changes the number of glycosylation sites. It has been shown that in the rat thymocyte form (lacking exon 4,5 and 6), all the O-linked carbohydrates were found within the first 32 amino acids (15), which were equivalent to the sequences encoded by mouse exon 3, 7 and 8. Although the overall homology of the alternate exon coded sequences among the three species (human, rat, and mouse) was found to be only 40%, the location of the O-linked sites was well conserved. Lefrancois has proposed that for some cell types the carbohydrates in this area contain N-acetylgalactosamine in a β1,4-linkage to galactose and sialic acid in an

Figure 2. Structure of CD45 glycoprotein. CD45 glycoprotein can be divided into three domains: extracellular, transmembrane and cytoplasmic domains. The cytoplasmic domain contains two protein tyrosine phosphatase -like subdomains. Exons 4, 5 and 6, are the alternate exons. Dark area represents the transmembrane region.

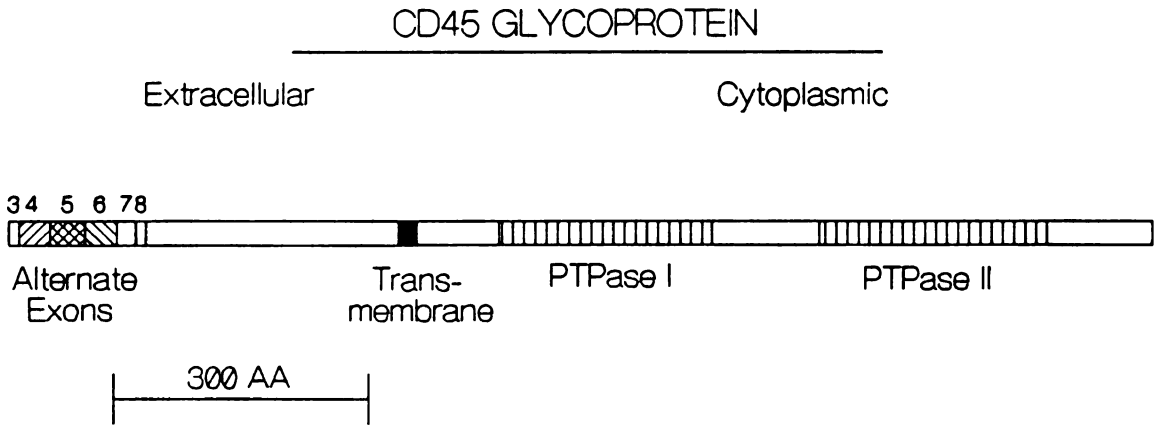
CD45 GLYCOPROTEIN
Extracellular
Cytoplasmic
3 4 5 6 7 8
Alternate Exons
Trans-membrane
PTPase I
PTPase II
300 AA

Figure 2.

α2,3 linkage (41, 42).

The peptide sequence coded by exons 9 to 15 was found to contain the cysteine-rich area. Although the homology among the three species was quite low (only 33%), many key conserved residues were found indicating that the exterior domain may have a conserved three-dimensional structure (43). This region was found to contain 16 cysteine residues in human and rat CD45 and 18 in mouse, and the positions were well conserved except for the two extra cysteines in mouse. These cysteines were believed to be disulfide-bonded (38), presumably to maintain the critical structure. There were also 11-17 potential N-linked sites (depending on the species) in the extracellular domain (15).

Since observations indicated a lack of protein sequence conservation in the exterior domain, Thomas et al. suggested that this sequence may act as a platform for the carbohydrate structure (19). The carbohydrates, rather than the protein sequence of the extracellular domain, may be crucial for the function of CD45. The observations supporting this are: first, the protein sequence homology was low among the species compared so far; second, the O-linked glycosylation was precisely regulated via alternate exon usage; and third, the locations and number of cysteine residues were well conserved. Additionally, there has been evidence showing the importance of carbohydrate residues in NK cell functions (44).

In contrast to the extracellular domain, the large cytoplasmic domain of CD45 was highly conserved among the species with >85% identity. There were sequences of over 100 amino acids that were 95% homologous. Thomas suggested that the discrepancy in conservation between the extracellular domain and the cytoplasmic domain implies that these domains perform different functions (43). In the cytoplasmic region two

subdomains were found, each of 300 amino acids in length, sharing 33% homology between each other (30). Each of the two cytoplasmic domains was found to have about 40% homology to placental protein tyrosine phosphatase (45). The cytoplasmic domain was also phosphorylated (46). This discovery raised the possibility that CD45 could serve as a signal transducer.

Function of CD45. CD45 has been proposed to function in the following cells; stem cells (47, 48), NK cells (11, 22, 44, 49-53) and lymphoid cells (2, 18, 20, 47, 54-61). Most of the hypothetical functions were based on observations involving the use of antibodies to disrupt cellular functions. Much of the data suggests that CD45 probably serves as the receptor/ligand molecule which transduces signals across the membrane resulting in cell lysis, proliferation, activation or anti-transformation. However, since the specificity of many of the antibodies is not clear, contradictory results using different antibodies were obtained. The development of new exon-specific or cell lineage-specific monoclonal antibodies with accurate epitope definition will greatly help our understanding of the function of CD45.

NK cells were found to express multiple forms of CD45 (22, 49). A number of antibodies against CD45 have been generated that have the ability to inhibit NK activity (11, 49, 52, 53, 62-65). Based upon the abundance of accumulated data, CD45 was proposed to be one of the critical molecules implicated in conjugate formation between the NK cells and target cells, and the ensuing target cell lysis (11, 52, 61, 66). Attempts to identify receptors on NK cells associated with conjugate formation or cytolytic activity by generating and screening monoclonal antibodies which blocked NK functions revealed that almost all such

antibodies have reacted with one or more epitopes of CD45 (67). Anti-Ly-5 sera and monoclonal antibody 13.1 against CD45 were examples of inhibitors of NK function in the mouse and human, respectively (44, 50, 52). Since there were multiple forms of CD45 on NK cells, it was difficult to assign a specific role to each individual isoform. It was not clear whether all the isoforms perform the same kind of function. To clarify this question, identification of all the isoforms expressed and definition of the epitopes recognized by the monoclonal antibodies for each CD45 isoform was considered imperative.

The involvement of CD45 carbohydrate structure in NK cell function has been explored. Gilbert et al, discovered that purified CD45 incorporated into liposomes inhibited conjugate formation between the NK cells and the target cells (44). However, no inhibition was observed if the liposomes were pretreated with Ly-5 antisera or enzymes to remove some of the carbohydrate (44). This research was among the first to demonstrate a connection between CD45 function and its carbohydrate structure.

When small resting human B cells were incubated with antibodies against CD45, the inducible proliferation by anti-IgM and T cell-replacing factors was abolished (55). Anti-Ly-5 sera greatly affect the generation of plaque-forming cells (plasma cells) specific to T cell-dependent antigens, but not T cell-independent antigens (54). The antibodies were also capable of inhibiting the IgG response when B cells were induced with lipopolysaccharide, whereas proliferation of IgM secreting cells or IgM response was not changed (56). The phenomenon was later rationalized as a decrease in the number of cells committed to class-switching rather than a defective clonal expansion of IgG-producing cells (57).

The involvement of CD45 in T cell functions is more complicated due to complex patterns of CD45 expression on T cell subsets (13-18, 21). The observation of the general correlation between the differential expression of CD45 and the distinct functions of T cell subsets, implied that CD45 was important in moderating their cellular functions (18). It has been observed that the ratio of T cell subsets, defined by the CD45 epitope expression, was altered in the course of some diseases such as rheumatoid arthritis (68), multiple sclerosis (69, 70), and multiple myeloma (71). In mice with recessive lymphoproliferative disorders, lpr or gld, lymph node T cells abnormally expressed CD45 antigen with more of the high molecular weight isoform (similar to that of the B cell form) than that in normal animals (72). As a result of abnormal proliferation, lymph nodes enlarged up to 50-fold. It remains unclear whether the diseases result from the irregularity of CD45 expression.

There has been evidence showing that CD45 was indeed involved in T cell activation or differentiation. In the mouse model, the addition of anti-Ly-5 sera to the culture resulted in the inhibitory effect on the generation and lytic ability of cytotoxic T lymphocytes in the mixed lymphocyte reaction (47, 58, 59). Another line of evidence was provided by the anti-CD45 monoclonal antibody 2H4, which distinguished $CD4^+$ T-helper cells and $CD4^+$ suppressor inducer T cells (23). When the suppressor inducer T cells were pretreated with 2H4 antibody, they lost the ability to induce suppression (73). In another experiment, the same antibody was added directly into the culture, but surprisingly it enhanced the suppression (60). Thus far, it is still not clear why the same antibody had the opposite effect under different conditions. In addition, antibodies against different epitopes of CD45 may have totally opposite

effects (2, 20). Monoclonal antibody against the restricted epitope on CD45 augmented the mitogenic response to suboptimal dose of PHA (2). On the other hand, antibody against the common epitope suppressed PHA and ConA responses (20). It is possible that antibodies against different epitopes act on a different subset of T cells, consequently inducing opposite functions. On the other hand cells may express more than one CD45 isoform and, depending on the isoform that the antibody binds, can be induced to perform diverse functions.

Several recent studies indicated that CD45 might be involved in early T cell activation. It was proposed that antibodies stimulate early T cell activation by making some alteration in the IL-2/IL-2 receptor pathway (2, 20). Monoclonal antibody against the common epitope on human CD45 together with Sepharose-coupled anti-CD3 antibody acted as a mitogen for T cells and resulted in T cell activation. Neither of the two alone was mitogenic (61). The most direct evidence showing the involvement of CD45 in the T cell response came from the work of Pingel and Thomas in which they found that a T cell mutant lacking CD45 could not proliferate in response to antigen even though they expressed other T cell surface proteins normally (74).

CD45 was found to be physically associated with the cytoskeletal protein fodrin through its cytoplasmic domain and participated in receptor patching and capping (75, 76). Although the significance of this association is not certain, it is probably an indication of a role in signal transduction or in regulation of cell membrane organization and mobility (43). CD45 was also found to be physically associated with CD2 in human T lymphocytes (77).

PTPase activity. The mechanism of function of CD45 was better

understood upon the discovery that CD45 was a protein tyrosine phosphatase (PTPase) (78). In lymphoid and myeloid cells, considerable interest has emerged from the recent discovery that the cytoplasmic domain of CD45 possesses two domains bearing significant amino acid sequence homology with placental protein tyrosine phosphatase 1B (PTPase-1B) (45). Tonk's group presented evidence showing that CD45 had PTPase activity (78). They suggested that the binding of CD45 to its ligand modulated the CD45 enzymatic activity as a protein tyrosine phosphatase, thus resulting in the regulation of protein tyrosine kinase activity, whose activity is related to cell proliferation and transformation (78). By using a CD45-negative mutant, Koretzky et al. concluded that CD45 was essential for coupling T cell antigen receptor to the phosphatidyl inositol (PtdIns) pathway to generate the PtdIns-derived second messenger (12). In addition, several investigators proposed that CD45 may dephosphorylate $p56^{lck}$ (79, 80), a protein tyrosine kinase encoded by the *lck* proto-oncogene. CD45 has been shown to activate $p56^{lck}$ in a lymphoma cell line by dephosphorylation of a regulatory site.

Perhaps the most interesting proposed function of CD45 is that of an anti-oncogene or tumor suppressor (81). This concept was derived from an experiment in which the results of transfection with mutated human *neu* oncogene were compared between normal mouse 3T3 cell line and the same cell line with over-expressed PTPase 1B activity. They found that introduction of the mutated human *neu* oncogene into the normal cells aggressively induced transformation presumably by acting as an unregulated tyrosine kinase. By contrast, a severe reduction in transformation was observed in the PTPase over-expressing cells. Since CD45 shares homology (about 40%) with PTPase 1B and has been shown to have PTPase activity, it

was thus suggested that CD45 may be an anti-oncogene.

Overall, the above evidence suggested a model in which CD45 extracellularly interacts with other molecules (or its ligand), transduces a signal inside through the cytoplasmic domain of CD45, which then regulates the activity of some cytoplasmic component, and finally results in the cellular response. There are, however, several unanswered questions. Are all the isoforms of CD45 capable of performing the functions as proposed, or is it just a privilege of certain isoforms? Since all the isoforms have the same cytoplasmic domain, upon interacting with their specific molecule (stimulus) on the outside, is the signal transduced inside of the same quality and intensity for all the isoforms? Perhaps, the difference in alternate exon usage and glycosylation of each isoform is to construct the specificity for the individual isoform so that each of them can react specifically with certain molecules or ligands. If every different isoform has its own distinct function, it will be essential to know both the exact isoform expression and the relative quantity of the isoforms. It should also be kept in mind that all the complicated functions proposed for CD45 probably involved the co-presence of other molecules.

Antibodies to CD45. There have been many antibodies developed against CD45, but the specific epitopes recognized by a given antibody is still not well defined. Initially, antibody specificity was determined by comparing the data obtained by Northern hybridization and by anti-CD45 cell surface labeling. The ambiguity of this approach resulted from the multiple expression of CD45 on a single cell. This was solved by the establishment of transformants obtained by infecting $CD45^-$ cell lines with expression vectors containing only one specific cDNA isoform of CD45 (82).

Based on these data, the epitope recognized by some of the monoclonal antibodies could be said, at most, to be dependent on the presence of a specific exon, but the property of the epitope was still obscure. This was because of the fact that the antibody may have recognized the sequence around the exon junction, or it may have recognized unique carbohydrate residues rather than the peptide sequence (41, 58, 83). It is possible that one particular isoform may have dissimilar glycosylation in different cell types. Evidence supporting the tissue-specific glycosylation of CD45 was provided by the existence of CT1 and CT2 antigens only on murine CTL (41), and the extraordinarily high molecular weight (260 kd) only observed in IEL (19, 20).

Antibodies or anti-sera believed to recognize the common epitopes on CD45 include I3/2 (8) and Ly-5.1 (84) in mice, and EO-1 in humans (25). Antibodies that have been claimed to be directed against a specific epitopes include: 2H4 for exon 4 in humans (60), UCHL1 for the junction between exon 3 and exon 7 in humans (85), NK-9 for carbohydrate on human NK cell CD45 (49), CT1 and CT2 for carbohydrate on CD45 of the murine CTL (41), 14.8 and RA3-2C2 for exon 4 in mice (86), C363.16A, MB23G2 and MB4B4 for exon 5 in mice (82, 86), and OX-22 for exon 5 on rat CD45 (31). It should be kept in mind that the epitopes assigned to a certain monoclonal antibody may be changed with the emergence of more conclusive data.

Tissue-specific expression of CD45. Although it is believed that the expression patterns of CD45 glycoproteins are not only cell lineage-specific but also related to the degree of maturation and activation (35, 82, 87), only small progress has been made on the characterization of CD45 expression during lymphoid cell development (21, 23, 31, 35, 82, 87-89). It has been reported that the size of mouse CD45 mRNA is 4.8 kb for

thymocytes, 5.2 kb for cytotoxic T lymphocytes, 4.8 kb for T_h clones, and 5.5 kb for B cells (21). Most of the study has been centered on T cells with very limited research on B cells (21, 23, 31, 35, 88), whereas study of non-lymphoid cells is almost absent.

The isoform expression on pre-B cells and immature thymocytes seems to be much simpler. Based on cDNA screening and sequencing, the former has the full length isoform with all the three alternate exons, namely exon 4, 5, and 6 (17), while the latter has the shortest isoform (13). Since some of the minor cDNA clones may be lost during library construction or colony screening, it is possible that by a more sensitive method, other minor isoforms may be detected. By primer extension, minor isoforms containing only one or two of the three exons were also detected in B cells in addition to the major isoform, which included all three exons (37).

Conclusions regarding the expression pattern on other cell types were either incomplete or sometimes conflicting in different reports. Most of these conclusions were based on Northern hybridization or antibody labeling. It was reported that when thymocytes became mature, some subsets expressed the larger CD45 isoforms (CD45R) including exon 4, 5 and/or 6 (35, 82, 87, 89). After stimulation with either antigens or ConA, the expression of larger isoforms was again down-regulated, while the smallest isoform was up-regulated (35, 89). Birkeland et al. (82) reported similar results. They found that when resting T cells (either $CD4^+$ or $CD8^+$) were stimulated with allogenic dendritic cells, they lost the 190 kDa isoform (presumably the exon 5 dependent isoform) with an increase of the 180 kd form (82). According to Bottomly et al., about 45% of $CD4^+$ T cells expressed exon 5 dependent antigen on the surface (90). Among

them, one third expressed lower levels of exon 5, which were the T_{h2} subpopulation, with the more brightly stained cells representing the T_{h1} subpopulation (90).

The majority of the results regarding expression were obtained from cDNA sequencing (13, 17), Northern hybridization with exon-specific oligo-probes, or monoclonal antibody labeling. These methods have certain disadvantages and limitations. Sequencing cDNA clones from the cell types to be studied certainly would be the most accurate way, but it is impractical. Northern hybridization with exon-specific probes or surface labeling with monoclonal antibodies can only offer accurate information about the presence and relative quantity of each particular exon, but it can not answer how many or which combinations of the exons are present. Furthermore, the epitopes recognized by some of the monoclonal antibodies are not well defined as discussed above. Consequently, the conclusions based upon the antibody labeling can be misleading.

Polymerase chain reaction. It was hypothesized that the polymerase chain reaction (PCR) could resolve problems about the exon usage of CD45. Although PCR is a newly developed technique (91, 92), its application has been found in a large variety of research areas as described in reviews (93). The widespread use of PCR was due to its ability to amplify very small amounts of DNA into sufficient quantities for later manipulation (92). In brief, PCR consisted of consecutive, multiple runs of DNA primer extension mediated by a thermal stable DNA polymerase (Taq polymerase, Thermus aquaticus) in the presence of a large excess of two specific primers. The primers were usually around 20-30 bases in length and synthesized according to the known sequences flanking the DNA segment of interest (target DNA). Under precise control, the PCR reagents were

subjected to about 30 cycles of the temperature changes. In each cycle, the double-stranded template DNA was first denatured into single-stranded DNA at about 94°C. When the temperature drops, the primers annealed to their own complementary strand of template DNA. After the annealing, the temperature was brought to 72°C, the optimal temperature for Taq polymerase, and the primers extended to the end of their sequence. The process of denaturing, annealing, and extension consisted of one PCR cycle. Both the original template DNA and the product DNA were available to be used as the template in the following cycle. Normally each PCR reaction included about 30 cycles. It has been estimated that at the end of 60 PCR cycles, amplification of up to a factor of 10^{12} can be achieved (94). This accounts for the usefulness of PCR in detecting the trace amount DNA extracted from tiny amount of samples, such as hair roots (95) or saliva (96) in forensic cases.

Since PCR was used with very specific primers to the flanking sequences, and the primer extension was carried out at high temperature, the chance of mispriming was very limited. Consequently, residual amounts of target sequence in a very noisy background still operated as templates without prior purification. A standard PCR reaction of 30 cycles took only about 3 hours and after that the product was ready for various assays such as electrophoresis, sequencing, or restriction enzyme digestion.

Nevertheless, there were several pitfalls and limitations recognized in the application of PCR. First, if a contaminant sequence was present in the reaction tubes, a false positive result might be obtained due to the high level of amplification. This problem was particularly obvious in experiments designed to detect rare sequences of pathogens in the early phase of infectious diseases such as human T cell leukaemia or AIDS (97,

98). However, this problem was avoided by taking precautions as described (93). Second, the error rate associated with Taq polymerase was about 1 in 9,000 for single base substitutions and 1 in 41,000 for frame-shift errors (99). Accordingly, extreme caution should be taken in sequencing DNA from PCR. Third, the sequences flanking both ends of the target DNA have to be known in order to design the primers for PCR. Another limitation was the length of sequences to be amplified. Although distance up to 10 kb have been successfully amplified (100), most of the applications have been for sequences within several kb. When the length of target DNA increased, PCR became much less efficient.

There have been successful examples of using PCR in mRNA phenotyping (92, 94) or for quantitation (101, 102). In those applications reverse transcription was necessary to convert mRNA into cDNA to function as the template. The primer for reverse transcription was either oligo $(dT)_{12-18}$ or the anti-sense PCR primer. After reverse transcription and PCR, the sequences of the products represent those of the mRNA in the corresponding segment. Thus, analysis of the PCR products enabled information for mRNA content to be obtained.

Since the entire sequence of CD45 mRNA was known (13, 17), it was feasible to use reverse transcription together with PCR to explore alternate exon usage in CD45 mRNA. It was possible to synthesize primers flanking the alternate exon region such that the PCR products were within 700 bp in length depending how many and which alternate exons were used. The PCR products were then subjected to more detailed analysis for characterization.

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Chapter Two

T200 ALTERNATE EXON USE IN MURINE LYMPHOID CELLS DETERMINED BY REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION[1]

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Running Title: T200 Alternate Exon Use Determined By RT-PCR

FOOTNOTES

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Abbreviations: PCR, polymerase chain reaction; RT, reverse transcriptase; RT-PCR, reverse transcription-polymerase chain reaction; bp, base-pairs; Ex, exon; M-MLV, Moloney murine leukemia virus; P2, a synthetic oligonucleotide that primes from a point beginning within Ex-2 of T200; P9, a synthetic oligonucleotide that primes from a point beginning within Ex-9 of T200.

ABSTRACT

T200 glycoproteins of lymphoid and myeloid cells exhibit cell lineage-specific structural heterogeneity. Peptide heterogeneity appears to arise from alternate 5'-exon use (Ex-4, 5 and 6), potentially giving rise to eight distinct forms of T200 mRNA containing 0-3 of these alternate exons. A method is described for determining the number and identity of the three alternate T200 exons expressed in cells using the polymerase chain reaction (PCR) and the reverse transcription-polymerase chain reaction (RT-PCR) without prior purification of RNA. Synthetic primers flanking the alternate exon region of T200 were designed to yield products for each possible exon combination having unique size and restriction enzyme sites. PCR amplification of plasmids containing T200 cDNA with none (pLy-5-68) or all three (p70Z/3-3) known alternate exons resulted in the amplification of 186 and 603 base-pair (bp) products, respectively. That the amplified products were derived from T200 cDNA was verified by restriction enzyme mapping of each PCR product. T200 cDNA prepared from cell lines utilizing no alternate exons (BW5147) or all three exons (70Z/3.12) were analyzed by RT-PCR and contained amplified products of 186 bp (zero alternate exons) and 603 bp (containing Ex-4+5+6), respectively. RT-PCR of EL4 cells revealed approximately 186 and 330 bp products suggestive of zero and one alternate exon forms. Restriction mapping confirmed that EL4 cells contained a zero-exon form and a one-exon form containing Ex-5. Analysis of the 3B3 pre-B cell line yielded 186, 330, 460 and 603 bp products; restriction mapping revealed T200 mRNA for a zero alternate exon form, two distinct one- and two-exon forms (Ex-4; Ex-5; Ex-4+5; Ex-5+6), and a three-exon form (Ex-4+5+6). Other lymphoid cell lines were heterogeneous in T200 alternate exon use,

with distinct patterns distinguishing B and T cells. RT-PCR can facilitate the analysis of variations in T200 alternate exon use among developmentally and functionally distinct lymphoid and myeloid cells.

INTRODUCTION

T200 (also called CD45, B220, Ly-5 and L-CA) is a molecularly heterogenous family of transmembrane glycoproteins ubiquitously expressed on the surface of all mammalian lymphoid and myeloid cells (1-3). Heterogeneity is believed to result from variations in N-terminal peptide sequences coded for by up to three alternately used exons, designated Ex-4, 5 and 6 (Figure 1), as well as by differences in glycosylation (4-8).

T200 has a number of unusual features that suggest an important role for the glycoprotein in lymphoid cell function. T200 has a large (705 aa) cytoplasmic domain (9) which is highly conserved (>90%) between mice and humans (5,10) and is a substrate for protein kinase-C-mediated phosphorylation (11). Recent structural and functional studies have suggested a possible regulatory role for T200 in the phosphorylation state of intracellular proteins. Specifically, the cytoplasmic domain of T200 has been shown to share significant sequence homology with placental tyrosine phosphatase (12). In addition, significant protein tyrosine phosphatase activity has been identified in purified preparations of T200 (13). These and other data suggest that the cytoplasmic domain of T200 may transduce or regulate the signal derived from cell surface interactions involved in the generation of intracellular responses. The N-terminal extracellular domain of T200 is more variable, suggesting that this segment may regulate interactions with other cells or molecules,

Figure 1. Strategy for PCR amplification of the region of alternate exon use in T200 cDNA. The complete T200 cDNA (top) includes the alternate exon region (thick segment) and coding region (*⟶ ⟵*). The expanded first kb (middle) contains the three alternately used exons (Ex-4, 5 and 6) and shows the position and orientation of the flanking PCR primers P2 and P9. The 603 bp amplified product is shown (bottom), including the three alternate exons. Exon positions are from Saga et al. (7), and are numbered according to Johnson et al. (29). Base positions of cleavage sites (474, 522) are given according to the T200 cDNA clone p70Z/3-3 (5).

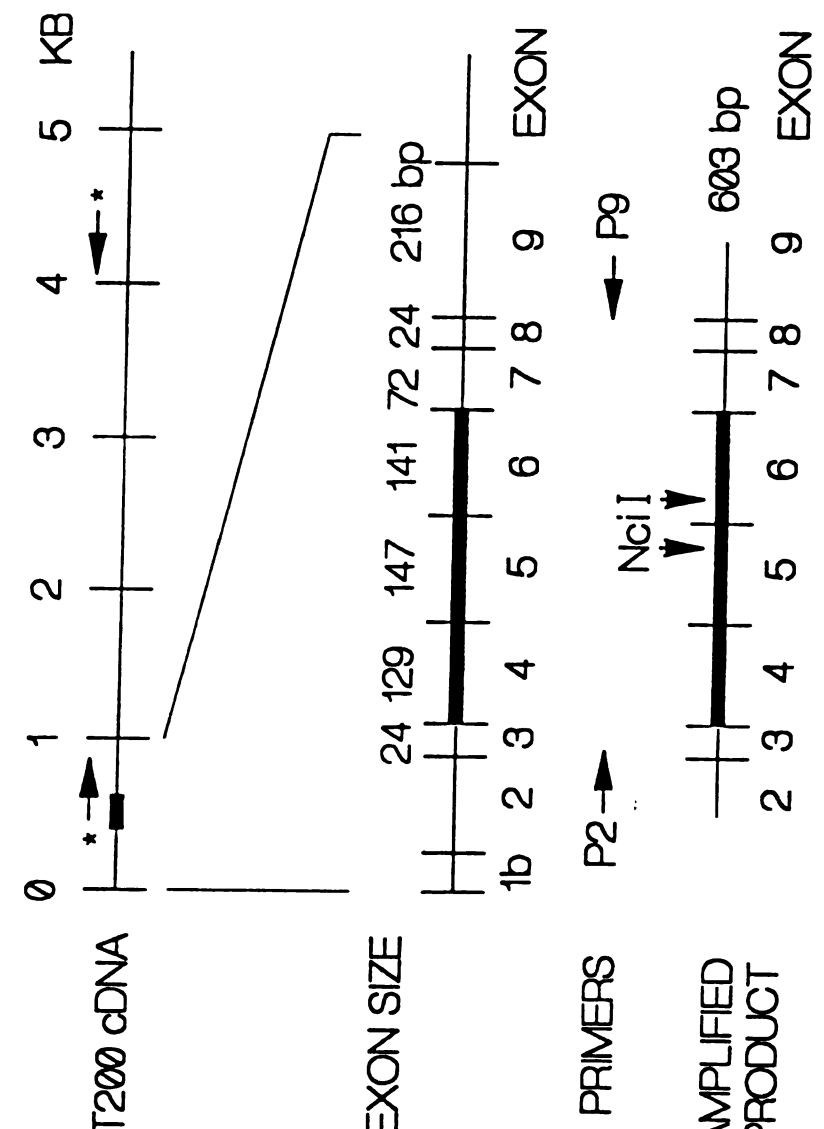
T200 cDNA
0
1
2
3
4
5 KB
EXON SIZE
1b
2
3
4
5
6
7
8
9 EXON
24
129
147
141
72
24
216 bp
PRIMERS
P2
P9
NciI
AMPLIFIED PRODUCT
603 bp

Figure 1.

thereby acting as a cell receptor for certain transmembrane signaling processes. This possibility is supported by previous work in this laboratory and others of a role for cell surface T200 in B cell recognition/processing of macrophage signals (14), B cell proliferation (15) and antibody responses (16), certain examples of CTL CMC (17,18), T cell proliferation (19,20), NK-target cell binding (21-24), and the Ca^{2+}-dependent programming phase of NK CMC (25-27). Since some epitope-specific T200 mAb affect the function of certain lymphoid cell types but not others (28), it seems likely that distinct epitopes on T200 are involved in the function of T200 in different cell systems.

The degree to which cell lineage-specific heterogeneity is observed in the T200 glycoprotein family remains to be determined, but appears to occur despite a common origin from a single gene (5,10), resulting in part from alternate exon shuffling involving three 5′ exons (Ex-4, 5 and 6 [7,29]; Figure 1). This process involves the synthesis of T200 containing one of up to eight possible combinations of from 0-3 exons, with cells of different origin often expressing different isoform phenotypes (4,6,30). Individual cell lines may also express more than one T200 isoform as a result of alternate exon shuffling, possibly including major and minor forms.

Specific determination of alternate exon usage has been reported for some tumor and continuous lymphoid cell lines using transfer blot techniques. Thomas et al. (5) used a probe derived from cDNA containing all three alternate exons to determine that murine B cells and some suppressor T cell line T200 mRNA contained alternate exons, while helper T cell lines and thymocytes did not. Similarly, a study of human T200 showed variable expression of T200 alternate exons in different human

tumor cell lines (6).

The polymerase chain reaction (PCR) is an important recent development in recombinant DNA technology which allows single copy genes from a few cells to be amplified to microgram quantities which may be easily detected by conventional agarose gel electrophoresis without the use of radioactive tracers (31-34). The amplified product is produced in adequate amounts for subcloning or for sequence analysis and may be directly gel-purified and evaluated by restriction enzyme analysis. In an extension of PCR designated reverse transcription-PCR (RT-PCR), purified mRNA may be analyzed by initial reverse transcription into cDNA which is used as the template in a subsequent PCR (35). This method is extremely sensitive and has been reported to be capable of revealing the specific mRNA content of a single cell (35).

In this report, we describe a sensitive technique involving RT-PCR to amplify the region of alternately-used exons of T200 which enables identification of specific exon usage in lymphoid cells. This method does not require purification of RNA prior to RT-PCR. The pattern of both major and minor alternate exon usage of B and T cell lines is presented.

METHODS

Cell lines and peptide analysis. 70Z/3.12, CTLL-2, D10, EL4, BW5147, YAC-1, P388D_1 and NIH 3T3 cells were obtained from the American Type Culture Collection (Rockville, MD). 3B3 cells were obtained from Dr. K. Brooks (Michigan State University, East Lansing, MI) and were grown without interleukins as described (36). CTLL-2 and D10 cells were grown with Con A-activated rat spleen conditioned supernatants or with

recombinant IL-2 (Cetus Inc, Emeryville, CA). Radioiodination, Ly-5.1 mAb immunoprecipitation and SDS-PAGE were preformed as described previously (24).

T200 plasmids. Plasmids containing T200 cDNA having none of the alternately-used exons (pLy-5-68) and bearing all three alternate exons (p70Z/3-3) were obtained from Drs. Y. Saga (10) and I. S. Trowbridge (5) respectively.

Oligonucleotide primers. The PCR and RT-PCR primers were made in the Macromolecule Synthesis Laboratory of Michigan State University by the phosphoramidite method with an Applied Biosystems 380B automated DNA synthesizer (Applied Biosystems, Foster City, CA). The P2 and P9 primers used to amplify the region of alternate exon use in T200 cDNA are described in Table I. Primer sequences were selected on the basis of the known sequence of T200 cDNA as described previously (5), and the expected restriction enzyme fragment size was predicted from the sequence data.

Polymerase chain reactions. The PCR protocol was adapted from methods previously described (31-34). The RT-PCR method used was a modification of a method of Drs. J.L. Yang, J. McCormick and V. Maher (personal communication). cDNA was prepared by addition of reverse transcriptase (M-MLV, Bethesda Research Laboratories, Inc., Gaithersburg, MD) to a pellet of 1000 to 3000 cells in 50 μl of 1X reverse transcription buffer containing 2% Triton X-100, 5 μg BSA, 500 μM spermidine, 36 units RNasin, 500 μM dNTPs and 0.5 μg anti-sense PCR oligonucleotide or oligo(dT)12-18 (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) as the RT primer. The mixture was incubated for 90 min at 37°C and one tenth of the resulting cDNA was amplified by 35 PCR cycles with 2.5 units Taq polymerase, 400 ng sense and anti-sense primers and 500 μM dNTPs in 1X Taq

Table I. PCR Primers Used to Amplify the Region of Alternate Exon Use in T200 cDNA.

PRIMER	ORIENTATION	EXON	SEQUENCE (base position.restriction site)
P2	SENSE	Ex-2	GGATCCCCTTCTGGACACAGAAGTCTTTGT (163-192[a], BamHI[b])
P9	ANTISENSE	Ex-9	GAATTCACAGTAATGTTCCCAAACATGGC (765-737, EcoRI)

a. Base position given according to cDNA clone p70Z/3-3 (5).

b. Restriction sites were incorporated into the 5' end of the primers.

buffer (50 mM KCl, 10 mM Tris-HCl pH 9.3, 3 mM $MgCl_2$ and 0.1% w/v gelatin). One PCR cycle consisted of incubations of 1 min at 94°C, 1 min at 50°C and 2 min at 72°C using a DNA Thermal Cycler (Perkin Elmer Cetus Inc., Norwalk, CT). The amplified products were analyzed by 2% agarose (Bethesda Research Laboratories) electrophoresis and visualized with ethidium bromide using a 123 ladder (Bethesda Research Laboratories) as the size standard.

Restriction enzyme analysis of PCR and RT-PCR products. Amplified fragments were gel-purified by electroelution in dialysis bags and analyzed by restriction enzyme digestion. NciI (Boehringer Mannheim Biochemicals, Inc., Indianapolis, IN), BanI and HinfI (New England Biolabs Inc., Beverly, MA) were used according to the manufacturer's protocols. The fragments were analyzed by 2% agarose electrophoresis as described above.

RESULTS

Strategy for PCR using plasmid T200 cDNA. The synthetic oligonucleotides, P2 (30 nucleotides, with a 5' BamHI site) and P9 (29 nucleotides, with a 5' EcoRI site) were prepared (Table I) which flanked the region of the T200 cDNA containing the three known alternately-used exons (Figure 1; Ex-4, 5 and 6). These primers were designed to have minimum possible mispriming sites and to yield PCR products for each of the eight possible combinations of 0-3 exons having unique size and restriction enzyme cleavage sites. PCR amplification of a plasmid containing T200 cDNA having none of the alternately-used exons (pLy-5-68; 10), yielded a 186 bp product (Figure 2B), while a second plasmid

containing a distinct form of T200 cDNA bearing all three alternate exons (p70Z/3-3; 5) resulted in the amplification of a 603 bp product (Figure 2H, depicted in Figure 1). These corresponded exactly to the predicted sizes for the zero and three alternate exon forms, respectively.

T200 mRNA phenotyping in lymphoid cells using RT-PCR. We then amplified T200 cDNA from 1000 to 3000 lymphoid cells using RT-PCR. The method involved the use of viral reverse transcriptase (M-MLV) to make cDNA copies from mRNA, with subsequent use of selective primers to amplify T200 cDNA. cDNA was prepared from cell lines that were expected, on the basis of T200 mAb immunoprecipitation and SDS-PAGE protein analysis, to utilize none of the alternate exons (BW5147; M_r = 180 kDa, Figure 3C), all three alternate exons (70Z/3.12; M_r = 220 kDa, Figure 3E), or multiple combinations of alternate exons (EL4 and 3B3; Figure 3B and D). Reverse transcriptase (M-MLV) was used with the PCR-primer (P9) as a RT primer and added to a pellet of 1000 to 3000 cells. The PCR primers and Taq polymerase were added after 90 min incubation and 35 cycles of the PCR reaction commenced. After determining the critically important optimal conditions of buffers, cycle temperature and time, purification of mRNA was found to be unnecessary prior to production of T200 cDNA.

RT-PCR resulted in amplification of 186 bp products for BW5147 and 603 bp product for 70Z/3.12 (Figure 2D and G, respectively). RT-PCR of EL4 cells revealed approximately 186 and 330 bp products suggestive of zero and one alternate exon forms (Figure 2E). RT-PCR of the 3B3 pre-B cell line yielded amplified products of approximately 186, 330, 460 and 603 bp (Figure 2F), consistent with the simultaneous presence in a single cell line of T200 mRNA coding for zero, one, two and three alternate exon forms, and raising the possibility of multiple forms within each alternate

Figure 2. PCR of T200 cDNA-containing plasmids pLy-5-68 and p70Z/3-3 and RT-PCR of cell lines. Ethidium bromide-stained agarose gel electorphoresis of PCR amplification products of T200 cDNA in plasmids as template; B, pLy-5-68 (no alternate exons); and H, p70Z/3-3 (three alternate exons). Results of RT-PCR with P2/P9 are for the following cells: C, NIH 3T3 (negative control); D, BW5147 (T cell lymphoma); E, EL4 (T cell thymoma); F, 3B3 (B cell lymphoma); and G, 70Z/3.12 (pre-B lymphoma). A size marker (123 ladder) is in lane A.

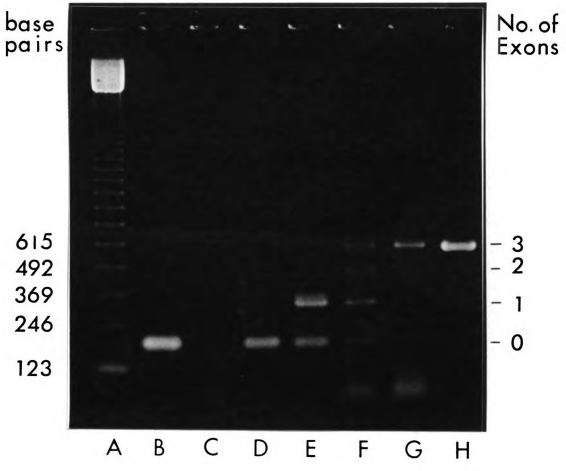

Figure 2.

Figure 3. Immunoprecipitation and SDS-PAGE analysis of T200 glycoprotein. Select cell lines used in this study were radioiodinated, lysed in detergent and immunoprecipitated with anti-Ly-5.1 (B to E) or unimmunoprecipated (A and F) for M_r analysis on an SDS-gradient polyacrylamide gel. A, EL4 whole cell lysate; B, EL4 T200; C, BW5147 T200; D, 3B3 T200; E, 70Z/3.12 T200; F, whole cell lysate of splenocytes. The position of size markers is given in kDa .

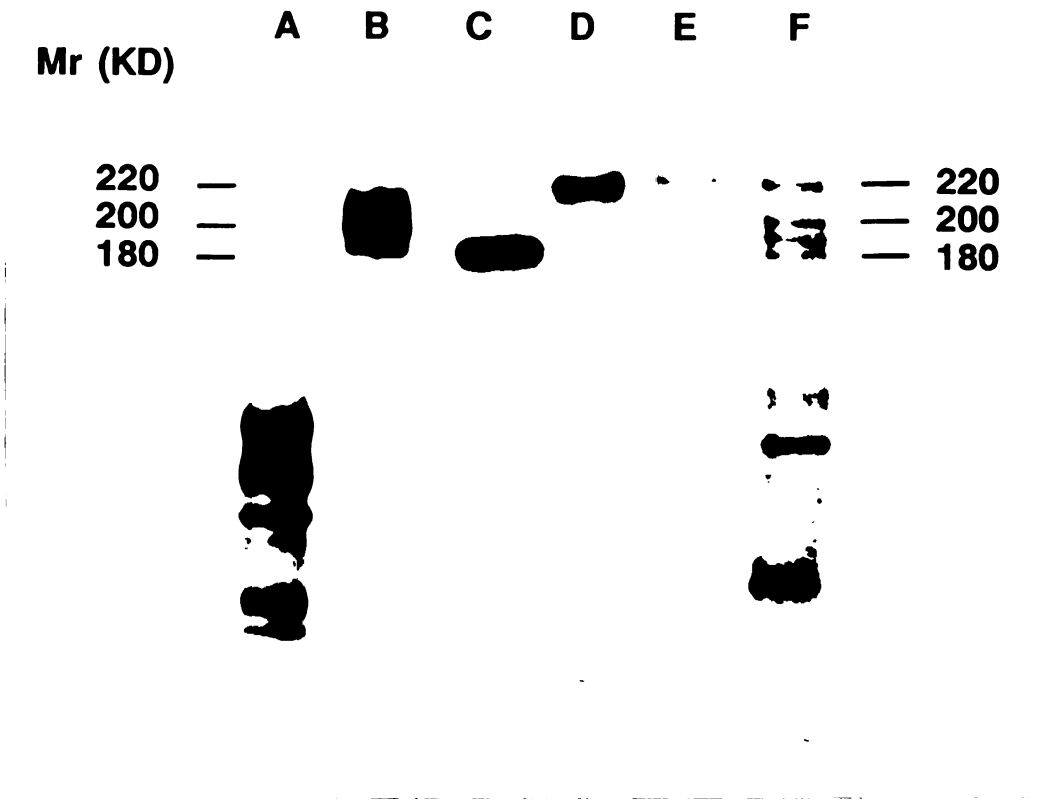

Figure 3.

exon group. NIH 3T3 cells (Figure 2C) were used as a negative control throughout the experiments.

Restriction enzyme confirmation of lymphoid cell T200 alternate exon use. That the amplified plasmid products were derived from T200 cDNA was verified by restriction enzyme digestion mapping of sites unique to each PCR product. The details of the strategy for PCR and restriction enzyme analysis of each amplified product are shown in Table II. The number and nature of alternate exons expressed in plasmid T200 cDNA could be readily determined from the predicted size of PCR-amplified products before and after restriction enzyme treatment with NciI (and other enzymes). P2/P9 primer-directed PCR-amplified products were gel-purified and treated with NciI, with the results shown in Figure 4. As predicted, the PCR product derived from p70Z/3-3 T200 cDNA with all three alternate exons (Figure 4L) was cleaved to fragments of 48, 243 and 312 base pairs (Figure 4K), while the product from the pLy-5-68 T200 cDNA having none of the alternately-used exons was digested with BanI but resisted digestion with NciI (data not shown). Digestion with other restriction enzymes further confirmed the identity of the amplified fragments (data not shown).

To determine and confirm the number and identity of the alternate T200 exons used by each of several lymphoid cell types, restriction enzyme mapping of the RT-PCR-amplified products from 70Z/3.12, BW5147, EL-4 and 3B3 cells was carried out. The usefulness of the RT-PCR method in producing and amplifying T200 cDNA was further confirmed by analysis of 3B3 cells which simultaneously contained T200 mRNA coding for zero, one, two and three alternate exon forms of T200 cDNA. Each RT-PCR amplified product of 3B3 cells was gel-purified and further characterized by size and NciI restriction enzyme mapping (Figure 4, Table II).

Table II. Analysis of alternative exon use and identification of RT-PCR products[a].

Alternate Exons	Product (bp)	NciI Fragments Predicted	Alternate Exon Observed BW5147	EL4	3B3[b]	70Z
Ex-4+5+6	603	48, 243, 312			++[c]	+++
Ex-4+5	462	150, 312			++	
Ex-4+6	456	213, 243				
Ex-5+6	474	48, 183, 243			++	
Ex-4	315	315[d]			+[e]	
Ex-5	333	150, 183		++	++	
Ex-6	327	84, 243				
none	186[f]	186[d]	+++	++	++	

a. Data are typical of at least two experiments with each cell line.

b. Electrophoresis data are shown in Figure 4.

c. Expression of alternate exon forms was determined by the size of RT-PCR products and NciI digested fragment(s), which were identical to those predicted from sequence data, within the resolution of the technique (± 10 bp). Key: +++, major form; ++, moderately expressed form; +, minor form. Blank spaces indicate that these forms were not observed or identified.

d. No change.

e. A very small amount of the one-exon form resisted digestion by NciI and was shown to contain Ex-4 by HinfI digestion.

f. The form without alternative exons was further identified by BanI digestion.

Figure 4. NciI and BanI restriction enzyme mapping of gel-purified products of RT-PCR of T200 alternate exon forms in 3B3 cells. Pairs of digested and undigested fragments are as follows: B, zero-exon form treated with BanI; C, zero-exon form: D, one-exon form treated with NciI; E, one-exon form: G, two-exon form treated with NciI: H, two-exon form: I, three-exon form treated with NciI; J, three-exon form: K, p70Z/3-3 product treated with NciI; L, PCR-amplified plasmid p70Z/3-3 product. The position of the faint, 48 bp fragment in Lanes G, I and K is indicated. Size standards, A, F and M. Fragment sizes are given in Table II.

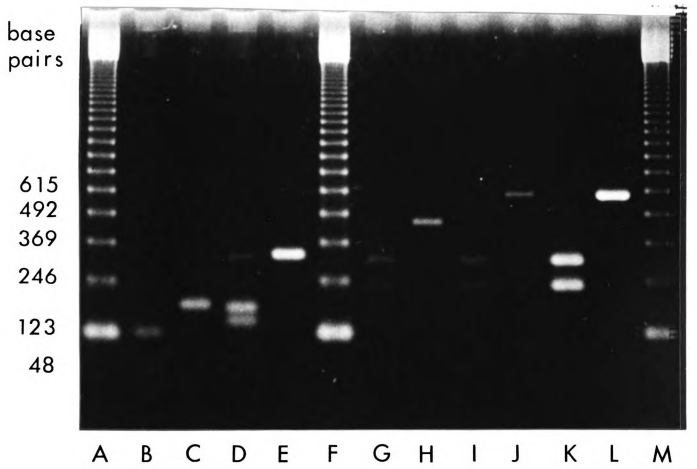

Figure4.

Digestion of the 3B3 three alternate exon form (Figure 4J) produced fragments (Figure 4I) which were indistinguishable in size to those produced by digestion of the p70Z/3-3 product, indicating the presence of one three-exon form, Ex-4+5+6 (Table II). In contrast, digestion of the two exon form (Figure 4H) produced five fragments (Figure 4G) which were indistinguishable in size to those predicted for the digestion of a mixture of two distinct two-exon forms containing Ex-4+5 and Ex-5+6. Digestion of the one-exon form (Figure 4E) produced two fragments (Figure 4D) which matched the size of those predicted for the digestion of a product containing Ex-5, along with a very small amount of NciI-resistant material consistent with a form containing Ex-4 (further confirmed by HinfI digestion). Finally, the zero-exon form of 3B3 cells (Figure 4C) was resistant to NciI digestion (data not shown) and was digested with BanI into fragments which agreed with the predicted size (59 and 127 bp) for the zero-exon form (Figure 4B). The BanI site is created at the junction between Ex-3 and 7 when Ex-4, 5 and 6 are not present. Hence, multiple forms for some of the alternate exon groups were observed, with six of the eight possible T200 alternate exon forms detected. Only the Ex-6 one-exon form and the Ex-4+6 two-exon form were not apparent.

The gel-purified zero alternate exon product of BW5147 cells (186 bp) resisted digestion with NciI but was cleaved into two appropriate size fragments by BanI which were indistinguishable to those shown in Figure 4B. Similarly, the amplified product of 70Z/3.12 cells was cleaved by NciI to fragments having sizes consistent with the expression of all three alternate exons as shown in Figure 4K and I.

Restriction mapping of gel-purified RT-PCR products from EL4 cells (from Figure 2E) revealed a zero alternate exon form; a one-exon form (Ex-

5) and very small amounts of a two exon form which could not be gel-purified in amounts adequate for further characterization.

Survey of T200 alternate exon use in other lymphoid cells. After demonstrating that the combination of RT-PCR and restriction enzyme analysis was suitable for precise identification of the alternate exon expression of T200 cDNA, we undertook to estimate the degree of heterogeneity of T200 alternate exon use in a variety of lymphoid and myeloid cells (Figure 5). T cell lines predominantly expressed the zero-exon form as well as smaller amounts of one-exon forms (Lanes D, F-J). Among T cell lines, BW5147 cells (D) almost exclusively expressed the zero-exon form, while YAC-1 cells (F) also expressed a small amount of an intermediate form (390 bp). This intermediate form was also observed in other T cell lines (Lanes G-I), P815 mastocytoma cells (C) and the $P388D_1$ macrophage cell line (B). The zero alternate exon T200 isoform also predominated in P815 and $P388D_1$ cell lines. While 70Z/3.12 cells exhibited predominant expression of the three exon form, smaller forms, including a zero-exon form, similar to those in 3B3 cells were evident in small amounts.

DISCUSSION

The method described herein permitted selective amplification of a small amount of mRNA coding for a portion of a complex lymphoid cell surface glycoprotein that is generated by a process involving alternative exon shuffling. The amplified products were identified by standard agarose electrophoresis and individually mapped by restriction enzymes. This method is a significant advance of PCR technology and to our knowledge is the first report describing the amplification of mRNA

Figure 5. Survey of T200 alternate exon use in lymphoid and myeloid cells as demonstrated by RT-PCR. The cells analyzed were as follows: B, P388D_1 (macrophage line); C, P815 (mastocytoma); D, BW5147 (T cell lymphoma); F, YAC-1 (T cell lymphoma); G, D10 (T_{h2} helper cell line); H, HT-2 (T cell line); I, CTLL-2 (cytotoxic T cell line); J, EL-4 (T cell thymoma); L, 3B3 (B cell lymphoma); M, 70Z/3.12 (pre-B lymphoma); A, E, and K, size standards (123 ladder).

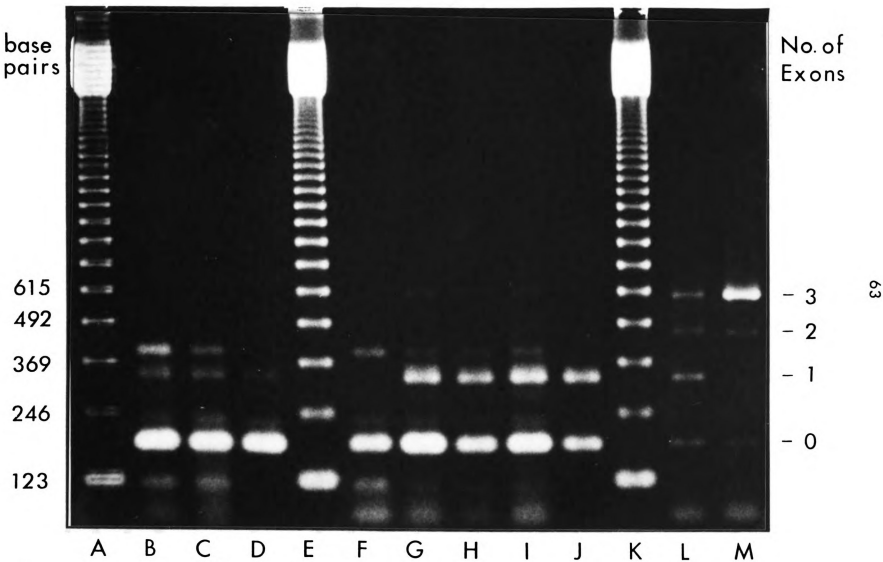
base pairs
No. of Exons
615
492
369
246
123
3
2
1
0
A
B
C
D
E
F
G
H
I
J
K
L
M

Figure 5.

containing alternately used exons without prior purification of RNA. This makes RT-PCR more sensitive and direct in the analysis of mRNA derived from small numbers of cells.

The features of this protocol that enabled direct cDNA production from mRNA without prior purification include the addition of reverse transcriptase directly to cell pellets (1000 to 3000 cells) with detergent, nucleotides and buffer. An aliquot of the resultant cDNA was then added to Taq polymerase, PCR primers, and a specific buffer system for PCR amplification. Other reagents and conditions that appear to contribute to the success of direct mRNA phenotyping using RT-PCR include the use of a pH of 9.3 for the PCR reaction, the use of primers approximately 30 nucleotides in length, and optimization of reaction temperatures and number of cycles. We observed that RT-PCR proceeded only in a small range around these optimal conditions, and expect that these reaction conditions may require adjustment for different template and primer systems. In addition, the use of one of the PCR primers for RT initiation proved superior to the use of oligo-dT (data not shown). Primer sequences were designed in positions which have no more than five matches out of ten 3′ nucleotides and no homology in the last few 3′ nucleotides in order to eliminate mispriming artifacts. The primers were also designed with 5′ restriction sites for subsequent subcloning.

RT-PCR was chosen for these studies because no other suitable method was available for determining T200 alternate exon usage in small numbers of cells. RT-PCR permits amplification of mRNA/cDNA from a few hundred cells, enabling visualization on ordinary agarose gels. This capability was felt to be important to advance current understanding regarding the relationship between T200 structural heterogeneity and its possible role

in the differentiation and function of various lymphoid and myeloid cells. We found mRNA phenotyping to be superior to Northern blot transfer analysis in the determination of T200 alternate exon expression (data not shown). T200 mRNA is large (5.5 kb), which can impair the stability and resolution of closely related isoforms. In this report, the RT-PCR method identified and distinguished apparent major and minor T200 alternate exon isoforms as well as complex mixtures, while such isoforms could not be clearly distinguished in Northern blots. In addition, the products of PCR are obtained in sufficient quantity for detailed analysis by restriction mapping, direct sequencing and subcloning.

Although analysis of lymphoid cell lines revealed considerable heterogeneity in T200 alternate exon use, several predictable patterns were observed. As predicted from SDS-PAGE analysis, cells expressing the lowest M_r (180 kDa) isoform of T200 glycoprotein (e.g., BW5147) generally showed a predominance of the zero alternate exon T200 isoform by RT-PCR analysis, while cells expressing the highest M_r isoform (B cell lines) expressed the highest alternate exon form (Ex-4+5+6). The pattern of alternate exon use was more complex in cell lines expressing several intermediate surface T200 glycoprotein isoforms (190 to 215 kDa). For example, EL4 cells expressing both one-exon and zero-exon forms by RT-PCR exhibited several T200 glycoprotein isoforms by SDS-PAGE, with M_r of 190-210 kDa (Figures 2, 3 and 5).

Correlation of T200 glycoprotein expression and alternate exon use in the 3B3 B cell line was even more complex. These cells exhibited mRNA yielding PCR products of zero- to three-exons and protein isoforms with M_r of 200-220 kDa. It is proposed that these higher M_r protein isoforms contained proteins translated from all of the alternate exon products.

The apparent M_r of a particular T200 isoform as determined by SDS-PAGE may have been more a function of the extent and complexity of glycosylation than of the number of alternate exons present. This possibility is supported by the finding that T200 isolated from B cells grown in the presence of tunicamycin had an apparent M_r of only 190 kDa, compared to 220 kDa in the absence of tunicamycin (8). Similarly, thymocyte T200 from tunicamycin-treated cells had an apparent M_r of only 160 kDa, while untreated thymocyte surface T200 had a M_r of 180 kDa. Consequently, for the 220 kDa isoform of B cell T200 using all three alternate exons, glycosylation accounts for about 30 kDa (14%) of apparent M_r while each alternate exon would only account for about 5 kDa (2%) of apparent M_r. If only peptide structural differences accounted for apparent M_r differences observed on SDS-PAGE, RT-PCR results for 3B3 cells would have predicted four T200 isoforms. That only higher M_r forms were observed suggests that the lower alternate exon isoforms are extensively glycosylated resulting in an apparent M_r between 200 and 220 kDa. Despite the apparent existence of multiple alternate exon isoforms, the major T200 glycoprotein expressed on 3B3 cells is a 220 kDa form. The M_r heterogeneity of T200 observed by SDS-PAGE is therefore contributed significantly to by variations in glycosylation (glycoforms), which may obscure minor differences due to alternate exon usage (protein isoforms). It is also possible that alternate exon use directs the extent and complexity of T200 glycosylation.

An alternative explanation for the above phenomena which requires further study is that not all of the mRNA isoforms are translated into cell surface T200 glycoproteins. It is also possible that unequal amplification of cDNA representing different alternate exon isoforms

occurred after reverse transcription, leading to the disproportionate appearance of minor forms. Since the precise relationship between the number of copies of mRNA for a particular isoform and the intensity of the band seen after RT-PCR is not known, we are unable to estimate the degree to which unequal amplification of cDNA contributed to variations in the amounts of final products produced in these experiments.

The observation of small amounts of intermediate size RT-PCR products (Figure 5) requires further study. These products (e.g. 390 bp, Figure 5B,C,F) do not correspond exactly to the predicted sizes for RT-PCR products of known alternate exon isoforms of T200 (approximately 330 or 460 bp). Of particular interest is the possibility that this intermediate form may include an as yet unproven additional alternate exon as suggested for a macrophage cell line by Saga et al. (7). The presence in trace amounts of this intermediate form in most of the T cell lines studied by RT-PCR may not have been previously identifiable using other less sensitive methods. Similarly, the occurrence of a minor product at approximately 115 bp in some cell lines (Figure 5B,C,F) also suggests the possible lack of an additional alternately used exon of approximately 70 bp (possibly Ex-7). The faint bands of lowest size are believed to represent excess primers. Mispriming artifacts may also be responsible for the generation of products of intermediate size. Sequence analysis should allow resolution of these possibilities.

The significance of the multiple T200 isoforms occurring in a particular cell line remains to be determined. The possibility that each mRNA isoform is translated into a functional member of the T200 membrane glycoprotein family further suggests that T200 alternate exon usage contributes to the generation of individual T200 isoforms which may have

distinct roles in the function of individual lymphoid cells. RT-PCR will be useful in determining T200 isoform expression in small numbers of lymphoid cells or even individual lymphocytes (35). The general method will also be useful in the study of mRNA and protein expression in a wide variety of other systems involving immune cell differentiation and function.

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Chapter Three

DEVELOPMENTAL EXPRESSION OF CD45 ALTERNATE EXONS IN MURINE T CELLS: EVIDENCE OF ADDITIONAL ALTERNATE EXON USE[1]

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Running Title: CD45 Alternate Exon Use

FOOTNOTES

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[2] Address correspondence to: Dr. W. J. Esselman, Department of Microbiology & Public Health, Michigan State University, 339 Giltner Hall, East Lansing, MI 48824-1101.

[3] Abbreviations: bp, base-pairs; Ex, exon; LCA, leukocyte-common antigen (one of several alternative designations for CD45); IEL, intraepithelial lymphocytes; LN, lymph node; M-MLV, Moloney murine leukemia virus; PCR, polymerase chain reaction; P2, P4, P5, P6, and P9, synthetic oligonucleotide primers from a point beginning within CD45 exons 2, 4, 5, 6 and 9 respectively; RT, reverse transcriptase; RT-PCR, reverse transcription-polymerase chain reaction.

ABSTRACT

The CD45 family of lymphoid and myeloid cell surface glycoproteins exhibits cell lineage-associated structural heterogeneity arising in part from alternate 5'-exon shuffling. Previous studies from other investigators about exons involved in the final glycoprotein structure have provided evidence of alternate exon use only for exons 4, 5 and 6. However, our prior RT-PCR data on CD45 alternate exon use implied the presence of additional alternate exons. We employed the reverse transcription-polymerase chain reaction (RT-PCR), Southern blotting using exon-specific or exon splice junction-specific oligonucleotide probes, and direct DNA sequencing of RT-PCR products to provide evidence that exons 7 and 8 are also alternately used. In order to estimate the relative amount of mRNA for each CD45 isoform present in lymphocytes, the effect of template length on RT-PCR amplification efficiency was tested by using radiolabeled primers and quantitating products at intervals of five PCR cycles. Smaller CD45 cDNA isoforms amplified more efficiently than larger ones when RT-PCR exceeded 25 cycles, but the amplification was proportional when PCR was limited to 20-25 cycles. Under these conditions, examination of CD45 isoform expression revealed that Stage I thymocytes ($CD4^-CD8^-$) expressed only trace amounts of minus-one [Ex(-1)] and minus-two exon [Ex(-2)] isoforms, with no other isoforms detected. A Ex(-2) isoform was also detected in all thymocytes and T cells analyzed but only in trace amounts. Stage II thymocytes ($CD4^+CD8^+$) expressed high and approximately equal amounts of Ex(-1) and zero alternate exon [Ex(0)] isoforms, with minor quantities of one exon [Ex(1)] and two exon [Ex(2)] isoforms. Among stage III thymocytes, both $CD4^+CD8^-$ and $CD4^-CD8^+$ cells expressed significant quantities of only the Ex(-1) and Ex(0) isoforms.

Comparison of CD45 alternate exon use in resting $CD4^+$ and $CD8^+$ lymph node T cells revealed evidence of divergent exon use, with $CD8^+$ cells expressing detectible quantities of an Ex(2) isoform, while producing proportionately less of the Ex(-1) and Ex(0) isoforms. Examination of allogeneically activated T cells revealed that the $CD4^+$ BC-3 helper T cell clone expressed less of the Ex(1) isoform, while the $CD8^+$ 8.2.2 CTL clone increased its production of higher alternate exon isoforms, including Ex(2) and Ex(3) isoforms. Our evidence of the alternate use of exons 7 and 8 suggests that at least five alternate exons exist in the CD45 glycoprotein family. Analysis of isoform expression among thymocytes and T cells suggests that shuffling of CD45 alternate exons occurs in an organized and predictable sequence during the process of T cell maturation and activation.

INTRODUCTION

The CD45 family of lymphoid and myeloid cell membrane-associated glycoproteins (also termed LCA, T200, and Ly-5) has attracted considerable interest recently as a potential participant and regulator of cellular activation and proliferation (1-3). Molecular heterogeneity in the CD45 glycoprotein family is known to result both from variations in the N-terminal extracellular peptide region and in glycosylation (4-10). Protein heterogeneity has been attributed to variable incorporation of N-terminal peptide sequences coded for by up to three alternately transcribed exons, designated Ex-4, 5 and 6 (Figure 1; 4, 6). These exons share in common a large number of potential O-linked glycosylation sites (11, 12) suggesting that alternate exon use directs the nature and extent of glycosylation of the extramembranous portion of CD45.

The constant cytoplasmic domain of CD45 possesses two tandemly repeating subdomains homologous to placental protein tyrosine phosphatase-

Figure 1. Illustration of the amplified region of CD45 and the relative position of RT-PCR primers and probes for Southern hybridization. The Ex-8/Ex-3 junction-specific probe is not shown. The thick line represents the alternate exon region and exons 7 and 8. Based upon published sequences (6), NciI has one cleavage site in each of exons 5 and 6 (nucleotide no. 473 and 521); HinfI has one cleavage site in each of exon 4 and 5 (nucleotide no. 287 and 392).

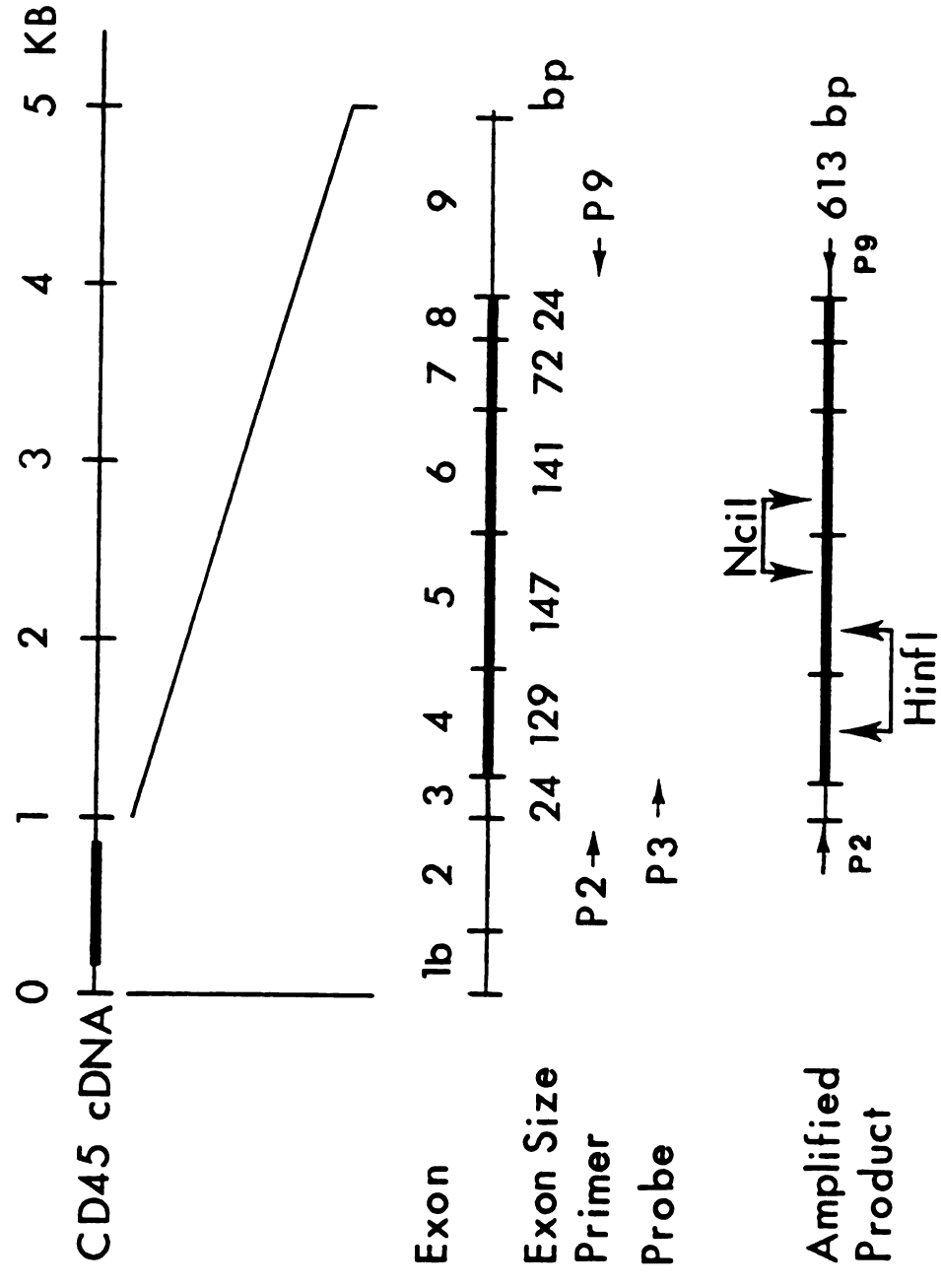
CD45 cDNA
0
1
2
3
4
5 KB
Exon
1b 2 3 4 5 6 7 8 9
Exon Size
24 129 147 141 72 24 bp
Primer
P2
P9
Probe
P3
Amplified Product
NciI
HinfI
P2
P9
613 bp

Figure 1.

1B (PTPase-1B; 13). The confirmation of PTPase activity in human CD45 (14) has prompted models for CD45 function in which the cytoplasmic region is proposed to affect transmembrane signaling through the dephosphorylation of critical tyrosyl residues on other membrane-associated proteins (1, 2). The cytoplasmic region of CD45 appears to be responsible for its biological function in modulating transmembrane signaling for cellular activation and proliferation. It has been proposed that the physical interaction of CD45 with other membrane-associated proteins to facilitate CD45/PTPase-mediated dephosphorylation reactions is determined by its extracellular N-terminal structure (15). This hypothesis was supported by the evidence for cell type-specific heterogeneity of CD45 expression (5, 7, 15, 16) and by evidence for modulation of Ca^{2+} flux by cross-linking antibodies (1). Further knowledge of CD45 isoform expression in specified lymphocyte populations may provide clues to the structure-function relationships in the CD45 glycoprotein family.

Murine CD45 (Ly-5) alternate exon use in individual cell populations has been studied using a limited number of characterized mAbs (17) specific for peptides coded by Ex-4 (14.8) and Ex-5 (C363.16A). Human CD45 has been studied using mAbs with specificity for certain of the alternate exons, as well as a mAb specific for the CD45 isoform lacking exons 4, 5 and 6 (UCHL1; 18). The use of specific mAbs has allowed identification of cellular subsets expressing individual exons but is limited in the recognition of CD45 isoforms containing multiple exons. This limitation is also true for Northern hybridization analysis using exon-specific probes. In addition, it is possible that variations in glycosylation of some CD45 isoforms could prevent antibody binding, thus

rendering mAb reactivity analyses unreliable.

We recently showed RT-PCR to be a sensitive and reliable alternative in the characterization of CD45 isoform expression in murine lymphoid cell lines (9). In the same report, we observed amplified products whose sizes on agarose electrophoresis suggested that additional alternately used exons may exist in the CD45 glycoprotein family. In this report, we present evidence that Ex-7 and Ex-8 are additional CD45 alternate exons, and that lymphocytes of the T cell lineage generally include increasing numbers of alternate exons during the processes of cellular maturation and activation.

METHODS

Monoclonal antibodies. Monoclonal clonal antibodies used in this study were 3.168 (anti-CD8; 19), AD4 (anti-CD8; 20), and RL172 (anti-CD4; 21).

Cells and cell lines. 70Z/3.12 cells were obtained from the American Type Culture Collection (Rockville, MD). 3B3 cells were obtained from Dr. K. Brooks (Michigan State University). Subsets of thymocytes and peripheral lymph node T cells were obtained from 8-10 week-old C57BL/6J mice by a previously described method (22). Cells were at least 95% pure with respect to a given phenotype. The alloreactive $CD8^+$ CTL clone, 8.2.2, was derived by limiting dilution from a mixed lymphocyte culture (MLC) generated by reacting C57BL/6J splenocytes with irradiated DBA/2 splenocytes. The alloreactive $CD4^+$ T cell clone, BC-3, was obtained from an MLC containing Balb/c splenocytes and irradiated C57BL/6J splenocytes. IEL were isolated as previously described (23). Briefly, small intestines from 4-8 C57BL/6J mice were cut into 5 mm pieces and stirred at 37°C in HBSS containing 0.1 mM EDTA. Supernatant suspensions containing

lymphocytes and epithelial cells were then subjected to centrifugation through a 44/67.5 percent Percoll gradient. Cells at the interface were collected and further purified by panning with an anti-CD8 mAb to a final purity of 88-95% $CD8^+$ cells.

T lymphocytes used for RT-PCR were washed twice in PBS, with 30,000 cells transferred into a 500 μl tube, pelleted and supernatants decanted. Cell pellets were stored at -20°C and thawed just before adding the RT reagents. Pellets thus prepared provided reproducible results for RT-PCR for at least 9 months.

Oligonucleotide primers. The RT, PCR and sequencing primers, and exon-specific oligonucleotide probes (Figure 1 and Table I) were made in the Macromolecular Synthesis Facility at Michigan State University. For 5'-end labeling, 40 pmol of oligonucleotides were added to a final volume of 50 μl of 1X kinase buffer with 20 U T4 DNA polynucleotide kinase (New England Biolab., Beverly, MD) and 300 μCi of $\gamma[^{32}P]$-ATP (ICN, Irvine, CA). This mixture was incubated at 37°C for 45 min. The unincorporated nucleotides were removed using a G-25 Sephadex (Pharmacia, Piscataway, NJ) spun column.

Reverse transcription-polymerase chain reaction. The RT-PCR protocol was performed as described previously (9) with modifications. Briefly, cDNA was prepared by adding 200 units M-MLV reverse transcriptase (BRL, Gaitherburg, MD) to a pellet of 30,000 cells in 20 μl of 1X RT buffer containing 2% Triton X-100, 2 μg BSA (BRL), 200 μM spermidine (Sigma, St. Louis, MO), 36 U RNasin (Promega, Madison, WI), 200 μg dNTPs (BMB, Indianapolis, IN), and 0.2 μg unlabeled anti-sense PCR primer (P9) as the RT primer. The mixture was incubated 60 min at 37°C. Approximately 5 μl of the reaction mixture was used directly as template for amplification in

Table I. Oligonucleotides used for amplification of the alternate exon region in CD45 cDNA and the probes used for Southern hybridization.

OLigonucleotide	Orientation	Sequence (base position)
P2	Sense	CTTTGGATCCGCCCTTCTGGACACAGAAGT(157-186[a])
P9	Antisense	GGCGGAATTCACAGTAATGTTCCCAAACAT(769-740[a])
P3	Sense	AACACCTACACCCAGTGATG(202-221[a])
J8-3	Antisense	GCTTCGTTGTGGTAGCATCACTGGG(725-711+221-212[a])

[a]Base position given according to cDNA clone p70Z/3-3 (6).

a total volume of 50 μl containing 5' end-labeled primers (P2 and P9) in addition to the regular PCR components (9). The reaction was performed in a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT) for the number of cycles specified. Each cycle consisted of 40 sec at 94°C for denaturation, 15 sec at 55°C for annealing and 30 sec at 72°C for elongation. The first cycle was preceded by a 5 min incubation at 94°C and the last followed by 6 min at 72°C. Approximately one-fifth of the total amount of each amplified product was analyzed by 8% native polyacrylamide gel electrophoresis (PAGE) and visualized either by autoradiography or ethidium bromide staining. For determination of amplification efficiency, eight identical RT-PCR reactions were set up, with one reaction stopped at the end of every 5 PCR cycles. After PAGE and autoradiography, each band was excised and the radioactivity determined by scintillation counting.

For isolation of RT-PCR fragments after autoradiography or direct viewing under UV light, the desired fragment was excised and placed in dialysis tubing for electroelution. The eluted DNA was further concentrated using a Centricon-30 tube (Amicon, Danvers, MA). In some cases, eluted DNA was used as template in subsequent PCR.

Enzymatic analysis. The exon composition of purified products was identified by restriction enzyme digestion using NciI, HinfI, and BanI as previously described (9). After digestion, the fragments were separated by 8% PAGE and visualized by autoradiography.

Southern hybridization. Purified DNA fragments were separated using a 1.8% agarose gel and blotted onto a nylon membrane (GeneScreen, DuPont, Boston, MA) in 10X SSPE buffer. After baking under vacuum, the membrane was pre-hybridized at 42°C for three hours in 6X SSPE buffer containing

10X Denhardt's solution, 1% SDS, and denatured salmon sperm DNA (Sigma) at 50 μg per ml. The membrane was then cut to generate two identical membranes. One of the membranes was hybridized overnight with Ex-3-specific probe (P3), the other with Ex-8/Ex-3 junction-specific probe (J8-3), both at $2x10^6$ cpm/ml in 6X SSPE, 1% SDS at their respective hybridization temperature (T_H), 57°C and 73°C. The hybridized membranes were washed twice in 2X SSPE, 1% SDS at room temperature for 20 min and once in 1 X SSPE, 1% SDS at T_H for 5 min.

DNA sequencing of RT-PCR products. About 0.5 μg of the DNA was sequenced according to the protocols by Dr. L. M. Latinwo (Michigan State University, personal communication). All the sequencing reagents were purchased from United States Biochemical Corporation (Cleveland, OH). Briefly, template DNA with 1 pmol ^{32}P end-labeled P9 was boiled 7 min in 10 μl 1X reaction buffer and chilled on ice 5 min. Then the following were added: 1 μl 0.1M DTT, 2 μl labeling mix, 1 μl Mn^{2+} buffer, and 2 μl of Sequenase at 1:4 dilution. Immediately after mixing, 3.5 μl aliquots were pipetted into pre-warmed tubes containing 2.5 μl termination mixture. Tubes were then incubated at 37°C for 5 min and the reaction stopped by the addition of 4 μl stop buffer. An aliquot of 3 μl of the final product was denatured at 85°C for 5 min, chilled on ice and electrophoresed in 6% sequencing gel.

RESULTS

CD45 alternate exon use involving exons 7 and 8. Previous studies have provided evidence for CD45 alternate exon use involving only the translated exons 4, 5 and 6 (4, 6), as well as the untranslated Ex-1a and 1b (4, 24). However, other observations using RT-PCR have indicated the presence of intermediate CD45 RT-PCR products (9), raising the possibility

that additional alternate exons may exist. This possibility was addressed by analysis of $CD4^+$ lymph node T cells from C57BL/6J mice using RT-PCR, Southern hybridization with exon- or junction-specific oligonucleotide probes and direct sequencing of individual bands purified by PAGE.

RT-PCR analysis using P2 and P9 as primers (Figure 2) revealed a prominent "zero" exon isoform as conventionally defined (i.e., lacking Ex-4, 5 and 6). $CD4^+$ lymph node T cells also expressed very low level of one exon isoform. Two additional products having sizes of about 124 and 100 bp were also apparent. The size of the 124 bp product was consistent with a zero-exon isoform that additionally lacked Ex-7 [i.e., a "minus-one" exon isoform; Ex(-1)], while the 100 bp product was of a size consistent with a "zero" exon isoform that also lacked both Ex-7 and Ex-8 [i.e., a "minus-two" exon isoform; Ex(-2)]. Southern hybridization analysis of gel-purified RT-PCR products using probes specific for Ex-3 and the Ex-8/Ex-3 junction supported this interpretation (Figure 3). The Ex-3-specific probe confirmed the CD45 identity of RT-PCR products, since it interacts with an exon of CD45 that is not subject to alternative splicing and was always contained within the sequences amplified by the primers used for these experiments (P2; P9). The Ex-3-specific probe reacted with each amplified band (Figure 3B, lanes 1-3), but not with the negative control sample (lane S), thereby confirming the CD45 origin of each RT-PCR product. The Ex-8/Ex-3 junction-specific probe reacted only with the RT-PCR product immediately below that of the size expected for the Ex(0) isoform of CD45 (Figure 3C, lane 2). The size of this product (124 bp) was as predicted for an isoform lacking Ex-4, 5, 6 and 7. This finding strongly suggested that the product was amplified from a CD45 cDNA species that excised all exons between Ex-3 and Ex-8, including Ex-7.

Figure 2. RT-PCR pattern of CD4+ LN T-cells showing the presence of the 124 bp and 100 bp isoforms. Lane S, 123 marker; lane 1, $CD4^+$ LN T cells.

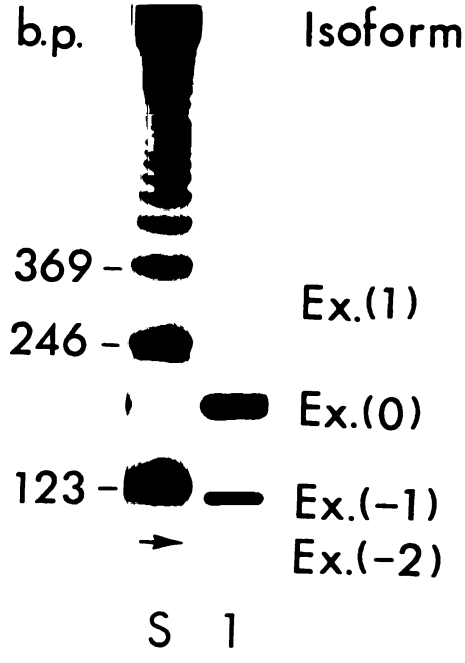

Figure 2.

Figure 3. Southern hybridization analysis of the Ex(0) isoform, and the 124 bp and 100 bp CD45 isoforms of $CD4^+$ lymph node T cells. Lane 1, 100 bp fragment; lane 2, 124 bp fragment; lane 3, Ex(0) form; and lane S, 123 size marker. (A). Electrophoresis of the re-amplified products, stained with ethidium bromide. (B). Southern hybridization with the Ex-3-specific probe, P3. (C). Southern hybridization with the Ex-8/Ex-3 junction-specific probe, J8-3.

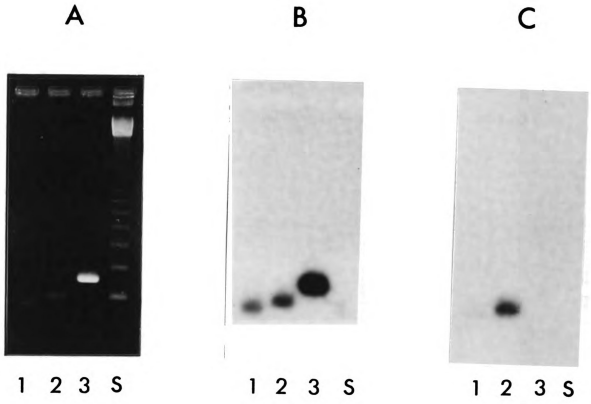

Figure 3.

To further confirm these results, the 124 bp CD45 RT-PCR product was isolated by electroelution and subjected to direct sequence analysis (Figure 4). The result confirmed that the last nucleotide of Ex-3 (nucleotide 221) was followed immediately by the first nucleotide of Ex-8 (nucleotide 711). Thus, the 124 base-pair product resulted from direct splicing of Ex-3 to Ex-8, thereby excluding Ex-7.

The size of the smallest RT-PCR fragment (100 bp) was as predicted for a CD45 isoform lacking all the exons between Ex-3 and Ex-9. The reactivity of this product with the Ex-3 specific probe confirmed its CD45 identity, while its appropriate size and failure to bind the Ex-8/Ex-3 junction probe strongly suggested that the product represented an isoform of CD45 that also lacks Ex-8. The very low level of expression of this product in cells studied thus far has complicated efforts to further confirm the absence of Ex-8 by direct sequence analysis.

CD45 amplification efficiency. Estimation of the relative abundance of the predominant CD45 isoforms expressed in lymphocyte populations was accomplished using radiolabeled primers and comparison of the radioactivity of products derived from RT-PCR. The reliability of this approach required experimental conditions in which each CD45 isoform could be shown to be amplified with comparable efficiency.

The relationship between CD45 cDNA template size and RT-PCR amplification efficiency was analyzed in C57BL/6J $CD4^+$ thymocytes. Use of ^{32}P-labeled primers enabled quantitation of RT-PCR products after every 5 cycles (Figures 5 and 6). Carrying out RT-PCR to 40 cycles resulted in clear evidence of unequal amplification of the CD45 cDNA, with smaller templates [Ex(-1) and Ex(0)] amplified approximately 10-fold more than thelarger Ex(1) template. Amplification of this larger template appeared

Figure 4. Sequencing data showing the absence of exon 7 in the 124 bp fragment isolated from $CD4^+$ lymph node T cells. Exon 3 is fused directly to exon 8. The nucleotide numbers are shown according to the sequence published by Trowbridge, et al. (6).

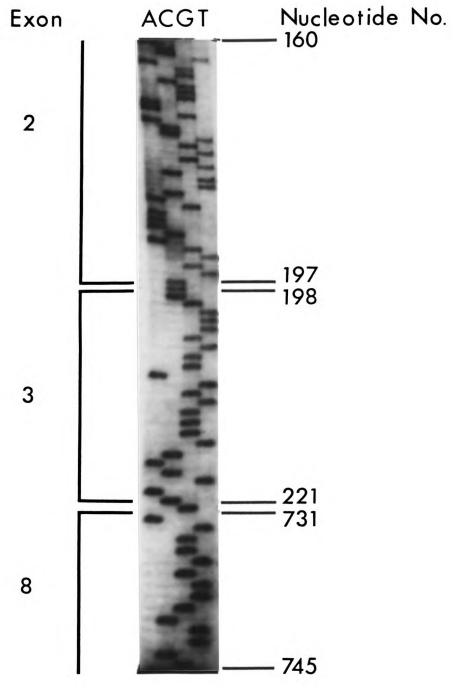

Figure 4.

Figure 5. Autoradiography of the RT-PCR pattern of $CD4^{+}CD8^{-}$ thymocytes at 5 PCR cycle intervals. RT-PCR was stopped at the end of 15th, 20th, 25th, 30th, 35th, and 40th cycle (lanes 1-6, respectively).

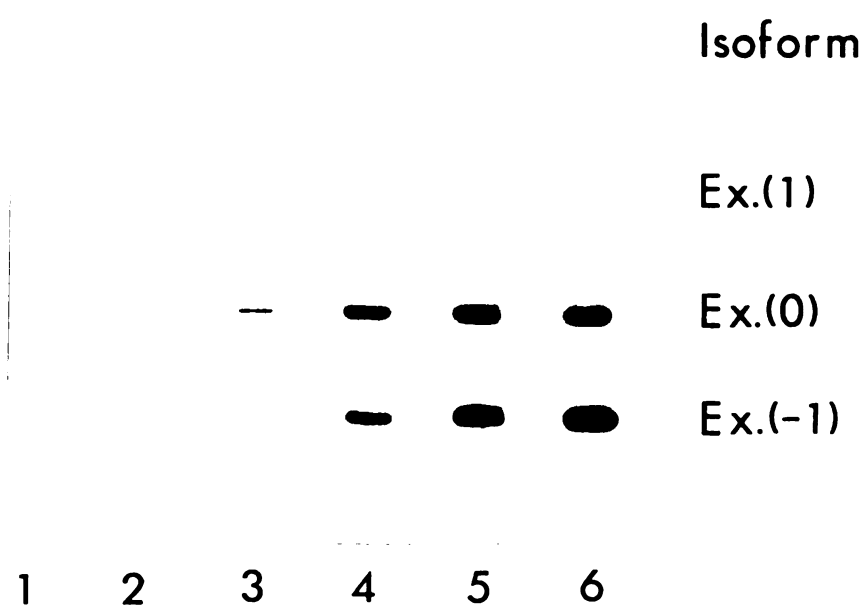

Figure 5.

Figure 6. RT-PCR amplification curves for CD45 isoforms from $CD4^+CD8^-$ thymocytes. Radioactivity (cpm) of the RT-PCR products was plotted against the cycle number at which the reaction was stopped. Ex(1) form, circle; Ex(0) form, triangle; and Ex(-1) form, square.

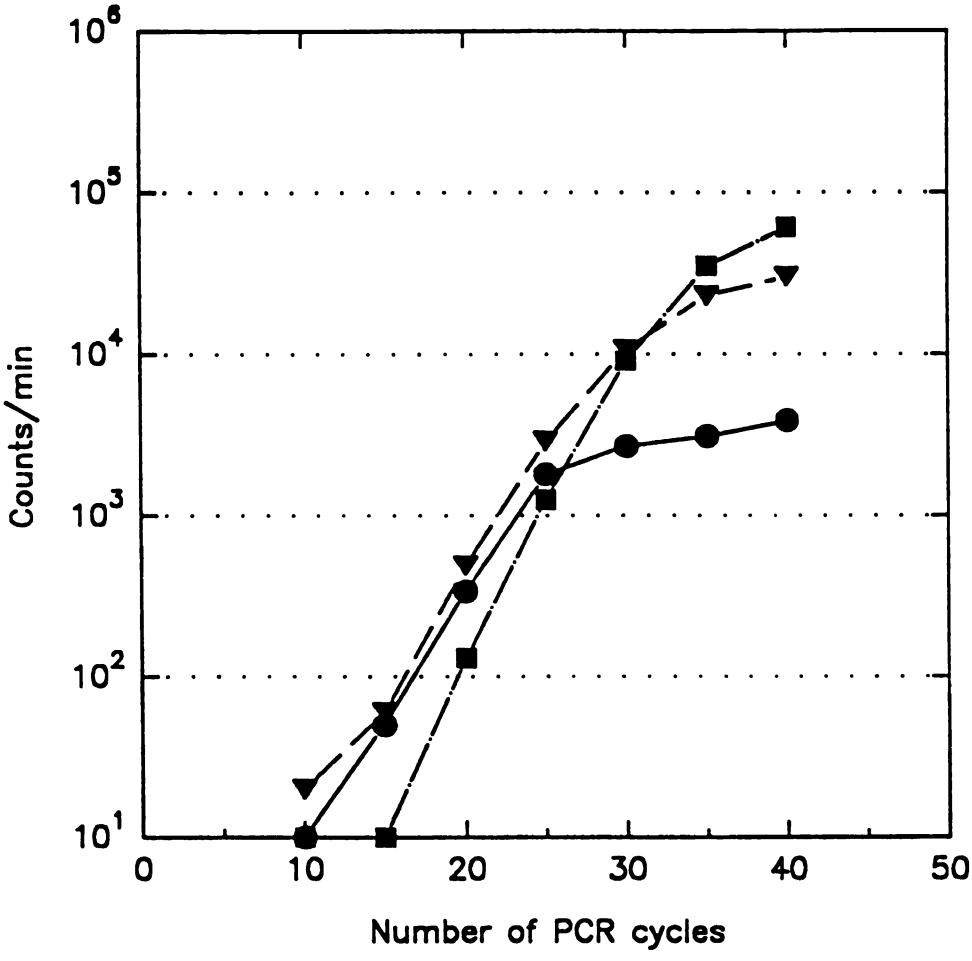
10^6
10^5
10^4
10^3
10^2
10^1
Counts/min
0
10
20
30
40
50
Number of PCR cycles

Figure 6.

to reach saturation at approximately 25 RT-PCR cycles. However, when PCR was limited to 25 cycles or less, each isoform was amplified at comparable rates independent of template size (Figure 6). As a result, RT-PCR was limited to 24 cycles or less to allow band intensity to reflect the amount of CD45 cDNA for each isoform present in the original cell population studied. We observed similar results for several lymphoid cell lines bearing all known CD45 alternate exon isoforms, and in PCR experiments using mixtures of the plasmids p70Z/3 (6) and pLy5-68 (5) as templates (data not shown).

CD45 alternate exon isoform expression in T cell populations. In view of the evidence that CD45 alternate exon use differs in lymphocytes of varying lineage (15, 22), and that such differences may affect the function of this cell surface glycoprotein (25), we employed RT-PCR to analyze CD45 isoform expression among T cells at different differentiation stages and activation states. C57BL/6J cell populations studied included: 1) immature thymocytes (Stage I: $CD4^-CD8^-$; Stage II: $CD4^+CD8^+$); 2) mature thymocytes (Stage III: $CD4^+CD8^-$, $CD4^-CD8^+$); 3) lymph node T cells. In addition, alloreactive $CD4^+$ and $CD8^+$ T-cell clones and IEL were examined.

Figure 7A compares CD45 alternate exon use in immature thymocyte populations. Stage I ($CD4^-CD8^-$) thymocytes (lane 1) expressed only low levels of Ex(-1) and Ex(-2) isoforms. Stage II ($CD4^+CD8^+$) thymocytes (lane 2) expressed much higher amounts of the Ex(-1) isoform, along with even more predominant expression of the Ex(0) isoform and small amounts of Ex(-2), Ex(1) and Ex(2) isoforms. Among mature (Stage III) thymocytes (Figure 7B, lanes 1 and 2), both $CD4^+CD8^-$ and $CD4^-CD8^+$ thymocytes were comparable in their expression of Ex(0) and Ex(-1) isoforms, with higher M_r isoforms not apparent. However, additional alternate exon use patterns were

Figure 7. CD45 RT-PCR pattern of T cell subsets and alloreactive T cell clones. (A). Immature thymocytes: lane S, 123 size marker; lane 1, Stage I ($CD4^-CD8^-$) thymocytes; lane 2, Stage II ($CD4^+CD8^+$) thymocytes. (B). Mature (Stage III) thymocytes and peripheral lymph node T cells: lane S, 123 size marker; lane 1, $CD4^+$ thymocytes; lane 2, $CD8^+$ thymocytes; lane 3, $CD4^+$ lymph node T cells, lane 4, $CD8^+$ lymph node T cells. (C). alloreactive T cell clones: lane S, 123 size marker; lane 1, T_h clone BC-3; lane 2, T_c clone 8.2.2. (D). IEL and control B cells: lane 1, 70Z/3; lane 2, 3B3; lane 3, IEL ($CD8^+$).

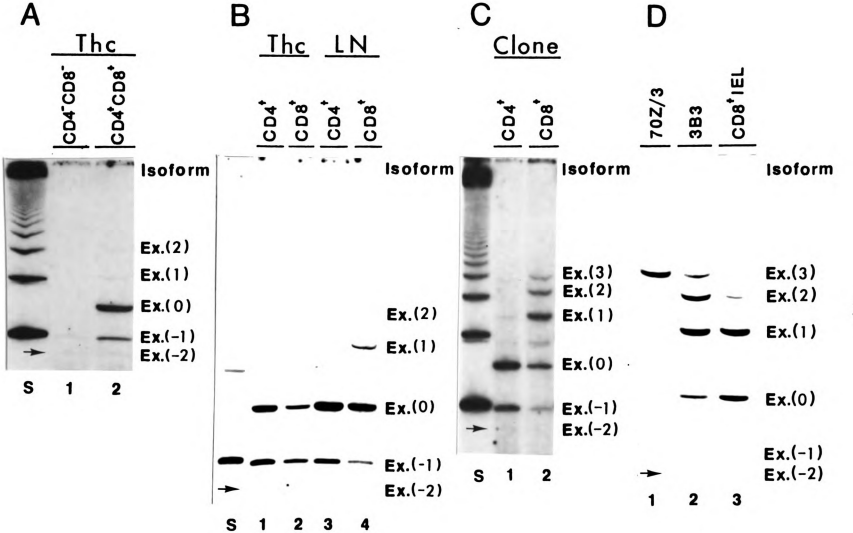
A
Thc
CD4-CD8-
CD4+CD8+
Isoform
Ex.(2)
Ex.(1)
Ex.(0)
Ex.(-1)
Ex.(-2)
S 1 2
B
Thc LN
CD4+
CD8+
CD4+
CD8+
Isoform
Ex.(2)
Ex.(1)
Ex.(0)
Ex.(-1)
Ex.(-2)
S 1 2 3 4
C
Clone
CD4+
CD8+
Isoform
Ex.(3)
Ex.(2)
Ex.(1)
Ex.(0)
Ex.(-1)
Ex.(-2)
S 1 2
D
70Z/3
3B3
CD8+IEL
Isoform
Ex.(3)
Ex.(2)
Ex.(1)
Ex.(0)
Ex.(-1)
Ex.(-2)
1 2 3

Figure 7.

observed among $CD4^+CD8^-$ and $CD4^-CD8^+$ T cells recovered from lymph nodes. $CD4^+CD8^-$ lymph node T cells continued to express Ex(0) and Ex(-1) isoforms, but also produced significant amounts of a Ex(1) isoform (Figure 7B, lane 3). $CD4^-CD8^+$ lymph node T cells produced all of these same isoforms, but additionally expressed an Ex(2) isoform.

RT-PCR analysis of the alloreactive T cell clones BC-3 ($CD4^+$) and 8.2.2 ($CD8^+$), revealed additional significant differences in alternate exon use (Figure 7C). $CD4^+$ BC-3 cells retained the CD45 phenotype of $CD4^+CD8^-$ lymph node T cells (Figure 7C, lane 1), with Ex(0) and Ex(-1) isoforms predominating and a smaller amount of an Ex(1) isoform also present. In contrast, $CD8^+$ 8.2.2 cells expressed significant amounts of Ex(-1), Ex(0), Ex(1), Ex(2) and Ex(3) isoforms. CD45 alternate exon use among IEL ($CD8^+$) was also compared to that of the T cell populations described above. IEL predominantly expressed Ex(0), Ex(1) and Ex(2) CD45 isoforms (Figure 7D, lane 3), but also produced small amounts of Ex(-1) and Ex(3) isoforms. In general, all T cells examined thus far have expressed the Ex(-1) and Ex(-2) isoforms, although the quantities have varied significantly.

Specific CD45 alternate exon use analysis. In order to determine which of the three previously identified alternate CD45 exons (exons 4, 5 and 6) is predominantly expressed in the Ex(1) isoforms identified in these experiments, Ex(1) isoforms were purified from the RT-PCR amplified products of $CD4^+$ and $CD8^+$ lymph node T cells, the CTL clone 8.2.2, and IEL, followed by PCR re-amplification using ^{32}P-labeled primers to yield quantities necessary for restriction enzyme analysis. In these experiments, P9 was labeled to a higher specific activity so that the resultant fragments containing the P9 sequence could be identified by their higher radioactivities following re-amplification and digestion.

Enzymes with unique CD45 alternate exon-specific digestion patterns (NciI, HinfI, and BanI) were used to digest the Ex(1) isoforms from $CD4^+$ lymph node T cells (Figure 8, lanes 1-3), $CD8^+$ lymph node T cells (lanes 4-6), 8.2.2 cells (lanes 7-9), and IEL (data not shown). The digestion pattern for all of these T cells was very similar. After NciI digestion, the appearance of 156 bp and 187 bp bands (lanes 2, 5 and 8) indicated the principal use of Ex-5. However, weaker bands of 249 bp and 88 bp, and the residual undigested fragment suggested the minor presence of Ex-6 and Ex-4, respectively. The dominant production of 237 bp and 106 bp fragments following HinfI digestion (lanes 3, 6 and 9), was also consistent with the primary expression of Ex-5, with additional bands indicating the presence of minor amounts of Ex(1) isoforms containing Ex-4 (95 bp; 130 bp) and Ex-6 (undigested). In order to determine if any of the undigested Ex(1) forms (containing either Ex-4 or Ex-6) represented unknown isoforms or non-specific products, we digested Ex(1) isoforms with BanI (cleaving at a site created at the junctions of Ex-4/Ex-7, Ex-5/Ex-7 and Ex-6/Ex-7). The results indicated no other isoforms or non-specific products, as all fragment sizes were as predicted for Ex(1) isoforms containing Ex-4, Ex-5 or Ex-6.

DISCUSSION

The present studies established the existence of additional alternately used exons in the murine CD45 glycoprotein family. From earlier investigations, only exons 4, 5 and 6 (as well as the untranslated exons, 1a and 1b) were known to be subject to alternate exon shuffling during the production of CD45 mRNA transcripts in lymphocytes (4, 6). The current data regarding the size of RT-PCR amplified products from murine thymocytes and T cells, as well as their reactivities with exon-specific

Figure 8. Restriction enzyme analysis of CD45 Ex(1) isoforms purified from $CD4^+$ lymph node T cells (lanes 1-3), $CD8^+$ lymph node T cells (lanes 4-6), and the T_c clone 8.2.2 (lanes 7-9). Lane S, 123 size marker; lanes 1, 4 and 7, undigested Ex(1) form (control); lanes 2, 5, and 8, NciI digestion; lanes 3, 6, and 9, HinfI digestion.

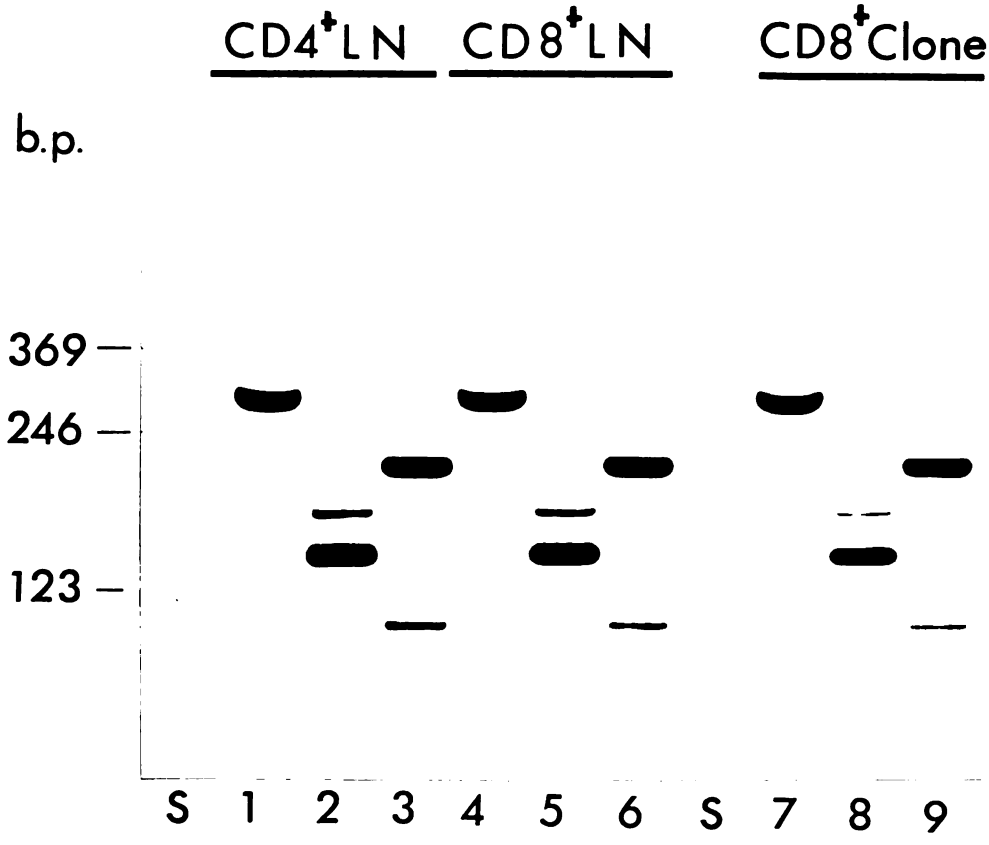

Figure 8.

oligonucleotide probes strongly suggested that both Ex-7 and Ex-8 are also alternately used. Direct sequencing data definitively confirmed the alternate use of Ex-7 and the creation of a splice junction between Ex-3 and Ex-8. These data indicated that at least five alternate exons exist in the CD45 glycoprotein family.

Analysis of CD45 isoform expression revealed a general trend toward incorporation of additional alternate exons into the CD45 mRNA of T cells as they matured and became more differentiated or activated (as summarized in Figure 9). An exception to this tendency was apparent in a $CD4^+$ allogenic Th clone, BC-3, which retained expression of the smaller CD45 isoforms after they were subject to activating stimuli (e.g., IL-2). $CD4^-$ $CD8^-$ thymocytes (Stage I), expressed only low levels of Ex(-1) and Ex(-2) isoforms. $CD4^+CD8^+$ thymocytes (Stage II), $CD4^+CD8^-$ and $CD4^-CD8^+$ thymocytes (Stage III), showed much higher amounts of Ex(0) and Ex(-1) isoforms.

Additional but distinctive alternate exon inclusion patterns were observed among $CD4^+$ and $CD8^+$ lymph node T cells. While both cell populations produced larger Ex(1) isoforms, $CD8^+$ cells also expressed even larger Ex(2) isoforms.

In activated T cell populations, further differences were observed. Following allogenic stimulation, the $CD8^+$ alloreactive CTL clone 8.2.2 expressed detectable quantities of isoforms containing from none [Ex(-2)] to all five [Ex(3)] of the alternate exons recognized in these experiments. Principal among these were the Ex(0), Ex(1) and Ex(2) isoforms. In contrast, CD45 isoform expression in the $CD4^+$ helper T cell clone BC-3 revealed less of the Ex(1) isoform seen in $CD4^+$ lymph node T cells, and a preponderance of Ex(-1) and Ex(0) isoforms that were prevalent in $CD4^+$ thymocytes. Hence, the pattern of CD45 alternate exon

Figure 9. Summary of developmental alterations of CD45 isoform expression in murine T cells. The arrow indicates the progression of maturation or activation.

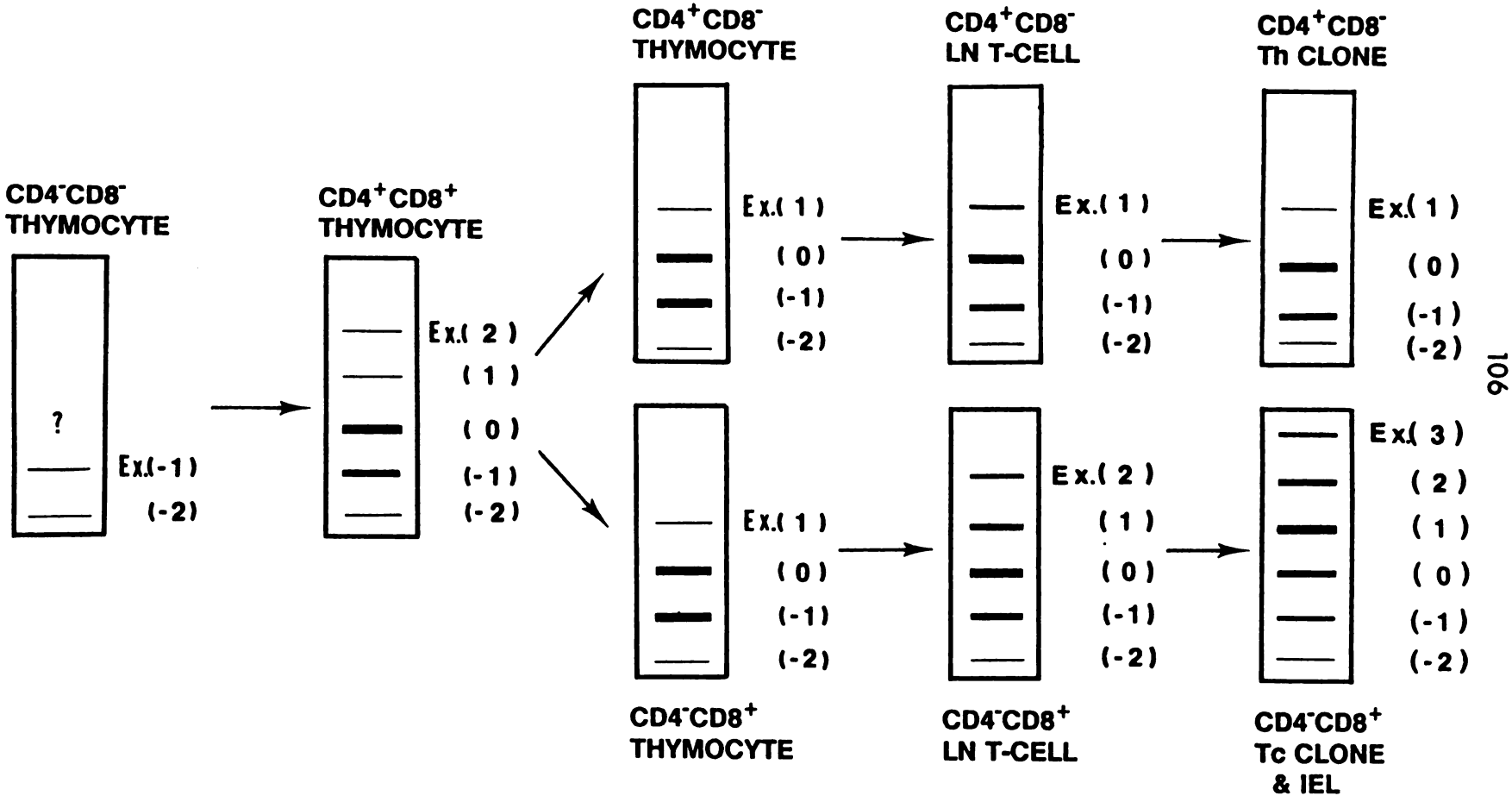
CD4-CD8-
THYMOCYTE
?
Ex.(-1)
(-2)
CD4+CD8+
THYMOCYTE
Ex.(2)
(1)
(0)
(-1)
(-2)
CD4+CD8-
THYMOCYTE
Ex.(1)
(0)
(-1)
(-2)
CD4+CD8-
LN T-CELL
Ex.(1)
(0)
(-1)
(-2)
CD4+CD8-
Th CLONE
Ex.(1)
(0)
(-1)
(-2)
CD4-CD8+
THYMOCYTE
Ex.(1)
(0)
(-1)
(-2)
CD4-CD8+
LN T-CELL
Ex.(2)
(1)
(0)
(-1)
(-2)
CD4-CD8+
Tc CLONE
& IEL
Ex.(3)
(2)
(1)
(0)
(-1)
(-2)

Figure 9.

use that emerges during activation of $CD4^+$ and $CD8^+$ T cells may be divergent, with $CD8^+$ T cells expressing additional CD45 alternate exons, while $CD4^+$ T cells may
instead delete certain alternate exons expressed more prominently in a less activated state.

The observation that, at high numbers of PCR cycles, smaller RT-PCR products were amplified more efficiently than larger ones made it important to identify conditions in which rates of amplification of each product were similar. The achievement of this goal in the present studies using radiolabeled primers and 24 cycles of PCR allowed estimation of the relative amount of mRNA present for each of the CD45 isoforms observed in a given cell population. While the Ex(-2) isoform of CD45 lacking all known alternate exons (Ex-4 to Ex-8) never predominated, the Ex(-1) isoform containing only Ex-8 was abundant in several of the cell populations studied.

Understanding variations in CD45 alternate exon use in cells of differing lineage, differentiation and activation states has taken on additional significance with recent evidence indicating that the highly conserved cytoplasmic portion of CD45 contains two PTPase domains (13, 26). Along with evidence of physiologically relevant membrane-associated phosphoprotein substrates for CD45 PTPase (e.g., $p56^{lck}$; 27, 28), these findings suggest that CD45 may play an important role in the regulation of lymphocyte activation and proliferation. The variably inhibitory and stimulatory effects observed upon treatment of different lymphocyte populations with antibodies directed against one or more epitopes of CD45 (29-32) underscore the complexity of the role of CD45 in lymphocyte responses to external stimuli. The employment of different combinations

of CD45 alternate exons in the extracellular portion of this major membrane glycoprotein may determine the ligand(s) with which CD45 interacts and the stimuli to which the lymphocyte responds. While dephosphorylation of $p56^{lck}$ by CD45 could be expected to result in cellular activation (27, 28), the PTPase activity of CD45 likely results in the dephosphorylation of proteins phosphorylated by protein tyrosine kinases and may thereby down-regulate these cellular activation signals.

RT-PCR data for CD45 isoform expression in lymphocyte populations in this study generally agreed with earlier SDS-PAGE analyses of CD45 immunoprecipitates from identical cell populations in the same murine strain (15, 22). Such analyses have demonstrated a 235 kDa glycoprotein isoform of CD45 in $CD8^+$ lymph node T cells not present in $CD8^+$ thymocytes. Similarly, a new CD45 glycoprotein isoform of 240 kDa was noted in activated $CD8^+$ CTL clones; whereas comparing allogenically activated $CD4^+$ clones to $CD4^+$ lymph node T cells revealed decreased expression of the high molecular weight isoform (220 kDa). Although it is theoretically possible that expression of higher or lower M_r CD45 isoforms resulted from changes in glycosylation rather than alternate exon use, the general correlation of RT-PCR and SDS-PAGE data suggests that the pattern of alternate exon use accurately predicts the Mr of CD45 isoforms observed on SDS-PAGE. Correlation of CD45 mRNA sizes with the sizes of nascent proteins determined by ^{35}S-methionine pulse-chase and endo-H treatment methods also supports the interpretation that increases in the size of CD45 mRNA produced by the use of additional alternate exons accounts for the emergence of higher M_r glycoprotein isoforms (22, 33).

While others have also reported similar losses of high M_r CD45 isoforms (CD45R) in $CD4^+$ T cells after allogenic stimulation, this has also

been observed in $CD8^+$ T cells (34). A similar finding has been made for human lymph node T cells (35). These disparate findings may be due to the use of mAb to infer the presence or absence of CD45 alternate exons without full knowledge of the epitopes recognized by these mAb. Further, increased O-glycosylation associated with activation may block mAb binding to alternate exon epitopes. Support for this possibility was demonstrated in CTL expressing highly glycosylated CD45 isoforms (M_r up to 260 kDa; 15, 36), with associated unique, O-glycan-associated carbohydrate antigens (CT1; CT2).

RT-PCR revealed differences in alternate exon use between Stage I ($CD4^-CD8^-$) and Stage II ($CD4^+CD8^+$) thymocytes not suggested by previous SDS-PAGE analyses (15, 22). This discrepancy may be explained by relatively minor differences in peptide length among the smallest CD45 alternate exon isoforms and the comparatively large effect of glycosylation on apparent M_r by SDS-PAGE.

Restriction enzyme analysis was used to determine whether any of the previously recognized alternate exons (Ex-4, 5 or 6) predominated when a single additional alternate exon was incorporated into the Ex(0) isoform. Regardless of lymphocyte population studied, Ex(1) isoforms principally involved insertion of Ex-5. Other Ex(1) isoforms containing Ex-4 or Ex-6 were present only in minor amounts. For lymph node T cells, this finding was consistent with conclusions based on mAb labeling studies (34).

In addition to evidence for alternate use of Ex-7 and Ex-8 in the CD45 glycoprotein family, our data indicated that a CD45 isoform lacking all alternate exons from Ex-4 through Ex-7 is significantly expressed in various members of the T lymphocyte lineage, including thymocytes, lymph node T cells and $CD4^+$ cloned helper T cells. However, only trace amounts

of this isoform were detected in the 8.2.2 CTL clone, with no significant expression in IEL. The as yet undetermined glycosylation pattern of this Ex(-1) isoform may eventually help to explain why it has not been identified as a discrete isoform by SDS-PAGE analysis of CD45 immunoprecipitates.

While data were presented that CD45 can also alternately exclude Ex-8, evidence of such exclusion was only seen in the context of exclusion of all known alternate exons (Ex-4 to 8), and then only in extremely small amounts. Alternate use of exons 7 and 8 may not have been previously recognized in part because only a small number of CD45 cDNAs have been directly sequenced. Further, other investigators using PCR to study CD45 isoform expression have selected primers in Ex-3 and Ex-7 (37), thereby only allowing for amplification of sequences contained within these exons.

To-date, we have found Ex-7 to be alternately excluded only when Ex-4, 5 and 6 were also absent. No isoforms lacking Ex-7 but containing Ex-4, 5 or 6 have yet been identified by Southern blotting or restriction enzyme analysis. Even considering the high sensitivity of the PCR method, we cannot entirely preclude the possibility that other minor CD45 isoforms may exist which alternately exclude Ex-7. However, the unique size of Ex-7 (74 bases) and the lack of evidence of major, intermediate size PCR products would seem to indicate that if any such isoforms exist, they are likely to be present only in minute quantities. Nevertheless, evidence exists to support the theoretical possibility that Ex-7 and Ex-8 may be used independently, since a study of the expression of CD45 mini-gene constructs revealed that the absence of Ex-3 and Ex-7 did not affect tissue-specific splicing of exons 4-6 (38).

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SUMMARY AND CONCLUSIONS

The method involving the combination of reverse transcription and the polymerase chain reaction (RT-PCR) followed by analysis by restriction enzymes, Southern blots and sequencing is the best strategy for studying CD45 alternate exon shuffling. This approach is more sensitive, more conclusive, and requires far less sample than any other assay. In addition, it is the only practical assay available for the study of very small numbers of T cell subsets and clones. The interpretation of the RT-PCR data was taken with caution since target DNA templates of unequal length were found to be amplified with different efficiencies under different conditions. The use of radiolabeled primers in conjunction with limiting the PCR reaction to the earliest possible cycle number was found to overcome this problem.

The use of RT-PCR in the analysis of T cell subsets confirmed that one cell type (or one cell line) expressed more than one CD45 mRNA isoform. In fact, cells that expressed only one single CD45 isoform have not been revealed by using RT-PCR in our study of CD45 expression. Murine T-cells express CD45 mRNA isoforms in a tissue-specific pattern. In general, the patterns observed by use of RT-PCR were in accord with the patterns of CD45 expression obtained by SDS-PAGE analysis. T cells tended to express more of the higher exon isoforms (two exon and three exon forms) in addition to the common isoforms (mainly zero exon and minus one exon isoforms) when they become more differentiated and more activated. Despite the fact that $CD4^+$ cells and $CD8^+$ cells expressed different levels

of the one exon isoform, the exon usage from both lineages appeared to be identical and contained mainly exon 5 with very little exon 4 and 6. Exons 7 and 8 are previously unrecognized alternate exons.

Since no other CD45 alternate exons have been discovered which could account for a large increase in the size of the peptide backbone, the appearance of the extraordinarily high molecular weight glycoprotein isoform (up to 260 kDa) of CD45 in IEL implies that there is tissue-specific glycosylation. The glycosylation of the alternate exons is likely to provide for highly unique characteristics when comparing isoforms containing alternate exons to the isoform without alternate exons. It is likely that CD45 molecular function involves interaction of the glycosylated portion on the outside of the cell with other membrane molecules. This interactiion would then bring the CD45 PTPase domains into proximity of substrates in the cytoplasm of the cell. Knowledge of the precise alternate exon use of different subsets of lymphoid and myeloid cells will no doubt lead to an better understanding of this function and will suggest future experiments.