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CELL CYCLE-RELATED ACTIVITY OF CD45 AND EXPRESSION OF A CD45-ASSOCIATED SERINE-THREONINE KINASE

presented by

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has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Microbiology

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Date 14 March 1995

CELL CYCLE-RELATED ACTIVITY OF CD45 AND EXPRESSION OF A CD45-ASSOCIATED SERINE-THREONINE KINASE

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology

1995

ABSTRACT

CELL CYCLE-RELATED ACTIVITY OF CD45 AND EXPRESSION OF A CD45-ASSOCIATED SERINE-THREONINE KINASE

By

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CD45 is an abundant cell-surface protein tyrosine phosphatase (PTPase) which is essential for antigen activation of B and T lymphocytes. CD45 is also expressed abundantly in many other hematopoietic cells indicating that it has additional functions. In particular, the expression of CD45 in all hematopoietic cells except erythrocytes and their immediate precursors suggests that CD45 may have a fundamental role in cellular processes, such as cellular stasis or cell cycle regulation. In the first study presented in this dissertation, the hypothesis that CD45 PTPase activity varies during progression through the cell cycle was tested. Then, having determined that the enzymatic activity of CD45 was elevated in mitosis, the second study was undertaken to determine if accessory proteins associate with CD45 when it exhibits peak PTPase activity late in the cell cycle. Centrifugal elutriation was used in both studies to fractionate logarithmically-growing asynchronous CTLL-2 cells into cell cycle stage-enriched subpopulations. For this cytotoxic T cell line, CD45 PTPase activity was elevated 2- to 5-fold in G_2+M fractions when compared to its activity in G_1 or S fractions. FACS and SDS-PAGE analyses indicated that there was only a minor increase in CD45 protein expression during progression through the cell cycle, suggesting that increased CD45 PTPase activity was principally due to increased enzymatic activity. The timing of this observation indicated that the peak PTPase

activity of CD45 occurred in mitosis. Treatment of cells with a chemical cross-linking reagent prior to immunoprecipitation of CD45 indicated that 60 to 70 kD proteins associated with CD45 in S and G_2+M. The association of 60 to 70 kD proteins with CD45 was confirmed by the analysis of CD45 immunoprecipitates from cells lysed with the mild detergent digitonin. When CD45 immunoprecipitate complexes from digitonin lysates were subjected to *in vitro* phosphorylation treatments, it was determined that an active kinase was present in the complex and that the activity of the kinase from G_2+M-enriched subpopulations was elevated. Phosphoamino acid analysis of kinase products demonstrated that the kinase was a serine-threonine kinase. The elevation during mitosis of the activity of both CD45 and a CD45-associated kinase supports the hypothesis that CD45 has a role in phosphorylation events during cell division.

This dissertation is dedicated to my family and the many educators who have helped me attain this goal.

ACKNOWLEDGEMENTS

I am greatly indebted to Dr. Walter Esselman for his guidance and support through my years in his research group. I wish to express my gratitude to Drs. Kathryn Brooks, Richard Schwartz, and Michael Zaroukian for their guidance and time. I am also grateful to Drs. Lyla Melkerson-Watson, Hsu-Lang Chang, Louis King, Sue Dagher, Laurie Iciek, and Jim Bretz and to Howard Beitenmiller and Mary Morrison for their invaluable assistance.

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CHAPTER ONE

LITERATURE REVIEW

Introduction

The murine leukocyte common antigen (LCA, which is also known as T200, B220, Ly-5, gp180, and CD45) was first identified in 1975 by Trowbridge *et al.* (1) as a group of high molecular weight species immunoprecipitated from T and B cell lysates by rabbit anti-mouse lymphocyte serum and by Komura *et al.* (2) as a T lymphocyte alloantigen system. Homologous proteins with highly conserved regions (3) have been identified on human (4) and rat (5) leukocytes, and analogous LCA have also been noted for sheep (6), chickens (7), cows, dogs, and sharks (8). CD45 was the first in a family of receptor-type protein tyrosine phosphatases (PTPases) to be identified (9).

CD45 is an abundant transmembrane protein with large intra- and extracellular regions (10-12). Murine CD45 is expressed by all hematopoietic cells except erythrocytes and their immediate precursors (13), but there is much cell lineage- and activation state-related heterogeneity of structure in this family of glycoproteins (reviewed in 3, 14). This heterogeneity occurs in the extracellular N-terminal region due to variable use of at least three exons (15-19). The alternate use of 5' exons gives rise not only to different primary structures of CD45 proteins but also to differences in glycosylation (14, 20). Because the primary structure of the intracellular and transmembrane regions is not varied among isoforms (16, 18), it

appears that the intracellular signal transduction mechanism of this putative receptor may be identical for the various cells expressing CD45, while the extracellular binding of ligands could be cell-type specific.

Full-length murine CD45 is produced as a 1291 amino acid protein (18). After the 23 residue leader sequence is removed, the extracellular region consists of 370-541 residues depending on exon usage, the transmembrane sequence of 22 residues, and the intracellular region of 705 amino acids (Fig. 1) (18).

CD45 Gene and mRNA Transcripts

The gene for murine CD45 is located on distal chromosome 1 in a large, conserved linkage group, which is homologous to a group localized to chromosome 1q21-32 in the human genome (21). The CD45 locus is referred to as *Ly-5*, and there are at three recognized alleles (21, 22). *Ly-5* spans approximately 120 kb and is comprised of 34 exons (19; nomenclature is from 23).

One of two alternate exons, denoted exons 1a and 1b, forms the 5' end of CD45 mRNA (19). Use of 1b is common in B cells, and in mRNA from T cells, exon 1a is more abundant. There are also mRNA transcripts in T cells which are exon-1a$^-$ and exon-1b$^-$, which may arise from an exon which is not yet identified. Primer extension analysis has revealed that transcription may initiate at any one of a number of sites at or near the 5' end of exon 1a or 1b (19). Although there is extremely high conservation of the nucleotide sequence 50 bp upstream of exon 1b between the human and the mouse, the TATA and CAAT boxes which have been

Figure 1. CD45 structure. The overall structure of the receptor-type PTPase, CD45, is shown depicting the extracellular domain, the transmembrane domain, and the cytoplasmic domain. The extracellular domain contains the alternate-exon-encoded sequences which generate the multiple isoforms. Glycosylation sites in the extracellular domain are indicated. The location of a fibronectin-III-like (Fn-III-like) repeat is shown. The cytoplasmic region contains two tandemly-repeated PTPase domains along with a membrane proximal spacer, an inter-PTPase domain spacer, and a C-terminal tail. Each cysteine essential for PTPase activity is depicted by C. The position of a regulatory tyrosine residue phosphorylated by $p50^{csk}$ is indicated. The numbers given in the figure refer to amino acid residue positions.

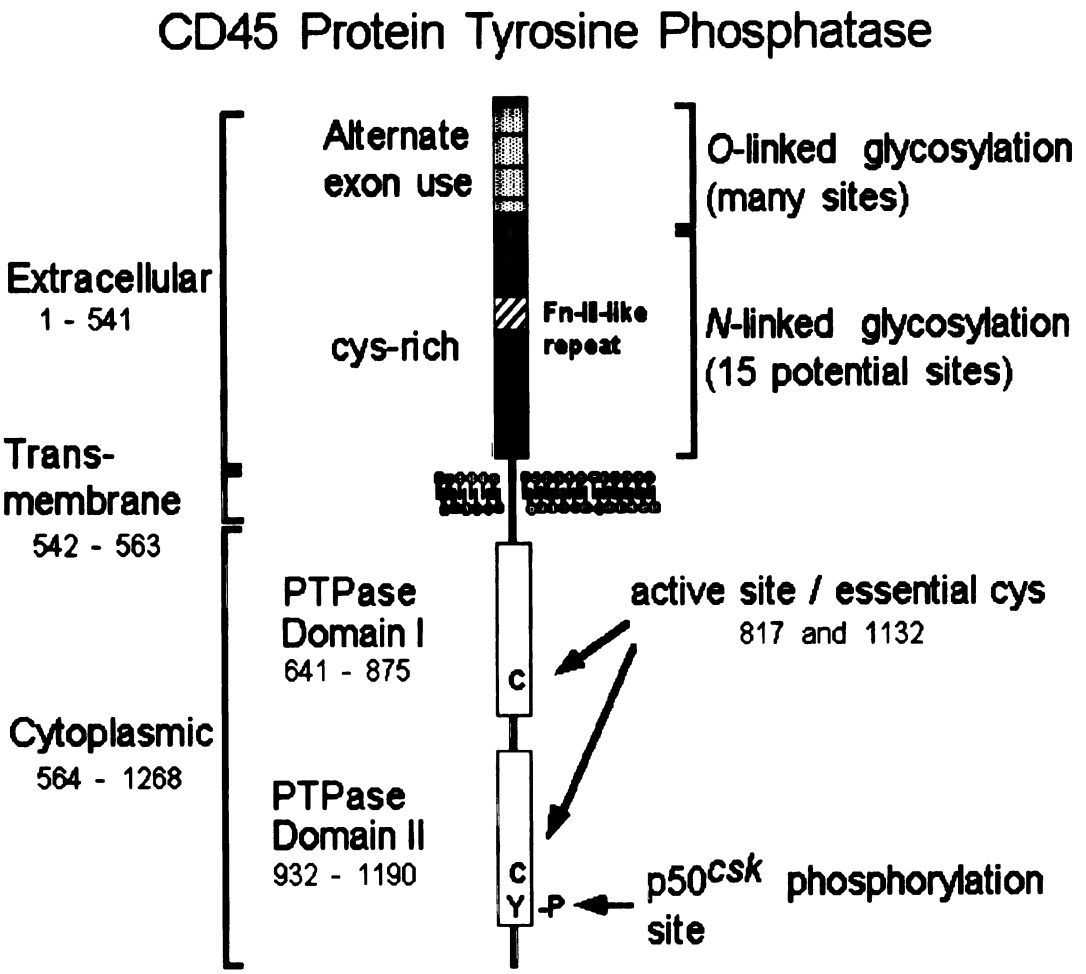
CD45 Protein Tyrosine Phosphatase
Alternate exon use
O-linked glycosylation (many sites)
Extracellular
1 - 541
cys-rich
Fn-II-like repeat
N-linked glycosylation (15 potential sites)
Trans-membrane
542 - 563
PTPase Domain I
641 - 875
active site / essential cys
817 and 1132
C
Cytoplasmic
564 - 1268
PTPase Domain II
932 - 1190
C
Y
-P
p50csk phosphorylation site

Figure 1.

identified in some eukaryotic promoters are not found upstream of this exon or exon 1a (19, 23). However, a number of TATA-like and CAAT-like sequences are located upstream of each of these exons (19, 23).

Translation begins in the middle of exon 2 which encodes the 23 residues of the signal sequence and is followed by an intron of about 50 kb (19). The seven residues which are found at the N terminus of all CD45 isoforms are encoded by exon 3. While the use of exons 2 and 3 is invariant, mRNA species lacking any combination of exons 4, 5, and 6 have been identified (17, 18, 24). Species also lacking exons 7 and 8 have been found (25), and two other studies have also noted mRNA species that cannot be explained by alternate use of exons 4, 5, and 6 (19, 24). Exon 16 encodes the transmembrane domain, and the termination of translation and the 3' untranslated region are encoded by exon 33 (19). There are two potential polyadenylation sites in the 3' untranslated region of exon 33, and it has been shown that either one can be used (16).

Alternate-Exon Usage

Because cell type-specific patterns of alternate-exon usage are conserved between human, mouse, and rat species, it is believed that CD45 isoform usage is highly regulated (14, 26). While alternate-exon usage in nonlymphoid hematopoietic cells has not been studied extensively (14), early studies of lymphoid cells led to the generalizations that: 1) B cells express the highest molecular weight isoform of 220 kD which is produced when all of the exons are incorporated into CD45 mRNA, 2)

thymocytes express the smallest isoform of 180 kD which is encoded by mRNA lacking exon 4, 5, and 6 sequences, and 3) T cells have complicated patterns of alternate-exon usage and, thus, isoform expression (3). Subsequently, the former two generalizations have been shown to be over-simplifications. Use of the polymerase chain reaction technique has shown that all of the eight mRNA species which are possible from the alternate use of exons 4, 5, and 6 can be found in a panel of B cell tumor lines (24). Also, a number of studies have demonstrated that thymocyte subsets can use alternate exons and that CD45 isoforms larger than 180 kD can be found on the cell surface of thymocytes (25, 27-30).

In the study of cell type-specific expression of CD45 isoforms by T cell subsets, anti-CD45 monoclonal antibodies (mAbs) with epitopes in the variably used regions have been utilized (28, 29, 31): antibodies with specificities to epitopes dependent on the expression of exon 4, also called exon A, are called CD45RA mAbs (R denotes restricted epitope); those dependent on the expression of exon 6, *i.e.* B, are CD45RB mAbs; those dependent on exon 7, *i.e.* C, are CD45RC mAbs; and those which recognize the isoform produced from the mRNA lacking exons 4, 5, and 6 are called CD45R0 mAbs (R0 refers to restriction to the "0 exon" form). CD45R is used to refer to any mAb with a restricted epitope, and antibodies which recognize all CD45 isoforms are called CD45 or pan-CD45 mAbs. These terms are also used to refer to the isoforms.

Rogers *et al.* (32) showed there is a high correlation of exon usage in CD45 mRNA in a cell line and the mAbs which react with CD45 on the cell surface. Of the alternate exons, exon B is the most commonly used in humans and mice, but

mouse cells use alternate exons less often than their human counterparts (32). Among the T helper subsets, alternate-exon use is more common in T_H2 than in T_H1 subsets although there are numerous patterns of expression in each group (24, 28, 32-36). $CD8^+$ T cells use alternate exons more often and typically express higher molecular weight isoforms than do $CD4^+$ T cells (14, 25).

CD45R0 and CD45R have been considered to be markers for memory and naive T cells, respectively (37-39). In the human system, CD45RA mAbs are frequently used in memory studies while in the mouse system, in which alternate exons are used less often, antibodies against epitopes from the most commonly-used alternate exon, exon B, are typically used. Decreased use of exon B-dependent epitopes by both $CD4^+$ and $CD8^+$ cells and a corresponding loss of 190 kD cell-surface CD45 have been detected four days after *in vivo* allostimulation (40). Also, Lee *et al.* (41) determined that splenic T cells from newborn mice or mice raised in aseptic environments are predominantly $CD45RB^{hi}$. Increases in antigenic exposure or age correlated with increases in the number of $CD45RB^{lo}$ T cells, and after long term-*in vivo* priming the majority of T cells were $CD45RB^{lo}$ (41). When either $CD45RB^{hi}$ or $CD45RB^{lo}$ splenic T cells from primed donors were transferred into nu/nu mice, recipients reconstituted with $CD45RB^{lo}$ cells exhibited an IgG response while recipients reconstituted with $CD45RB^{hi}$ exhibited an IgM response (41). When splenic $CD4^+$ cells sorted into $CD45RB^{hi}$ and $CD45RB^{lo}$ populations were challenged *in vitro* with antigen, from unprimed mice $CD45RB^{hi}$ cells proliferated well while from mice previously primed with the antigen, $CD45RB^{lo}$ cells proliferated to a

greater degree (42). Hence, CD45RB^{hi} cells appear to be naive T cells and a switch to the CD45RB^{lo} phenotype appears to occur after priming.

The same correlation of B-exon usage and age Lee and his co-workers (41) reported was likewise observed by Ernst *et al.* (43). In the latter study, however, although the CD45RB^{lo} CD4^{+} T cells recovered from the spleens of older mice appeared to produce more IL-4 than the cells from younger mice in response to *in vitro* stimulation, the CD45RB^{lo} CD4^{+} cells showed decreased proliferation when compared to the cells from younger mice. Thus, in this study CD45RB^{lo} cells did not display the proliferative response typical of primed/memory cells.

The switch from CD45R expression to CD45R0 expression was considered to be irreversible and unidirectional (44). The conversion of CD45R$^{lo/-}$ to the CD45R$^{hi/+}$ phenotype, first observed in human and rat systems, has now also been observed in mouse cells (36, 45-48). Thus, CD45R$^{hi/+}$ and CD45R$^{lo/-}$ phenotypes may not be reliable markers for naive and memory cells (49-51). Instead, the CD45R0^{+} CD45R^{-} phenotype, which reflects the omission of the alternate exons from mRNA, may be a marker for recently primed T cells.

Regulation of Splicing of the Alternate Exons

The regulation of splicing of the alternate exons was studied by Streuli and Saito (52) using mini-LCA gene constructs encoding 5' exons in an SV40 transcription unit. *Cis*-elements within or flanking the alternate exons 4 and 6 were able to direct the tissue-specific splicing of the human gene constructs in murine T

(EL4) and pre-B (300-19) cell lines: in EL4 cells, each alternate-exon sequence was omitted from cytoplasmic RNA and in 300-19 cells, it was included regardless of the LCA exon flanking the alternate exon (52). A mini-gene construct with exon 5 did not give a clear tissue-specific pattern of use, which may reflect its more common use in T cells (32). When exon 9 was placed in the mini-gene construct instead of an alternate exon, it was included in the mRNA of both cell types. The splicing pattern typical of thymocytes is seen in non-hematopoietic cell lines: murine NIH3T3 and L cells spliced out the alternate exons in the production of mRNA from the mini-LCA gene constructs; in human HeLa cells, though, a small amount of mRNA which included exon 4 was detected (52). Several studies have determined that several sequences within each alternate exon and in its nearby flanking sequences are required to direct the tissue-specific splicing of the CD45 RNA (52-54). It is interesting to note that the *trans*-acting factor which directs the alternate splicing of the exons is apparently conserved between mice and humans because the factors produced by the murine cells were able to faithfully splice the human gene constructs (52).

It was proposed that positive *trans*-acting factors produced by B cells direct their use of the alternate exons based on the exon usage of T/B cell hybrids: the B cell-splicing pattern was seen after stable hybrid lines were selected (54). However, in the study of *trans*-acting factors, it is preferable to study the heterokaryons shortly after fusion when the activities in one nucleus may (or may not) be altered by factors clearly produced by the other nucleus, *i.e. trans*-acting factors (55). When murine T cells and cells expressing the exon-usage pattern of human B cells were fused and the products were analyzed within hours after the fusions for the presence of mRNA from

the human gene, increases in the splice products lacking alternate exons and decreases in the products containing two or three of the alternate exons were noted (26). Hence, T cells contain a regulatory signal which directs the splicing out of the alternate exons. When murine B cells were fused with human T cells and likewise analyzed, there was a slight, inconsistent increase in two-exon forms of human CD45 mRNA and no three-exon mRNA was detected. Thus, B cells do not appear to have a positive regulatory signal to incorporate the alternate exons into mRNA. When NIH3T3 cells were fused to human B cells, there was a shift to the production of mRNA containing fewer alternate exons, which indicates that the negative regulatory factor detected in T cells is also present in non-hematopoietic cells. The effect of cycloheximide, a protein synthesis inhibitor, on the splicing patterns of normal human thymocytes and peripheral T cells supported the theory that T cells express a negative regulatory *trans*-acting factor which directs the removal of alternate exons from RNA transcripts (26).

CD45 PTPase Domain

Mutational analysis of the murine cytoplasmic region expressed *in vitro* indicated that the membrane proximal spacer and both PTPase domains must be present for activity while the deletion of the cytoplasmic tail did not affect activity (56). The deletion of the C terminus including 13 residues from the PTPase II domain, though, did abolish activity. Within the conserved domains of several PTPases, there are several highly conserved features: a cysteine residue and its

surrounding 10-15 amino acids, a GXGXXG motif, and in PTPase II domains, a region of about 20 acidic amino acids (56, 57). Since mutation of Cys^{817}, the highly conserved cysteine residue in domain I of murine CD45, but not of Cys^{1132}, its counterpart in domain II, abolished PTPase activity, it was determined that the PTPase I domain was responsible for the activity (56). Also, mutation of any of the three glycine residues in the GXGXXG motif in PTPase I decreased activity (5-fold or more) while only alteration of the first glycine in the motif in the second domain affected activity (2-fold decrease). Deletion of the acidic region of PTPase II also reduced activity (4-fold). Alteration of Tyr^{729} to phenylalanine abolished activity while similar mutations of several other conserved tyrosine residues did not affect activity (56). Analysis of human CD45 cytoplasmic sequences expressed in bacteria determined that PTPase I is active by itself (57). The second PTPase domain of CD45 has also been shown to become active in highly-specialized circumstances involving phosphorylation of the cytoplasmic domain (58-60).

CD45 Function

Many of the early studies undertaken to determine the function of CD45 were performed by observing the effects of anti-CD45 antibodies on cellular activities. From determinations that such antibodies inhibit or enhance cytolytic functions of antibody-dependent cell-mediated cytotoxicity, natural killer cells, and cytolytic T cells (61-64), and proliferation and differentiation of B and T cells (63, 65-68), a role for CD45 in a variety of signalling processes had been indicated.

Subsequently, two discoveries have entirely changed the methods used to study CD45 function. The first revolution in CD45 experimentation occurred in 1988 when Charbonneau and his colleagues proposed on the basis of amino acid sequence similarity to a human placental enzyme that CD45 may be a PTPase (9). Shortly thereafter, several groups confirmed that CD45 is indeed a PTPase (69-71). The second discovery that changed the manner in which CD45 is studied was that CD45-deficient lymphocytes have impaired immunological responses (72). This finding has led to the extensive use of $CD45^-$ cell lines to study CD45 function.

CD45 is essential in both B and T cells to couple stimulation of the antigen receptor to second messenger pathways. The earliest intracellular responses to ligation of antigen receptors are tyrosine phosphorylation and phospholipase C activation, which results in Ca^{2+} mobilization (73-76). In $CD45^-$ cell lines both tyrosine phosphorylation and Ca^{2+} flux responses are diminished or absent (77-81). Normal tyrosine phosphorylation and Ca^{2+} flux responses to antigen receptor stimulation are restored to $CD45^-$ cells when the intracellular region of CD45 is expressed in the cells, even when the external CD45 region is absent or replaced by MHC class I or epidermal growth factor receptor extracellular sequences (79, 81-84).

CD45 appears to regulate the phosphorylation and activity of src family kinases in lymphocytes. Both $p56^{lck}$ and $p59^{fyn}$ are typically hyperphosphorylated in $CD45^-$ T cells, but the resulting effect on the protein tyrosine kinase (PTK) activity of $p56^{lck}$ and $p59^{fyn}$ is variable (71, 76, 81, 85-89). Data derived from other experimental approaches also provide evidence that CD45 can up- or down-regulate $p56^{lck}$ and $p59^{fyn}$ PTK activity (71, 90-92). Perhaps further studies based on the

observation that CD45 regulates specific pools of $p56^{lck}$ and $p59^{fyn}$ (89) will clarify the seemingly contradictory data on the effect of CD45 on the activity of the src family kinases.

In addition to its role in the activation of lymphocytes, CD45 appears to have a role in mitosis. The PTPase activity of exponentially-growing cultures is higher than that of stationary cultures (93, 94). Using centrifugal elutriation to obtain subpopulations of asynchronous B and T cell cultures which were enriched for cells in various stages of the cell cycle, it was determined that CD45 PTPase activity is elevated 2- to 5-fold in G_2+M when compared to its activity during other stages of the cell cycle.

CD45 Interactions with Other Molecules

The B lymphocyte adhesion molecule CD22 has been identified as a potential ligand of CD45 (95). Although it has been determined that several human CD45 isoforms can interact with CD22β (95, 96), this interaction is not highly specific since CD22β is a lectin which recognizes a number of glycoproteins on B and T cell membranes with α-2,6-linked sialic acids (97, 98).

In studies using immunoprecipitation following chemical cross-linking or lysis with the mild detergent digitonin, CD45 has been shown to associate with several other molecules on lymphocyte cell surfaces including Thy-1, CD2, CD3, and CD26 (99-101). In addition, CD45 co-localization with capped CD4 (but not other T cell

surface molecules) has been observed (102). This CD4/CD45 interaction appeared to be limited to low molecular weight isoforms of CD45 as CD45RB did not co-cap.

CD45 interacts with the cytoskeleton, and this interaction increases after receptor patching and/or capping (103-105). In particular, the cytoskeletal element fodrin appears to form a complex with CD45 (103, 104, 106).

In resting splenic murine B cells membrane IgM, $p53^{lyn}$, and the antigen receptor complex components MB-1 and B29 have been coprecipitated with CD45 from digitonin lysates (107). In this study, many proteins were labelled in *in vitro* phosphorylation reactions of CD45 immunoprecipitates from digitonin, CHAPS, and Brij 35 lysates. Although the authors reported that the immunoprecipitates were washed extensively before further analyses, they did not report the use of a high salt solution in the washing process, a practice commonly used to reduce the presence of nonspecifically bound material. Thus, care must be taken in the consideration of these findings. Despite the lack of a high salt wash, however, the presence of the src family kinase $p53^{lyn}$ in the CD45 complex was judged to be specific since other src-related kinases which were expressed in the cells were not detected in the CD45 immunoprecipitates.

In the activation of T cells, CD45 may regulate the activity of both $p56^{lck}$ and $p59^{fyn}$, two src family PTKs which are involved in the response to TCR/CD3 stimulation (71, 81, 85, 86, 88-92). Thus, an interaction of CD45 with each of these PTKs can be inferred, and although binding of $p56^{lck}$ to CD45 has been observed, similar data for $p59^{fyn}$ is yet to be presented.

The interaction of CD45 with $p56^{lck}$ appears to occur via the src homology region 2 (SH2) domain of the PTK and a phosphotyrosine residue on CD45 (60). Although $p56^{lck}$ had some affinity for nonphosphorylated CD45, treatment of CD45 with $p50^{csk}$, a PTK, enhanced the association. In addition, the binding of $p56^{lck}$ to CD45 was inhibited by the presence of recombinant SH2 domain.

Analyses of CD45 immunoprecipitates from digitonin or Brij 96 lysates of human peripheral blood T lymphocytes and cultured human and murine T cells have shown that CD45 associates with $p56^{lck}$ and a group of phosphoproteins of variable molecular weights, referred to as pp29-34 or the lymphocyte phosphatase-associated phosphoprotein (LPAP) (108-114). LPAP can act as a substrate for both $p56^{lck}$ and CD45 *in vitro* (108). *In vivo*, LPAP is phosphorylated almost exclusively on serine, and although it underwent change in electrophoretic mobility upon activation of cells, no change in its phosphorylation could be detected (108, 109, 111, 112). It should be noted the *in vivo* phosphorylation analysis was performed without the use of the potent PTPase inhibitor phenylarsine oxide, which was essential in the detection of phosphotyrosine in CD45 (60, 115). The association of $p56^{lck}$ with CD45 is not mediated by CD4: even after CD4 was precleared from lysates, $p56^{lck}$ still coprecipitated with CD45 (112). Also, LPAP can bind to CD45 even in the absence of $p56^{lck}$ (112). Interestingly, although when CD45 is immunoprecipitated from digitonin lysates, $p56^{lck}$ can be identified in the CD45 complex both by immunoblotting and re-precipitation analyses (108, 111, 112), LPAP is phosphorylated almost exclusively on serine after *in vitro* phosphorylation treatments of the CD45 immunoprecipitates from Jurkat T cells (111). Thus, a serine-threonine

kinase must also be present in the CD45 immunoprecipitates. It was determined that the association of the serine-threonine kinase with the immunoprecipitate was specific to CD45 because serine-threonine kinase activity was not found in mock CD45 immunoprecipitates from a $CD45^-$ clone (111).

Regulation of the PTPase Activity of CD45

CD45 PTPase activity is increased 7.5-fold after the binding of fodrin *in vitro* (106). Thus, interaction with the cytoskeleton may play a role in regulating the PTPase activity of CD45.

Dimerization may also regulate the activity of CD45. A chimeric form of CD45, in which the external region was replaced by the external region of the epidermal growth factor receptor, restored normal tyrosine phosphorylation and Ca^{2+} flux responses to a $CD45^-$ cell line. However, these responses were inactivated when the chimeric CD45 was dimerized with the ligand epidermal growth factor (84). Dimerization of CD45 may be a naturally-occurring phenomenon as dimeric forms of CD45 have been recovered from the murine T cell line YAC-1 (110).

Studies on the regulation of CD45 PTPase activity by phosphorylation indicate that both serine and tyrosine phosphorylation are involved. Decreased PTPase activity has been shown to correlate with decreased serine phosphorylation after Ca^{2+} ionophore treatment of T cells (116). In contrast, increased serine phosphorylation of CD45 was not associated with modulation of its enzymatic activity after either *in vitro* treatment with casein kinase II and other serine-threonine kinases or after IL-2

stimulation of CTLL-2.4 cells (117, 118). The PTPase activity of CD45 can be increased up to 8-fold *in vitro* by treatment with $p50^{csk}$, a PTK (60). In another study, sequential *in vitro* phosphorylation of CD45 first on tyrosine with v-abl, then on serine with casein kinase II was necessary to produce maximal enhancement of its PTPase activity (59). Stover and Walsh (59) attributed the increased PTPase activity they noted to activation of the second PTPase domain of CD45.

REFERENCES

1. Trowbridge, I. S., P. Ralph, and M. J. Bevan. 1975. Differences in the surface proteins of mouse B and T cells. *Proc. Natl. Acad. Sci. U. S. A.* 72:157.

2. Komuro, K., K. Itakura, E. A. Boyse, and M. John. 1975. Ly-5: a new T-lymphocyte antigen system. *Immunogenetics.* 1:452.

3. Thomas, M. L., and L. Lefrancois. 1988. Differential expression of the leucocyte-common antigen family. *Immunol. Today.* 9:320.

4. Andersson, L. C., C. Wasastjerna, and C. G. Gahmberg. 1976. Different surface glycoprotein patterns on human T-, B- and leukemic-lymphocytes. *Int. J. Cancer.* 17:40.

5. Fabre, J. W., and A. F. Williams. 1977. Quantitative serological analysis of a rabbit anti-rat lymphocyte serum and preliminary biochemical characterisation of the major antigen recognised. *Transplantation.* 23:349.

6. Maddox, J. F., C. R. Mackay, and M. R. Brandon. 1985. The sheep analogue of leucocyte common antigen (LCA). *Immunology.* 55:347.

7. Houssaint, E., S. Tobin, J. Cihak, and U. Losch. 1987. A chicken leukocyte common antigen: biochemical characterization and ontogenetic study. *Eur. J. Immunol.* 17:287.

8. Matthews, R. J. and M. L. Thomas. 1990. Evolutionary conservation of the leukocyte common antigen. Fed. Proc. Abs.: A2199.

9. Charbonneau, H., N. K. Tonks, K. A. Walsh, and E. H. Fischer. 1988. The leukocyte common antigen (CD45): a putative receptor-linked protein tyrosine phosphatase. *Proc. Natl. Acad. Sci. U. S. A.* 85:7182.

10. Trowbridge, I. S. 1978. Interspecies spleen-myeloma hybrid producing monoclonal antibodies against mouse lymphocyte surface glycoprotein, T200. *J. Exp. Med.* 148:313.

11. Omary, M. B., and I. S. Trowbridge. 1980. Disposition of T200 glycoprotein in the plasma membrane of a murine lymphoma cell line. *J. Biol. Chem.* 255:1662.

12. Williams, A. F., and A. N. Barclay. 1986. Glycoprotein antigens of the lymphocyte surface and their purification by antibody affinity chromatography. In *Handbook of Experimental Immunology in Four Volumes, 4th ed., vol. 1.* D. M. Weir, ed. Blackwell Scientific Publications, Oxford, p. 22.1.

13. Scheid, M. P., and D. Triglia. 1979. Further description of the Ly-5 system. *Immunogenetics.* 9:423.

14. Thomas, M. L. 1989. The leukocyte common antigen family. *Annu. Rev. Immunol.* 7:339.

15. Lefrancois, L., M. L. Thomas, M. J. Bevan, and I. S. Trowbridge. 1986. Different classes of T lymphocytes have different mRNAs for the leukocyte-common antigen, T200. *J. Exp. Med.* 163:1337.

16. Saga, Y., J. S. Tung, F. W. Shen, and E. A. Boyse. 1986. Sequences of Ly-5 cDNA: isoform-related diversity of Ly-5 mRNA [published erratum appears in Proc Natl Acad Sci U S A 1987 Apr;84(7):1991]. *Proc. Natl. Acad. Sci. U. S. A.* 83:6940.

17. Saga, Y., J. S. Tung, F. W. Shen, and E. A. Boyse. 1987. Alternative use of 5' exons in the specification of Ly-5 isoforms distinguishing hematopoietic cell lineages. *Proc. Natl. Acad. Sci. U. S. A.* 84:5364.

18. Thomas, M. L., P. J. Reynolds, A. Chain, Y. Ben-Neriah, and I. S. Trowbridge. 1987. B-cell variant of mouse T200 (Ly-5): evidence for alternative mRNA splicing. *Proc. Natl. Acad. Sci. U. S. A.* 84:5360.

19. Saga, Y., J. S. Tung, F. W. Shen, T. C. Pancoast, and E. A. Boyse. 1988. Organization of the Ly-5 gene. *Mol. Cell. Biol.* 8:4889.

20. Ewald, S. J., and P. H. Refling. 1985. Analysis of structural differences between Ly-5 molecules of T- and B-cells. *Mol. Immunol.* 22:581.

21. Seldin, M. F., H. C. Morse, R. C. LeBoeuf, and A. D. Steinberg. 1988. Establishment of a molecular genetic map of distal mouse chromosome 1: further definition of a conserved linkage group syntenic with human chromosome 1q. *Genomics.* 2:48.

22. Morse, H. C., 3d., F. W. Shen, and U. Hammerling. 1987. Genetic nomenclature for loci controlling mouse lymphocyte antigens. *Immunogenetics.* 25:71.

23. Johnson, N. A., C. M. Meyer, J. T. Pingel, and M. L. Thomas. 1989. Sequence conservation in potential regulatory regions of the mouse and human leukocyte common antigen gene. *J. Biol. Chem.* 264:6220.

24. Saga, Y., K. Furukawa, P. Rogers, J. S. Tung, D. Parker, and E. A. Boyse. 1990. Further data on the selective expression of Ly-5 isoforms. *Immunogenetics.* 31:296.

25. Chang, H. L., L. Lefrancois, M. H. Zaroukian, and W. J. Esselman. 1991. Developmental expression of CD45 alternate exons in murine T cells. Evidence of additional alternate exon use. *J. Immunol.* 147:1687.

26. Rothstein, D. M., H. Saito, M. Streuli, S. F. Schlossman, and C. Morimoto. 1992. The alternative splicing of the CD45 tyrosine phosphatase is controlled by negative regulatory trans-acting splicing factors. *J. Biol. Chem.* 267:7139.

27. Lefrancois, L., and T. Goodman. 1987. Developmental sequence of T200 antigen modifications in murine T cells. *J. Immunol.* 139:3718.

28. Birkeland, M. L., J. Metlay, V. M. Sanders, R. Fernandez-Botran, E. S. Vitetta, R. M. Steinman, and E. Pure. 1988. Epitopes on CD45R [T200] molecules define differentiation antigens on murine B and T lymphocytes. *J. Mol. Cell. Immunol.* 4:71.

29. Hathcock, K. S., G. Laszlo, H. B. Dickler, S. O. Sharrow, P. Johnson, I. S. Trowbridge, and R. J. Hodes. 1992. Expression of variable exon A-, B-, and C-specific CD45 determinants on peripheral and thymic T cell populations. *J. Immunol.* 148:19.

30. Goff, L. K., L. Larsson, and A. G. Fisher. 1990. Expression of high molecular weight isoforms of CD45 by mouse thymic progenitor cells. *Eur. J. Immunol.* 20:665.

31. Johnson, P., L. Greenbaum, K. Bottomly, and I. S. Trowbridge. 1989. Identification of the alternatively spliced exons of murine CD45 (T200) required for reactivity with B220 and other T200-restricted antibodies. *J. Exp. Med.* 169:1179.

32. Rogers, P. R., S. Pilapil, K. Hayakawa, P. L. Romain, and D. C. Parker. 1992. CD45 alternative exon expression in murine and human CD4+ T cell subsets. *J. Immunol.* 148:4054.

33. Bottomly, K. 1988. A functional dichotomy in CD4+ T lymphocytes. *Immunol. Today.* 9:268.

34. Bottomly, K., M. Luqman, L. Greenbaum, S. Carding, J. West, T. Pasqualini, and D. B. Murphy. 1989. A monoclonal antibody to murine CD45R distinguishes CD4 T cell populations that produce different cytokines. *Eur. J. Immunol.* 19:617.

35. Luqman, M., P. Johnson, I. Trowbridge, and K. Bottomly. 1991. Differential expression of the alternatively spliced exons of murine CD45 in Th1 and Th2 cell clones. *Eur. J. Immunol.* 21:17.

36. Lee, W. T., and E. S. Vitetta. 1992. Changes in expression of CD45R during the development of Th1 and Th2 cell lines. *Eur. J. Immunol.* 22:1455.

37. Beverley, P. C. 1991. CD45 isoform expression: implications for recirculation of naive and memory cells. *Immunol. Res.* 10:196.

38. Luqman, M., and K. Bottomly. 1992. Activation requirements for CD4+ T cells differing in CD45R expression. *J. Immunol.* 149:2300.

39. Ernst, D. N., W. O. Weigle, D. J. Noonan, D. N. McQuitty, and M. V. Hobbs. 1993. The age-associated increase in IFN-gamma synthesis by mouse CD8+ T cells correlates with shifts in the frequencies of cell subsets defined by membrane CD44, CD45RB, 3G11, and MEL-14 expression. *J. Immunol.* 151:575.

40. Birkeland, M. L., P. Johnson, I. S. Trowbridge, and E. Pure. 1989. Changes in CD45 isoform expression accompany antigen-induced murine T-cell activation. *Proc. Natl. Acad. Sci. U. S. A.* 86:6734.

41. Lee, W. T., X. M. Yin, and E. S. Vitetta. 1990. Functional and ontogenetic analysis of murine CD45Rhi and CD45Rlo CD4+ T cells. *J. Immunol.* 144:3288.

42. Lee, W. T., and E. S. Vitetta. 1990. Limiting dilution analysis of CD45Rhi and CD45Rlo T cells: further evidence that CD45Rlo cells are memory cells. *Cell. Immunol.* 130:459.

43. Ernst, D. N., M. V. Hobbs, B. E. Torbett, A. L. Glasebrook, M. A. Rehse, K. Bottomly, K. Hayakawa, R. R. Hardy, and W. O. Weigle. 1990. Differences in the expression profiles of CD45RB, Pgp-1, and 3G11 membrane antigens and in the patterns of lymphokine secretion by splenic CD4+ T cells from young and aged mice. *J. Immunol.* 145:1295.

44. Deans, J. P., A. W. Boyd, and L. M. Pilarski. 1989. Transitions from high to low molecular weight isoforms of CD45 (T200) involve rapid activation of alternate mRNA splicing and slow turnover of surface CD45R. *J. Immunol.* 143:1233.

45. Bell, E. B., and S. M. Sparshott. 1990. Interconversion of CD45R subsets of CD4 T cells in vivo. *Nature.* 348:163.

46. Rothstein, D. M., A. Yamada, S. F. Schlossman, and C. Morimoto. 1991. Cyclic regulation of CD45 isoform expression in a long term human CD4+CD45RA+ T cell line. *J. Immunol.* 146:1175.

47. Sparshott, S. M., E. B. Bell, and S. R. Sarawar. 1991. CD45R CD4 T cell subset-reconstituted nude rats: subset-dependent survival of recipients and bi-directional isoform switching. *Eur. J. Immunol.* 21:993.

48. Warren, H. S., and L. J. Skipsey. 1991. Loss of activation-induced CD45RO with maintenance of CD45RA expression during prolonged culture of T cells and NK cells. *Immunology.* 74:78.

49. Bell, E. B., C. P. Yang, S. R. Sarawar, and S. M. Sparshott. 1992. The cyclic expression of CD45R isoforms on CD4 T cells. *Biochem. Soc. Trans.* 20:198.

50. Gray, D. 1993. Immunological memory. *Annu. Rev. Immunol.* 11:49.

51. Lightstone, L., and J. Marvel. 1993. CD45RA+ T cells: not simple virgins. *Clin. Sci. Colch.* 85:515.

52. Streuli, M., and H. Saito. 1989. Regulation of tissue-specific alternative splicing: exon-specific cis-elements govern the splicing of leukocyte common antigen pre-mRNA. *Embo. J.* 8:787.

53. Tsai, A. Y., M. Streuli, and H. Saito. 1989. Integrity of the exon 6 sequence is essential for tissue-specific alternative splicing of human leukocyte common antigen pre-mRNA. *Mol. Cell. Biol.* 9:4550.

54. Saga, Y., J. S. Lee, C. Saraiya, and E. A. Boyse. 1990. Regulation of alternative splicing in the generation of isoforms of the mouse Ly-5 (CD45) glycoprotein. *Proc. Natl. Acad. Sci. U. S. A.* 87:3728.

55. Baron, M. H., and T. Maniatis. 1986. Rapid reprogramming of globin gene expression in transient heterokaryons. *Cell.* 46:591.

56. Johnson, P., H. L. Ostergaard, C. Wasden, and I. S. Trowbridge. 1992. Mutational analysis of CD45. A leukocyte-specific protein tyrosine phosphatase. *J. Biol. Chem.* 267:8035.

57. Streuli, M., N. X. Krueger, T. Thai, M. Tang, and H. Saito. 1990. Distinct functional roles of the two intracellular phosphatase like domains of the receptor-linked protein tyrosine phosphatases LCA and LAR. *Embo. J.* 9:2399.

58. Tan, X., D. R. Stover, and K. A. Walsh. 1993. Demonstration of protein tyrosine phosphatase activity in the second of two homologous domains of CD45. *J. Biol. Chem.* 268:6835.

59. Stover, D. R., and K. A. Walsh. 1994. Protein-tyrosine phosphatase activity of CD45 is activated by sequential phosphorylation by two kinases. *Mol. Cell. Biol.* 14:5523.

60. Autero, M., J. Saharinen, T. Pessa-Morikawa, M. Soula-Rothhut, C. Oetken, M. Gassmann, M. Bergman, K. Alitalo, P. Burn, C. G. Gahmberg, et al. 1994. Tyrosine phosphorylation of CD45 phosphotyrosine phosphatase by p50csk kinase creates a binding site for p56lck tyrosine kinase and activates the phosphatase. *Mol. Cell. Biol.* 14:1308.

61. Kasai, M., J. C. Leclerc, F. W. Shen, and H. Cantor. 1979. Identification of Ly-5 on the surface of "natural killer" cells in normal and athymic inbred mouse strains. *Immunogenetics.* 8:153.

62. Nakayama, E. 1982. Blocking of effector cell cytotoxicity and T-cell proliferation by Lyt antisera. *Immunological Reviews* 68:117.

63. Harp, J. A., B. S. Davis, and S. J. Ewald. 1984. Inhibition of T cell responses to alloantigens and polyclonal mitogens by Ly-5 antisera. *J. Immunol.* 133:10.

64. Small, R. M., S. M. Walden, and S. J. Ewald. 1987. Effects of Ly-5 antibodies on antibody-dependent cell-mediated cytotoxicity (ADCC). *Immunology.* 60:159.

65. Ledbetter, J. A., L. M. Rose, C. E. Spooner, P. G. Beatty, P. J. Martin, and E. A. Clark. 1985. Antibodies to common leukocyte antigen p220 influence human T cell proliferation by modifying IL 2 receptor expression. *J. Immunol.* 135:1819.

66. Gruber, M. F., J. M. Bjorndahl, S. Nakamura, and S. M. Fu. 1989. Anti-CD45 inhibition of human B cell proliferation depends on the nature of

activation signals and the state of B cell activation. A study with anti-IgM and anti-CDw40 antibodies. *J. Immunol.* 142:4144.

67. Yakura, H., F. W. Shen, E. Bourcet, and E. A. Boyse. 1983. On the function of Ly-5 in the regulation of antigen-driven B cell differentiation. Comparison and contrast with Lyb-2. *J. Exp. Med.* 157:1077.

68. Yakura, H., I. Kawabata, F. W. Shen, and M. Katagiri. 1986. Selective inhibition of lipopolysaccharide-induced polyclonal IgG response by monoclonal Ly-5 antibody. *J. Immunol.* 136:2729.

69. Tonks, N. K., H. Charbonneau, C. D. Diltz, E. H. Fischer, and K. A. Walsh. 1988. Demonstration that the leukocyte common antigen CD45 is a protein tyrosine phosphatase. *Biochemistry.* 27:8695.

70. Kiener, P. A., and R. S. Mittler. 1989. CD45-protein tyrosine phosphatase cross-linking inhibits T cell receptor CD3-mediated activation in human T cells. *J. Immunol.* 143:23.

71. Mustelin, T., K. M. Coggeshall, and A. Altman. 1989. Rapid activation of the T-cell tyrosine protein kinase pp56lck by the CD45 phosphotyrosine phosphatase. *Proc. Natl. Acad. Sci. U. S. A.* 86:6302.

72. Pingel, J. T., and M. L. Thomas. 1989. Evidence that the leukocyte-common antigen is required for antigen-induced T lymphocyte proliferation. *Cell.* 58:1055.

73. June, C. H., M. C. Fletcher, J. A. Ledbetter, and L. E. Samelson. 1990. Increases in tyrosine phosphorylation are detectable before phospholipase C activation after T cell receptor stimulation. *J. Immunol.* 144:1591.

74. Lane, P. J., J. A. Ledbetter, F. M. McConnell, K. Draves, J. Deans, G. L. Schieven, and E. A. Clark. 1991. The role of tyrosine phosphorylation in signal transduction through surface Ig in human B cells. Inhibition of tyrosine phosphorylation prevents intracellular calcium release. *J. Immunol.* 146:715.

75. Cambier, J. C., D. C. Morrison, M. M. Chien, and K. R. Lehmann. 1991. Modeling of T cell contact-dependent B cell activation. IL-4 and antigen receptor ligation primes quiescent B cells to mobilize calcium in response to Ia cross-linking. *J. Immunol.* 146:2075.

76. Weiss, A., and D. R. Littman. 1994. Signal transduction by lymphocyte antigen receptors. *Cell.* 76:263.

77. Koretzky, G. A., J. Picus, M. L. Thomas, and A. Weiss. 1990. Tyrosine phosphatase CD45 is essential for coupling T-cell antigen receptor to the phosphatidyl inositol pathway. *Nature.* 346:66.

78. Koretzky, G. A., J. Picus, T. Schultz, and A. Weiss. 1991. Tyrosine phosphatase CD45 is required for T-cell antigen receptor and CD2-mediated activation of a protein tyrosine kinase and interleukin 2 production. *Proc. Natl. Acad. Sci. U. S. A.* 88:2037.

79. Justement, L. B., K. S. Campbell, N. C. Chien, and J. C. Cambier. 1991. Regulation of B cell antigen receptor signal transduction and phosphorylation by CD45. *Science.* 252:1839.

80. Volarevic, S., B. B. Niklinska, C. M. Burns, H. Yamada, C. H. June, F. J. Dumont, and J. D. Ashwell. 1992. The CD45 tyrosine phosphatase regulates phosphotyrosine homeostasis and its loss reveals a novel pattern of late T cell receptor-induced Ca2+ oscillations. *J. Exp. Med.* 176:835.

81. Shiroo, M., L. Goff, M. Biffen, E. Shivnan, and D. Alexander. 1992. CD45 tyrosine phosphatase-activated p59fyn couples the T cell antigen receptor to pathways of diacylglycerol production, protein kinase C activation and calcium influx. *Embo. J.* 11:4887.

82. Volarevic, S., B. B. Niklinska, C. M. Burns, C. H. June, A. M. Weissman, and J. D. Ashwell. 1993. Regulation of TCR signaling by CD45 lacking transmembrane and extracellular domains. *Science.* 260:541.

83. Hovis, R. R., J. A. Donovan, M. A. Musci, D. G. Motto, F. D. Goldman, S. E. Ross, and G. A. Koretzky. 1993. Rescue of signaling by a chimeric protein containing the cytoplasmic domain of CD45. *Science.* 260:544.

84. Desai, D. M., J. Sap, J. Schlessinger, and A. Weiss. 1993. Ligand-mediated negative regulation of a chimeric transmembrane receptor tyrosine phosphatase. *Cell.* 73:541.

85. Ostergaard, H. L., D. A. Shackelford, T. R. Hurley, P. Johnson, R. Hyman, B. M. Sefton, and I. S. Trowbridge. 1989. Expression of CD45 alters phosphorylation of the lck-encoded tyrosine protein kinase in murine lymphoma T-cell lines. *Proc. Natl. Acad. Sci. U. S. A.* 86:8959.

86. McFarland, E. D., T. R. Hurley, J. T. Pingel, B. M. Sefton, A. Shaw, and M. L. Thomas. 1993. Correlation between Src family member regulation by the protein-tyrosine-phosphatase CD45 and transmembrane signaling through the T-cell receptor. *Proc. Natl. Acad. Sci. U. S. A.* 90:1402.

87. Hurley, T. R., R. Hyman, and B. M. Sefton. 1993. Differential effects of expression of the CD45 tyrosine protein phosphatase on the tyrosine phosphorylation of the lck, fyn, and c-src tyrosine protein kinases. *Mol. Cell. Biol.* 13:1651.

88. Burns, C. M., K. Sakaguchi, E. Appella, and J. D. Ashwell. 1994. CD45 regulation of tyrosine phosphorylation and enzyme activity of src family kinases. *J. Biol. Chem.* 269:13594.

89. Biffen, M., D. McMichael-Phillips, T. Larson, A. Venkitaraman, and D. Alexander. 1994. The CD45 tyrosine phosphatase regulates specific pools of antigen receptor-associated p59fyn and CD4-associated p56lck tyrosine in human T-cells. *Embo. J.* 13:1920.

90. Ostergaard, H. L., and I. S. Trowbridge. 1990. Coclustering CD45 with CD4 or CD8 alters the phosphorylation and kinase activity of p56lck. *J. Exp. Med.* 172:347.

91. Mustelin, T., and A. Altman. 1990. Dephosphorylation and activation of the T cell tyrosine kinase pp56lck by the leukocyte common antigen (CD45). *Oncogene.* 5:809.

92. Mustelin, T., T. Pessa-Morikawa, M. Autero, M. Gassmann, L. C. Andersson, C. G. Gahmberg, and P. Burn. 1992. Regulation of the p59fyn protein tyrosine kinase by the CD45 phosphotyrosine phosphatase. *Eur. J. Immunol.* 22:1173.

93. Ohta, T., K. Kitamura, A. L. Maizel, and A. Takeda. 1994. Alterations in CD45 glycosylation pattern accompanying different cell proliferation states. *Biochem. Biophys. Res. Commun.* 200:1283.

94. Melkerson-Watson, L. J., M. E. Waldmann, A. D. Gunter, M. H. Zaroukian, and W. J. Esselman. 1994. Elevation of lymphocyte CD45 protein tyrosine phosphatase activity during mitosis. *J. Immunol.* 153:2004.

95. Stamenkovic, I., D. Sgroi, A. Aruffo, M. S. Sy, and T. Anderson. 1991. The B lymphocyte adhesion molecule CD22 interacts with leukocyte common antigen CD45RO on T cells and alpha 2-6 sialyltransferase, CD75, on B cells. *Cell.* 66:1133.

96. Aruffo, A., S. B. Kanner, D. Sgroi, J. A. Ledbetter, and I. Stamenkovic. 1992. CD22-mediated stimulation of T cells regulates T-cell receptor/CD3-induced signaling. *Proc. Natl. Acad. Sci. U. S. A.* 89:10242.

97. Sgroi, D., A. Varki, S. Braesch-Andersen, and I. Stamenkovic. 1993. CD22, a B cell-specific immunoglobulin superfamily member, is a sialic acid-binding lectin. *J. Biol. Chem.* 268:7011.

98. Powell, L. D., D. Sgroi, E. R. Sjoberg, I. Stamenkovic, and A. Varki. 1993. Natural ligands of the B cell adhesion molecule CD22 beta carry N-linked oligosaccharides with alpha-2,6-linked sialic acids that are required for recognition. *J. Biol. Chem.* 268:7019.

99. Volarevic, S., C. M. Burns, J. J. Sussman, and J. D. Ashwell. 1990. Intimate association of Thy-1 and the T-cell antigen receptor with the CD45 tyrosine phosphatase. *Proc. Natl. Acad. Sci. U. S. A.* 87:7085.

100. Schraven, B., Y. Samstag, P. Altevogt, and S. C. Meuer. 1990. Association of CD2 and CD45 on human T lymphocytes. *Nature.* 345:71.

101. Torimoto, Y., N. H. Dang, E. Vivier, T. Tanaka, S. F. Schlossman, and C. Morimoto. 1991. Coassociation of CD26 (dipeptidyl peptidase IV) with CD45 on the surface of human T lymphocytes. *J. Immunol.* 147:2514.

102. Dianzani, U., M. Luqman, J. Rojo, J. Yagi, J. L. Baron, A. Woods, C. A. Janeway Jr., and K. Bottomly. 1990. Molecular associations on the T cell surface correlate with immunological memory. *Eur. J. Immunol.* 20:2249.

103. Bourguignon, L. Y., S. J. Suchard, M. L. Nagpal, and J. R. Glenney Jr. 1985. A T-lymphoma transmembrane glycoprotein (gp180) is linked to the cytoskeletal protein, fodrin. *J. Cell. Biol.* 101:477.

104. Suchard, S. J., and L. Y. Bourguignon. 1987. Further characterization of a fodrin-containing transmembrane complex from mouse T-lymphoma cells. *Biochim. Biophys. Acta.* 896:35.

105. Lin, J., V. K. Brown, and L. B. Justement. 1992. Regulation of basal tyrosine phosphorylation of the B cell antigen receptor complex by the protein tyrosine phosphatase, CD45. *J. Immunol.* 149:3182.

106. Lokeshwar, V. B., and L. Y. Bourguignon. 1992. Tyrosine phosphatase activity of lymphoma CD45 (GP180) is regulated by a direct interaction with the cytoskeleton. *J. Biol. Chem.* 267:21551.

107. Brown, V. K., E. W. Ogle, A. L. Burkhardt, R. B. Rowley, J. B. Bolen, and L. B. Justement. 1994. Multiple components of the B cell antigen receptor complex associate with the protein tyrosine phosphatase, CD45. *J. Biol. Chem.* 269:17238.

108. Schraven, B., H. Kirchgessner, B. Gaber, Y. Samstag, and S. Meuer. 1991. A functional complex is formed in human T lymphocytes between the protein tyrosine phosphatase CD45, the protein tyrosine kinase p56lck and pp32, a possible common substrate. *Eur. J. Immunol.* 21:2469.

109. Schraven, B., A. Schirren, H. Kirchgessner, B. Siebert, and S. C. Meuer. 1992. Four CD45/P56lck-associated phosphorproteins (pp29-pp32) undergo alterations in human T cell activation. *Eur. J. Immunol.* 22:1857.

110. Takeda, A., J. J. Wu, and A. L. Maizel. 1992. Evidence for monomeric and dimeric forms of CD45 associated with a 30-kDa phosphorylated protein. *J. Biol. Chem.* 267:16651.

111. Koretzky, G. A., M. Kohmetscher, and S. Ross. 1993. CD45-associated kinase activity requires lck but not T cell receptor expression in the Jurkat T cell line. *J. Biol. Chem.* 268:8958.

112. Ross, S. E., B. Schraven, F. D. Goldman, J. Crabtree, and G. A. Koretzky. 1994. The association between CD45 and lck does not require CD4 or CD8 and is independent of T cell receptor stimulation. *Biochem. Biophys. Res. Commun.* 198:88.

113. Takeda, A., A. L. Maizel, K. Kitamura, T. Ohta, and S. Kimura. 1994. Molecular cloning of the CD45-associated 30-kDa protein. *J. Biol. Chem.* 269:2357.

114. Schraven, B., D. Schoenhaut, E. Bruyns, G. Koretzky, C. Eckerskorn, R. Wallich, H. Kirchgessner, P. Sakorafas, B. Labkovsky, S. Ratnofsky, and S. Meuer. 1994. LPAP, a novel 32-Kda phophoprotein that interacts with CD45 in human lymphocytes. *J. Biol. Chem.* 269(46):29102.

115. Stover, D. R., H. Charbonneau, N. K. Tonks, and K. A. Walsh. 1991. Protein-tyrosine-phosphatase CD45 is phosphorylated transiently on tyrosine upon activation of Jurkat T cells. *Proc. Natl. Acad. Sci. U. S. A.* 88:7704.

116. Ostergaard, H. L., and I. S. Trowbridge. 1991. Negative regulation of CD45 protein tyrosine phosphatase activity by ionomycin in T cells. *Science.* 253:1423.

117. Tonks, N. K., C. D. Diltz, and E. H. Fischer. 1990. CD45, an integral membrane protein tyrosine phosphatase. Characterization of enzyme activity. *J. Biol. Chem.* 265:10674.

118. Valentine, M. A., M. B. Widmer, J. A. Ledbetter, F. Pinault, R. Voice, E. A. Clark, B. Gallis, and D. L. Brautigan. 1991. Interleukin 2 stimulates serine phosphorylation of CD45 in CTLL-2.4 cells. *Eur. J. Immunol.* 21:913.

CHAPTER TWO

ELEVATION OF LYMPHOCYTE CD45 PTPASE ACTIVITY DURING MITOSIS[1]

FOOTNOTES

[1] This work was supported by NIH grant GM35774 and by a grant from the Children's Leukemia Foundation of Michigan.

[2] Abbreviations used in this paper: PTPase, protein tyrosine phosphatase; PTK, protein tyrosine kinase; PI, propidium iodide; DAPI, 4',6-diamidino-2-phenylindole; MPF, mitosis promoting factor.

ABSTRACT

CD45 is an abundant cell-surface protein tyrosine phosphatase (PTPase) of hematopoietic cells which mediates specific tyrosine dephosphorylation in lymphocyte antigen activation. The hypothesis that CD45 PTPase activity varies during progression through the cell cycle was examined in this study. CD45 PTPase activity was determined in the cytolytic T lymphocyte cell line CTLL-2 after fractionation by counterflow centrifugal elutriation into cell cycle stage-enriched subpopulations. For this cell line, CD45 PTPase activity was elevated 2- to 5-fold in G_2+M fractions compared to its PTPase activity in G_1 and S fractions. FACS and SDS-PAGE analyses indicated that there was only a minor increase in CD45 protein expression during progression through the cell cycle, suggesting that increased CD45 PTPase activity was due to increased enzymatic activity. The timing of this observation indicated that the peak PTPase activity of CD45 occurred in G_2+M or possibly in cytokinesis. The elevation of CD45 PTPase activity in G_2+M supports the hypothesis that CD45 has a role in phosphorylation events occurring during cell division.

INTRODUCTION

The level of cellular tyrosine phosphorylation is a consequence of the opposing actions of PTPases[2] and PTKs. Previous research has focused on the roles played by PTKs in cellular regulation. However, since the identification of the PTPase family, considerable effort has also been devoted to establishing the role of this class of soluble and membrane-associated enzymes in the regulation of signal transduction and cell cycle progression.

CD45 PTPase, a heterogeneous family of plasma membrane-associated proteins, is ubiquitously and prominently expressed on the surface of all nucleated hematopoietic cells (1). The intracellular region of CD45 consists of two tandemly-repeated PTPase domains with enzyme activity residing primarily in the first domain (2, 3). The level of CD45 expressed on the surface of a single lymphocyte can range as high as 50,000 to 100,000 molecules (4), making it one of the major membrane glycoproteins of the hematopoietic lineage. Deletion of CD45 severely impairs TCR- and membrane IgM-mediated signal transduction (5-9). CD45 dephosphorylates src family kinases such as $p56^{lck}$, which are essential for facilitation of T cell activation (6, 7, 10, 11). Studies with $CD45^-$ cell lines show that CD45 is essential for coupling antibody-mediated TCR stimulation to both the phosphatidyl inositol cascade and tyrosine phosphorylation signalling pathways (7). The PTPase activity of T cell

CD45 has also been shown to correlate with serine or tyrosine phosphorylation of the molecule (12, 13).

It has been proposed that the PTPase activity of CD45 is influenced by its association with cytoskeletal elements (14, 15). CD45 contains fodrin/spectrin binding domains and when fodrin or spectrin was added to purified CD45, its PTPase activity increased up to 7.5 fold (14). Association of fodrin with other cytoskeletal elements is believed to involve the phosphorylation state of these molecules. Further, in B cells, cross-linking of CD45 has been shown to induce association of CD45 with the cytoskeleton, resulting in capping of CD45 and increased tyrosine phosphorylation of membrane Ig-associated proteins (15). These reports suggest a regulatory role for the CD45/cytoskeleton complex during lymphocyte activation and proliferation.

Although CD45 clearly has a specific role in lymphocyte signal transduction and subsequent activation, the abundant expression of CD45 in many hematopoietic cell types suggests that it may also play a fundamental role in other immunological or cellular processes, such as maintenance of cellular stasis or in cell cycle regulation. Using counterflow centrifugal elutriation and immunoanalysis, this study tested the hypothesis that CD45 PTPase activity varies with cell cycle stage in a manner consistent with its expected association with cytoskeletal elements.

MATERIALS AND METHODS

Cell culture

The cytolytic T cell line CTLL-2 was obtained from the American Type Culture Collection (ATCC; Rockville, MD) and maintained at 37°C in a humidified environment with 7.5% CO_2 in RPMI 1640 (GIBCO/BRL, Gaithersburg, MD) containing 10% FBS (Biologos, Naperville, IL), 50 μM 2-ME (Sigma Chemical Co., St. Louis, MO), and 7 U/ml recombinant IL-2 (Cetus Corporation, Emeryville, CA) (complete medium). Cells were maintained in a log exponential growth state (0.25 to 5.0 x 10^5 cells/ml). Cultures were typically harvested upon attaining a density of 1-4 x 10^5 cells/ml.

Counterflow centrifugal elutriation

Cells were separated using counterflow centrifugal elutriation carried out at 12°C and 2250 rpm in a JE-6B elutriation rotor in a J2-21M/E elutriation centrifuge system (Beckman Instruments, Palo Alto, CA). Log phase cells were concentrated by centrifugation at 1000 x g and resuspended at 4 x 10^6 cells/ml in complete medium. Typically, 2-4 x 10^8 cells were loaded into the elutriation chamber with a peristaltic pump at an initial flow rate of 4.5 ml/min with the elutriation buffer, Hank's balanced salt solution (GIBCO/BRL), pH 7.4, containing 1 mg/ml BSA (Sigma). After all of the cells had entered the elutriation chamber, the system was equilibrated at 4.5

ml/min for 10-15 min. Elutriation fractions were obtained by increasing the flow rate 1-2 ml/min per fraction. Five minute fractions at each flow rate were collected in 50 ml conical tubes on ice and then pelleted and resuspended in minimal volumes for analysis. The possibility that cells attached to the apparatus and bled out over time was minimized by treating the elutriation chamber and tubing with silicone.

Immunoprecipitation of CD45

Viable cell counts were obtained for concentrated cell fractions using trypan blue exclusion. Cells were pelleted at 500 x g at 4°C for 5-10 min and were washed twice with cold PBS (8 mM Na_2HPO_4, 1 mM KH_2PO_4, 137 mM NaCl, 3 mM KCl, pH 7.3). Following the final wash, cells were lysed by suspending the pellet in 1% (v/v) Triton X-100 (Boehringer Mannheim, Indianapolis, IN); 0.5% (w/v) sodium deoxycholate (Sigma); 0.1% (w/v) SDS (Bio-Rad Laboratories Inc., Melville, NY); 0.1 M NaCl; 10 mM phosphate buffer, pH 7.5; 0.01% NaN_3 (1 x 10^7 cells/ml lysis buffer). After 45 min on ice, the suspension was centrifuged at 12,000 x g for 15 min at 4°C. Postnuclear lysates were precleared with 25 μl packed protein G-agarose (Pharmacia, Piscataway, NJ, or Boehringer Mannheim) per 10^7 cells.

A 10 μl aliquot was removed for determining protein concentration (16). CD45 and CD8 were immunoprecipitated from the remaining lysate by incubation with anti-CD45 M1/9.3.4 mAb (ATCC) or with isotype-matched anti-CD8α 53-6.72 (ATCC) for 60 min at 4°C, followed by addition of protein G-agarose with continuous mixing for an additional 30 min at 4°C. Immunoprecipitates were washed two times with 250 mM HEPES (Sigma), pH 7.3, and stored in 10X PTPase assay buffer (250 mM HEPES, pH 7.3; 50 mM EDTA; 100 mM DTT) at -70°C.

Phosphatase assay

The PTPase activity of immunoprecipitated CD45 was determined using a modification of established protocol (17). In brief, raytide (Oncogene Science, Manhasset, NY) was labelled with [γ-^{32}P]-ATP (>6000 mCi/mmol; Dupont/New England Nuclear, Boston, MA) using recombinant src tyrosine kinase (a gift of J. Dixon, University of Michigan, Ann Arbor). The final substrate labelling reaction contained 50 μg of raytide; 30 μM ATP (Sigma); 10 mM $MgCl_2$; 16 mM HEPES, pH 7.5; 0.03 mM EDTA; 0.07% 2-ME; 400-800 ng src kinase; and 50 μCi [γ -^{32}P]-ATP. After incubation at 30°C for 40 min, the ^{32}P-raytide was recovered by TCA precipitation and dissolved in 200 μl 200 mM Tris (Sigma), pH 8. Each PTPase assay contained up to 5 μl immunoprecipitated CD45, 5 μl 10X phosphatase assay buffer (250 mM HEPES, pH 7.3; 50 mM EDTA; 100 mM DTT), 5 μl ^{32}P-raytide (1-2 x 10^5 cpm) and H_2O to 50 μl. Reactions were allowed to proceed for 5-20 min at 37°C and then terminated by adding 750 μl of acidic charcoal suspension (0.9 M HCl, 90 mM $Na_4P_2O_7$, 2 mM NaH_2PO_4, 4% [v/v] Norit A [Sigma]). After centrifugation for 3 min, 400 μl of the supernatant were removed and the ^{32}P radioactivity of the sample was determined using liquid scintillation. The amount of $^{32}P_i$ released was expressed as total cpm released per min per mg protein of the lysate from which the CD45 was immunoprecipitated. A standard curve using CD45 immunoprecipitated from asynchronous cultures was used in each independent experiment for establishing the linear range for the assay. CD45 PTPase activity was measured in the linear range.

Immunofluorescent staining

For flow cytometry, cells were fixed in PBS/FBS/70% ethanol (0.2:0.2:1.2, v/v/v) for 18-36 hrs at -20°C, washed twice with PBS containing 5% (v/v) FBS, and immunolabelled on ice for 30 min with anti-CD45 mAb (M1/9.3.4). The cells were washed twice with PBS containing 5% (v/v) FBS and incubated with affinity-purified FITC-conjugated goat anti-rat IgG (preabsorbed with murine IgG, Cappel Laboratories, Durham, NC) for 30 min. The FITC mAb-labelled cells were washed twice with ice-cold PBS and resuspended in PI staining solution (PBS with 0.1% Triton X-100, 0.1 mM EDTA, 0.05 mg/ml RNase A, and 50 μg/ml PI [Sigma]). For fluorescent microscopy, cells were fixed in 2% formaldehyde in PBS and stained with 1% DAPI (Molecular Probes Inc., Eugene OR) in PBS.

Cell cycle analysis by FACS

The cell cycle states of CTLL-2 elutriation fractions were determined by FACS analyses of the DNA content of PI-stained, FITC-anti-CD45$^+$ cell populations. PI and FITC fluorescence were analyzed at 488 nm excitation on a Becton Dickinson Vantage FACS (San Jose, CA). PI emission was taken at 630 nm bandpass and FITC fluorescence was read at 530 nm bandpass. Data were analyzed using PC-Lysys version 1.0 software (Becton Dickinson, San Jose, CA). Mean FITC fluorescence was determined following singlet discrimination based on PI staining.

SDS-PAGE and silver staining

For each elutriation sample, the amount of CD45 or CD8 immunoprecipitate obtained from 380 μg protein in the lysate was subjected to SDS-PAGE in a 4-15% gel for approximately 1 hr at 125V (18). A variation of silver staining (19) was performed

as follows. Gels were first stained with Coomassie blue then destained. Gels were equilibrated to H_2O and treated with 5 μg DTT/ml H_2O for 30 min then with 1 mg $AgNO_3$/ml H_2O for 30 min. The stain was developed with 3% (w/v) $NaCO_3$, 0.05% (v/v) HCHO, and development was stopped with 10% (v/v) CH_3COOH.

RESULTS

The hypothesis of this study was that CD45 PTPase activity varied with cell cycle stage. This hypothesis was based on the observation that CD45 PTPase activity was influenced by culture density (data not shown). In addition, previous work with chemical blocks suggested that there may be an increase in CD45 PTPase activity in mitotic cells (data not shown). Therefore, further investigation utilizing counterflow centrifugal elutriation was initiated to isolate subpopulations of cells representing each of the phases of the cell cycle.

Counterflow centrifugal elutriation separates cells on the basis of velocity sedimentation rates, permitting the study of cell cycle-dependent phenomena with minimal perturbation of normal *in vitro* growth conditions (20-23). Distinct subpopulations from an asynchronously-proliferating population of cells were separated, stained with PI and FITC-anti-CD45, and analyzed by FACS. This procedure resulted in effective isolation of subpopulations enriched for the various cell cycle phases (Fig. 1). Debris and dead cells were recovered at less than 10 ml/min. Early G_1 cells were concentrated in fractions obtained from flow rates of about 12 ml/min, late G_1 from 14-20 ml/min, S from 21-27 ml/min, early G_2+M from 28-31 ml/min, and late G_2+M from 32-37 ml/min. The percentage of cells recovered in each fraction is shown in Table I. Elutriation flow rates varied slightly between

Figure 1. FACS analysis of the DNA content of elutriated CTLL-2 cells. Two-color FACS was performed on elutriation fractions from an exponentially-growing CTLL-2 culture. Cells from each sample were treated with FITC-anti-CD45 (M1/9.3.4) followed by PI and analyzed by flow cytometry. PI fluorescence histograms of FITC-anti-CD45$^+$ cells are shown for (*A*) the asynchronous starting population of CTLL-2 cells and (*B-P*) elutriation fractions 8-25. *Panel Q*, noted with an asterisk, corresponds to the maximal CD45 PTPase activity. The DNA staining patterns were interpreted as follows: *B-D*, fractions 8-10, G_1 phase; *E-H*, fractions 11-14, G_1/S phase; *I-K*, fractions 15-17, S phase; *L-M*, fractions 18 and 19, S/G_2 phase; and *N-S*, fractions 20-25, G_2+M phase. The flow rates and cell recoveries for each elutriation fraction are shown in Table I. The cell cycle profiles are from a single experiment and are representative of six independent experiments.

Table I. *Elutriation flow rates and recovery of CTLL-2 cells*

Elutriation Fraction	Flow Rate (ml/min)	Percent Recovered	Cell Cycle Phase
8	12	1.0	
9	14	10.2	
10	15.5	7.7	
11	17	7.2	G_1
12	18	11.8	
13	19.5	6.1	
14	21	8.5	
15	22.5	9.3	
16	24.5	4.9	S
17	25.5	4.9	
18	26.5	4.0	
19	28.5	5.3	
20	30	5.3	
21	31	4.2	G_2+M
22	32	2.8	
23[a]	34	4.1	
24	35.5	1.2	
25	37	1.4	

[a]Peak of CD45 PTPase activity.

experiments because of limitations in the precision of the peristaltic pump. In the asynchronous culture used in the elutriation analyzed in this paper, 43% of the cells were in G_1, 29% in S, and 28% in G_2+M (Fig. 1*A*).

The PTPase activity of CD45 immunoprecipitates was measured for the elutriated fractions and, at flow rates from 34-36 ml/min, was consistently 2- to 5-fold higher than in other fractions. A representative CD45 PTPase enzymatic profile obtained from six independently-elutriated log-phase cultures showed recovery of maximal CD45 PTPase activity as a peak (Fig. 2, fraction 23). Additionally, CD8 was immunoprecipitated as a control. Anti-CD8 immunoprecipitates of each elutriation fraction were found to lack PTPase activity thus confirming that PTPase activity did not nonspecifically associate with protein G/antibody complexes (Fig. 2). A fairly high basal level of CD45 PTPase activity was observed in these cells. Analysis of the DNA content of cells from these elutriation fractions by FACS indicated that the peak of CD45 PTPase activity occurred in the G_2+M population (Fig. 3).

FACS analysis of FITC-anti-CD45-labelled cells from each elutriation fraction showed that the increase in CD45 PTPase activity was not simply the result of an increase in the amount of CD45 present in the cells of the G_2+M fractions (Fig. 4). The relative amount of CD45 was estimated by mean fluorescence measured by FACS analysis (24). There was a relatively minor increase in mean green fluorescence of elutriated cells during cell cycle progression. The method used for fixation of the cells resulted in permeabilization of the plasma membrane and permitted effective labelling of both cytoplasmic and membrane-associated CD45 protein. Cell size, as

Figure 2. CTLL-2 CD45 PTPase activity during cell cycle progression. The relative PTPase activity of CD45 in each elutriation fraction (■) is expressed as the cpm of the $^{32}P_i$ released from ^{32}P-raytide per min per mg protein in the lysate from which CD45 was immunoprecipitated. The PTPase activities of the isotype-matched control immunoprecipitates, *i.e.* CD8 immunoprecipitates, are also presented (▲). Cell cycle histograms for fractions 8-25 are given in Fig. 1 *panels B-S*, respectively.

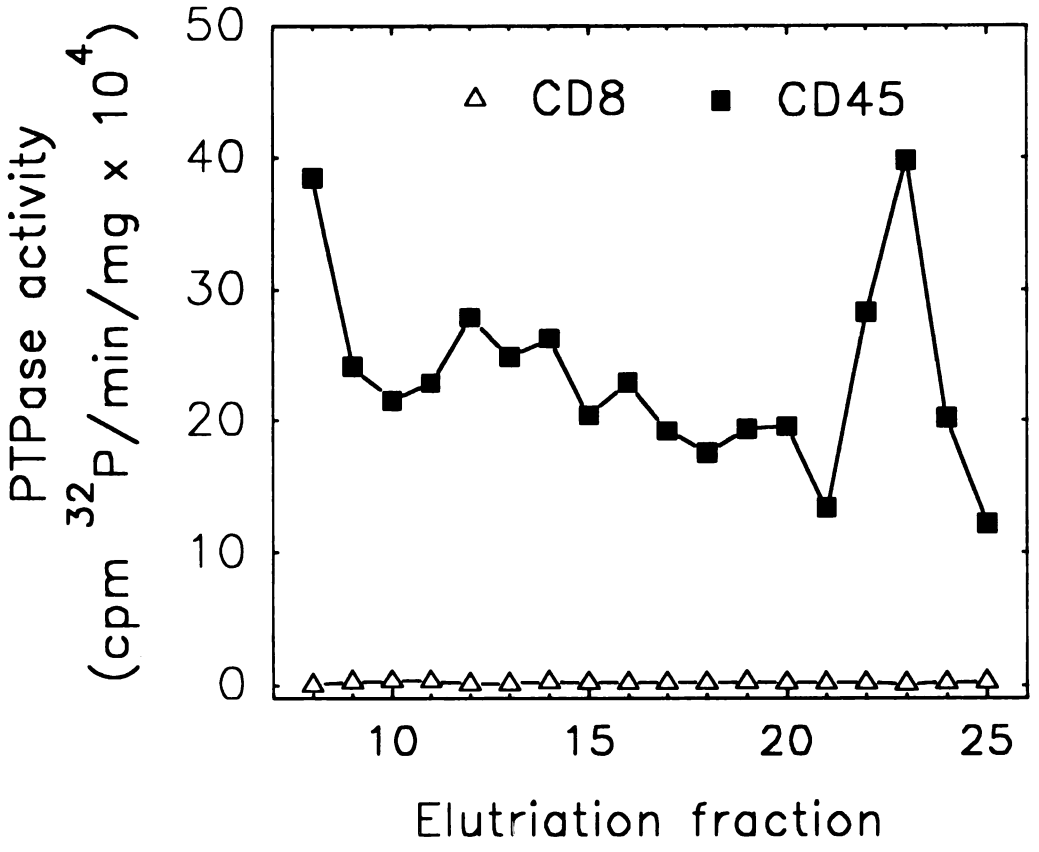
PTPase activity
(cpm ^{32}P/min/mg x 10^4)
50
40
30
20
10
0
CD8
CD45
10
15
20
25
Elutriation fraction

Figure 2.

Figure 3. Cell cycle stage analysis of CTLL-2 elutriation fractions. The percent of cells in each stage of the cell cycle, based on PI fluorescence depicted in Fig. 1, is presented for each elutriation fraction analyzed in Fig. 2. The fraction with the peak CD45 PTPase activity is noted with an asterisk.

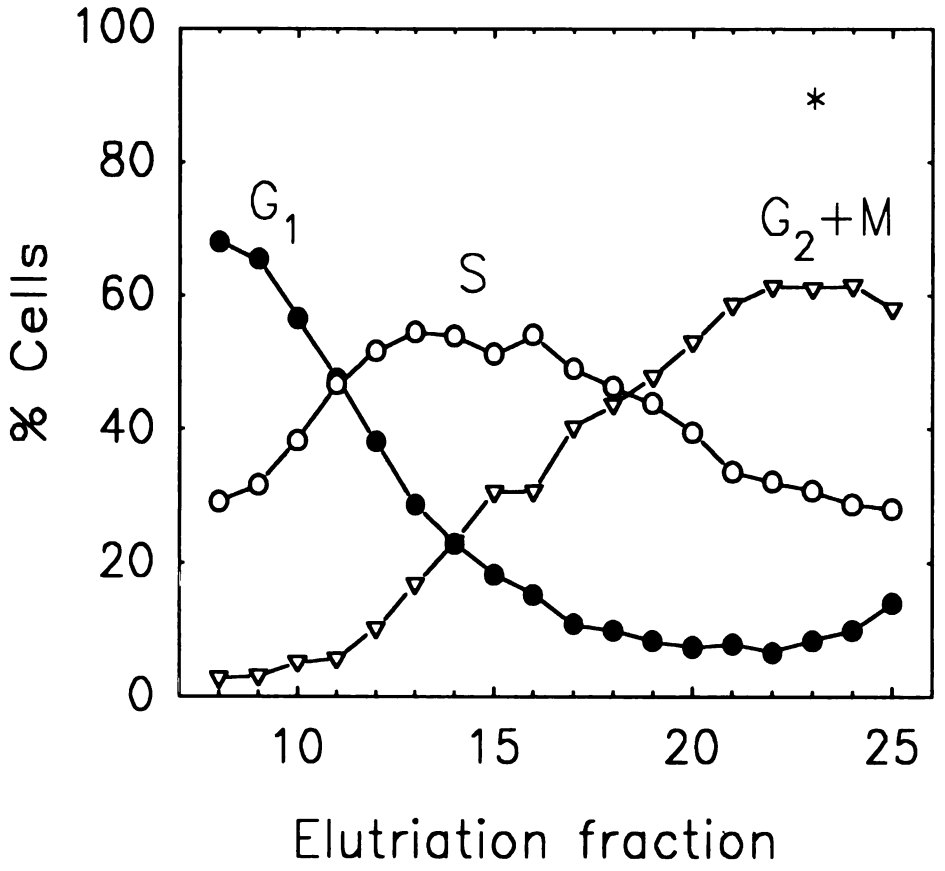
100
80
60
40
20
0
% Cells
G1
S
G2+M
*
10
15
20
25
Elutriation fraction

Figure 3.

Figure 4. CD45 fluorescence of elutriation fractions. CD45 expression of elutriated populations was compared by analysis of the mean FITC-anti-CD45 fluorescence exhibited by each elutriated fraction. This analysis is representative of six independent experiments. Peak CD45 PTPase activity occurred in fraction 23, denoted by an asterisk.

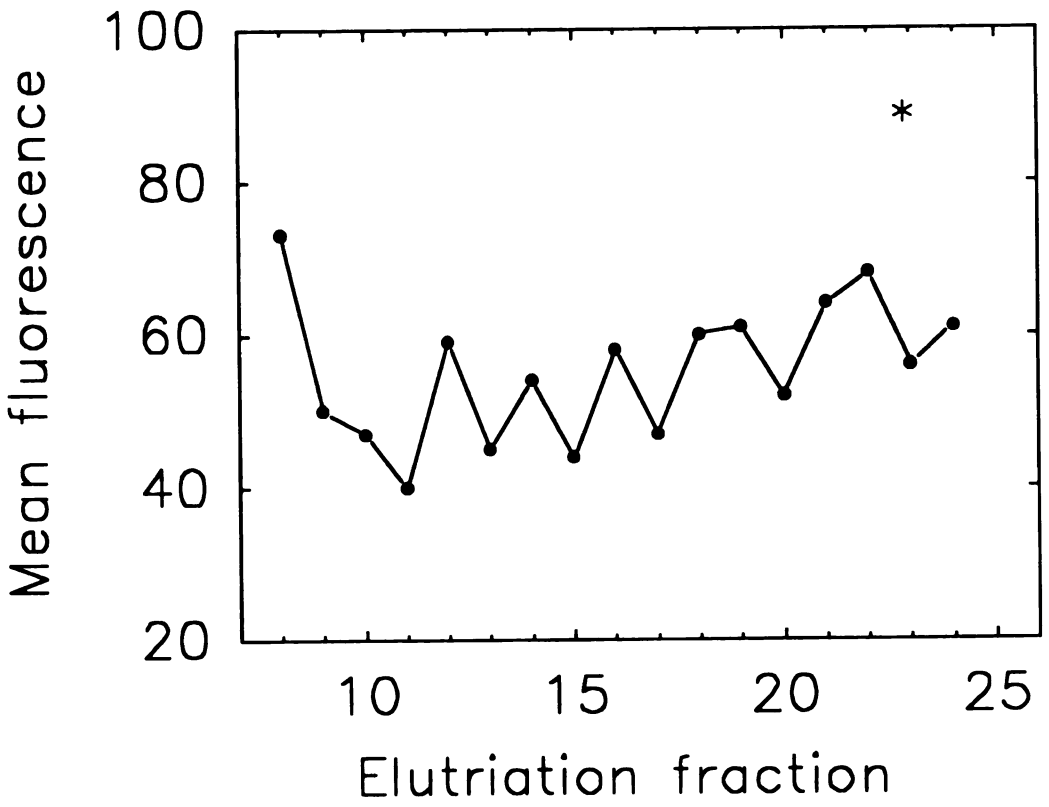
100
80
60
40
20
Mean fluorescence
*
10
15
20
25
Elutriation fraction

Figure 4.

estimated by mean forward scatter, also increased proportionately, indicating that the relative amount of CD45 did not increase more than would correspond to the size increase of the cells (data not shown).

The amount of CD45 present in cells from each elutriation fraction was also determined by SDS-PAGE analysis of CD45 immunoprecipitates. Immunoprecipitates that were used for the PTPase assay were separated by SDS-PAGE and proteins were visualized by silver staining (Fig. 5). This analysis demonstrated that CD45 immunoprecipitates contained only CD45 along with immunoglobulin heavy (about 50 kD) and light chains (about 23 kD) from the monoclonal antibody (Fig. 5*A*). Importantly, no significant increases in CD45 protein levels were observed upon comparison of elutriation fractions with basal levels of CD45 PTPase (Fig. 5*A*, fractions up to 21) to those with elevated CD45 PTPase (Fig. 5*A*, fractions 22 and 23). For comparison, SDS-PAGE analysis of the PTPase-negative, anti-CD8 immunoprecipitates indicated the presence of CD8α along with immunoglobulin heavy and light chains and only a few other contaminating proteins (Fig. 5*B*).

These results confirm that the increase in CD45 PTPase activity in G_2+M resulted primarily from an increase in relative PTPase activity rather than an increase in protein expression. A consistent amount of CD45 was immunoprecipitated from samples since all the conditions for immunoprecipitation were performed on a per cell basis and each PTPase activity measurement was adjusted to the level of protein present in the cell lysate.

Following fractionation by elutriation, cells were examined by staining with DAPI to ascertain the mitotic nature of the cells in the fraction exhibiting elevated

Figure 5. CTLL-2 expression of CD45 and CD8 through the cell cycle. The amounts of CD45 and CD8 proteins present in immunoprecipitates were determined by silver staining of SDS-PAGE-separated proteins. (*A*) CD45 immunoprecipitates from elutriation fractions showing CD45 (arrow) accompanied by immunoglobulin heavy (about 50 kD) and light chains (about 23 kD). (*B*) Control immunoprecipitates from elutriation fractions showing the position of CD8α (arrow) with immunoglobulin heavy and light chains.

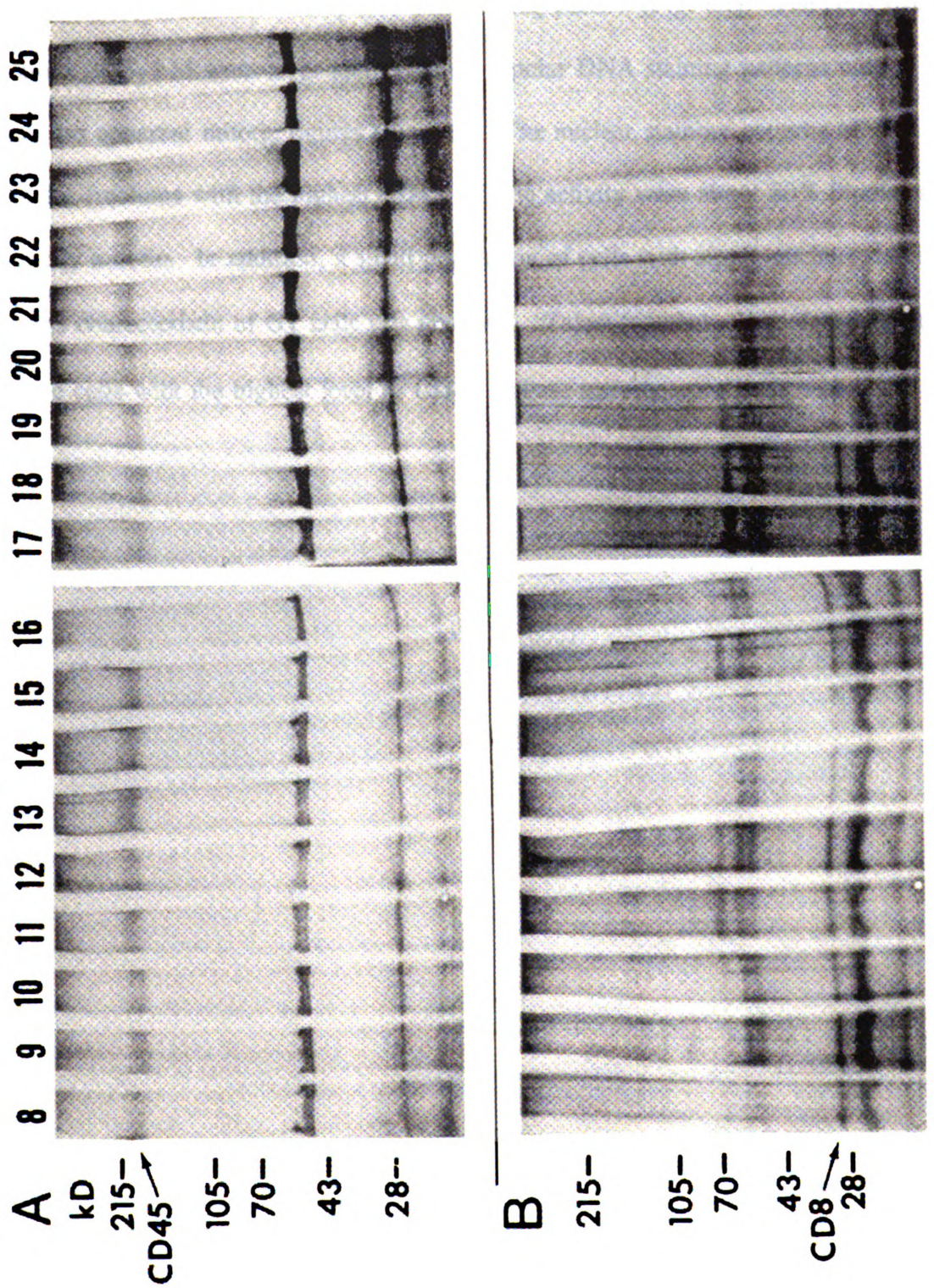
A
kD
215–
CD45
105–
70–
43–
28–
8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
B
215–
105–
70–
43–
CD8
28–

Figure 5.

CD45 PTPase activity. Cells recovered in early G_1, as determined by PI analysis, were small and exhibited a compact DNA staining pattern (Fig. 6*A*). The cells from fractions in G_2+M were large and exhibited bipolar DNA staining patterns with numerous apparent mitotic figures (Fig. 6*B*). The nuclear staining patterns of cells from the fractions with the highest CD45 PTPase activity were about 50% bipolar and distinctly mitotic. In addition, a small population of cells with single-nucleus staining patterns characteristic of G_1 cells was observed. Taken together, these data indicated that the cells with the highest level of CD45 PTPase activity were in G_2+M.

Figure 6. Fluorescent microscopy images of elutriated CTLL-2 cell populations. The DNA of elutriated cell populations was stained with DAPI and analyzed by microscopy. (*A*) Representative cells from a G_1 phase-enriched fraction. (*B*) Representative cells from the G_2+M fraction with the peak CD45 PTPase activity.

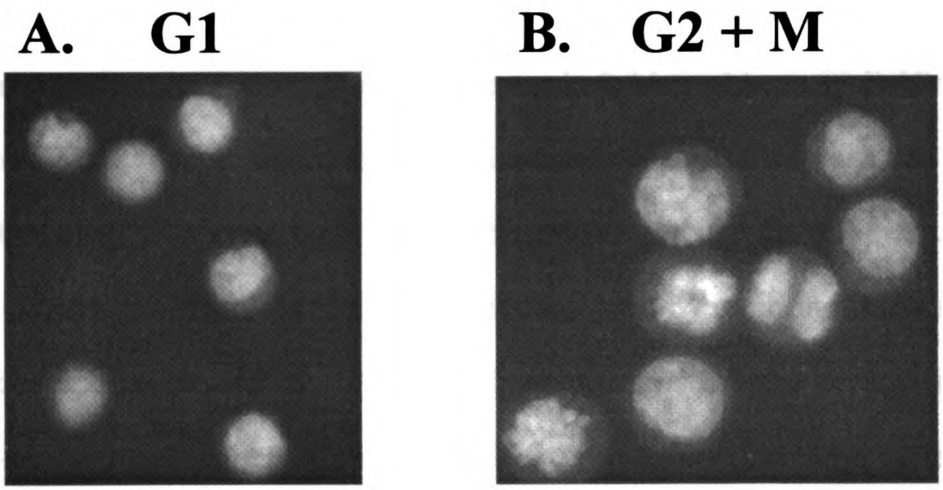

Figure 6.

DISCUSSION

Although CD45 clearly functions in antigen activation of B and T lymphocytes, its abundance on other hematopoietic cells suggests that it may also play a role in other basic immunological and cellular phenomena. Since it is believed that CD45 interacts with cytoskeletal microfilaments via fodrin and because dividing cells undergo extensive microfilament reorganization, we investigated the potential modulation of CD45 PTPase activity in the progression through the cell cycle. CD45 PTPase activity was examined in CTLL-2 cells separated by centrifugal elutriation. Cell cycle-dependent expression of CD45 PTPase activity reached a maximum in G_2+M-enriched fraction. This fraction also contained cells with bipolar nuclear staining patterns and some small G_1-type cells. The small cells in these fractions were likely cells that had just completed cytokinesis during the time of separation in the elutriator and during preparation of the cells for analysis. Once initiated, cytokinesis is believed to proceed in a very short period of time. The possibility that doublets were in the analyzed populations was eliminated by microscopic examination of the elutriated subpopulations. Typically, doublets appeared after the flow rate reached 36-40 ml/min and were not present in significant numbers in the subpopulations analyzed for CD45 PTPase activity. These observations suggest that peak CD45 PTPase activity occurred in G_2+M cells and may have occurred in cells

undergoing cytokinesis. The presence of elevated CD45 PTPase activity during cytokinesis would be consistent with a role for CD45 in the regulation of microfilament organization.

In other studies with 70Z cells, it was determined that the increase in CD45 PTPase activity was placed among events occurring after anaphase by the observation that the highest PTPase activity was found in elutriation fractions immediately following the disappearance of the 62 kD cyclin B protein (data not shown). Cyclin B accumulates within a cell as it proceeds through the cell cycle, associating with $p34^{cdc2}$ kinase forming MPF or M-phase specific histone H1 kinase (25, 26). The inactive MPF kinase complex attains a maximum level at prophase and becomes activated via the dephosphorylation of $p34^{cdc2}$ (27-29). Following activation, cells enter metaphase and the cyclin B/$p34^{cdc2}$ complex degrades by the completion of anaphase (30). The observation that the peak of CD45 PTPase activity was apparent only after cyclin B degradation supports the hypothesis that CD45 PTPase participates in cytokinesis.

The results of analysis of cell cycle-dependent CD45 PTPase activity in the CTLL-2 T cell line supports the hypothesis that CD45 plays a fundamental role in mitosis. However, the CTLL-2 line consistently displayed a high basal level of CD45 PTPase activity throughout the cell cycle. The specificity of the observed CD45 PTPase activity in CTLL-2 cells was confirmed by the parallel precipitation with the isotype-matched control antibody, anti-CD8. Even though CD8 was effectively precipitated with this antibody, there was no PTPase activity associated with these

immunoprecipitates. The PTPase activity measured in these experiments was found to be an accurate and reproducible measure of relative activity for each experiment.

In recent studies, CD45 was shown to contain a fodrin/spectrin binding domain (14). Fodrin has been shown to mediate association of actin filaments with the plasma membrane, leading to the proposal that it serves in membrane stabilization by linking the cytoskeleton to the plasma membrane (31, 32). During cellular activation, fodrin has been shown to serve as a kinase substrate, and this cytoskeletal protein is isolated in close association with CD45 (14). Importantly, fodrin binding to CD45 enhanced CD45 PTPase activity 7.5 fold (14). Thus, the increase in CD45 PTPase activity observed during late mitosis in this study may have resulted from binding to a cytoskeletal element such as fodrin. This increased activity may cause the dephosphorylation of fodrin or other microfilament proteins associated with reorganization of actin microfilaments during cytokinesis.

Alternatively, elevation of CD45 PTPase activity could be due to modification of phosphorylation of CD45 itself. Phosphorylation of tyrosine residues within CD45 has been detected after lectin or anti-CD3 activation of Jurkat cells (33) and after *in vitro* treatment of CD45 with $p50^{csk}$ PTK (13). Importantly, in the latter report, the activity of CD45 increased several-fold after tyrosine phosphorylation by $p50^{csk}$ (13). Serine residues on CD45 have also been shown to be phosphorylated in response to T cell treatment with phorbol esters via protein kinase C (34), after *in vitro* treatment with casein kinase 2 and other serine/threonine kinases (35), and after IL-2 treatment of CTLL-2.4 cells (36). No modulation of CD45 PTPase activity was observed after phosphorylation in the latter two reports. However, down-regulation of CD45

PTPase activity after addition of Ca^{2+} ionophores, *i.e.* ionomycin or A23187, to T cells coincided with a decrease in serine phosphorylation (12). Thus, the increase in CD45 activity observed in mitosis may be a consequence of serine/threonine or tyrosine phosphorylation. Further experiments will be necessary to clarify this issue.

Recent investigation into the role of PTPases in the cell cycle has led to the discovery that cdc25, a PTPase, was essential for activation of MPF and for allowing entry into and progression through mitosis (28-30). In addition, over-expression of a truncated form of another PTPase, TC.PTP, has been shown to result in cytokinetic failure (37). These data support the potential involvement of tyrosine phosphorylation in signalling events coordinating cell division.

These data and others support the concept that CD45 may have an important role in the cellular mechanism regulating mitosis or cytokinesis. The observation of a 2- to 5-fold increase in CD45 PTPase activity during mitosis in CTLL-2 and 70Z cell lines suggests that phosphorylation of CD45 or association of CD45 with fodrin (or fodrin-like molecules) during mitosis may result in the activation of a CD45 PTPase domain. Further investigation into the phosphorylation state of CD45 and associated proteins such as fodrin should provide information regarding the regulation of CD45 PTPase activity in mitosis.

ACKNOWLEDGMENTS

I thank Dr. L. Melkerson-Watson (Michigan State University, East Lansing, MI) for her advice and assistance in this work, Dr. S. Conrad (Michigan State University, East Lansing, MI) for use of the JE-6B elutriation system, Dr. L. King (Michigan State University, East Lansing, MI) for technical support and assistance with the FACS, and Dr. J. Dixon (University of Michigan, Ann Arbor, MI) for his generous gift of src kinase.

REFERENCES

1. Trowbridge, I. S. 1991. CD45. A prototype for transmembrane protein tyrosine phosphatases. *J. Biol. Chem.* 266:23517.

2. Tonks, N. K., H. Charbonneau, C. D. Diltz, E. H. Fischer, and K. A. Walsh. 1988. Demonstration that the leukocyte common antigen CD45 is a protein tyrosine phosphatase. *Biochemistry.* 27:8695.

3. Johnson, P., H. L. Ostergaard, C. Wasden, and I. S. Trowbridge. 1992. Mutational analysis of CD45. A leukocyte-specific protein tyrosine phosphatase. *J. Biol. Chem.* 267:8035.

4. Williams, A. F., and A. N. Barclay. 1986. Glycoprotein antigens of the lymphocyte surface and their purification by antibody affinity chromatography. In *Handbook of Experimental Immunology, 4th ed., vol. 1.* D. M. Weir, L. A. Herzenberg, C. Blackwell, and L. A. Herzenberg, eds. Blackwell Scientific Publications, Oxford, p. 22.1.

5. Koretzky, G. A. 1993. Role of the CD45 tyrosine phosphatase in signal transduction in the immune system. *Faseb. J.* 7:420.

6. Pingel, J. T., and M. L. Thomas. 1989. Evidence that the leukocyte-common antigen is required for antigen-induced T lymphocyte proliferation. *Cell.* 58:1055.

7. Koretzky, G. A., J. Picus, M. L. Thomas, and A. Weiss. 1990. Tyrosine phosphatase CD45 is essential for coupling T-cell antigen receptor to the phosphatidyl inositol pathway. *Nature.* 346:66.

8. Justement, L. B., K. S. Campbell, N. C. Chien, and J. C. Cambier. 1991. Regulation of B cell antigen receptor signal transduction and phosphorylation by CD45. *Science.* 252:1839.

9. Weaver, C. T., J. T. Pingel, J. O. Nelson, and M. L. Thomas. 1991. CD8+ T-cell clones deficient in the expression of the CD45 protein tyrosine

phosphatase have impaired responses to T-cell receptor stimuli. *Mol. Cell. Biol.* 11:4415.

10. Schraven, B., A. Schirren, H. Kirchgessner, B. Siebert, and S. C. Meuer. 1992. Four CD45/P56^{lck}-associated phosphorproteins (pp29-pp32) undergo alterations in human T cell activation. *Eur. J. Immunol.* 22:1857.

11. Ostergaard, H. L., and I. S. Trowbridge. 1990. Coclustering CD45 with CD4 or CD8 alters the phosphorylation and kinase activity of p56^{lck}. *J. Exp. Med.* 172:347.

12. Ostergaard, H. L., and I. S. Trowbridge. 1991. Negative regulation of CD45 protein tyrosine phosphatase activity by ionomycin in T cells. *Science.* 253:1423.

13. Autero, M., J. Saharinen, T. Pessa-Morikawa, M. Soula-Rothhut, C. Oetken, M. Gassmann, M. Bergman, K. Alitalo, P. Burn, C. G. Gahmberg, and T. Mustelin. 1994. Tyrosine phosphorylation of CD45 phosphotyrosine phosphatase by p50^{csk} kinase creates a binding site for p56^{lck} tyrosine kinase and activates the phosphatase. *Mol. Cell. Biol.* 14:1308.

14. Lokeshwar, V. B., and L. Y. Bourguignon. 1992. Tyrosine phosphatase activity of lymphoma CD45 (GP180) is regulated by a direct interaction with the cytoskeleton. *J. Biol. Chem.* 267:21551.

15. Lin, J., V. K. Brown, and L. B. Justement. 1992. Regulation of basal tyrosine phosphorylation of the B cell antigen receptor complex by the protein tyrosine phosphatase, CD45. *J. Immunol.* 149:3182.

16. Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt. Biochem.* 72:248.

17. Streuli, M., N. X. Krueger, A. Y. Tsai, and H. Saito. 1989. A family of receptor-linked protein tyrosine phosphatases in humans and Drosophila. *Proc. Natl. Acad. Sci. U. S. A.* 86:8698.

18. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227:680.

19. Gorg, A., W. Postel, J. Weser, H. W. Schiwara, and W. M. Boesken. 1985. Horizontal SDS electrophoresis in ultrathin pore-gradient gels for the analysis of urinary proteins. *Science Tools (Sweden)* 32:5.

20. Feder, J. N., C. J. Guidos, B. Kusler, C. Carswell, D. Lewis, and R. T. Schimke. 1990. A cell cycle analysis of growth-related genes expressed during T lymphocyte maturation. *J. Cell. Biol.* 111:2693.

21. Catron, K. M., J. M. Purkerson, P. C. Isakson, and T. P. Bender. 1992. Constitutive versus cell cycle regulation of c-myb mRNA expression correlates with developmental stages in murine B lymphoid tumors. *J. Immunol.* 148:934.

22. Bruyninckx, W. J., W. H. Vanneste, P. J. Leijh, R. Van-Furth, and R. E. Vercauteren. 1990. Characterization of the efficiency of a cell separation process by the extent of elimination of a contaminating cell type. *Anal. Biochem.* 191:144.

23. Page, D. M., and A. L. DeFranco. 1990. Antigen receptor-induced cell cycle arrest in WEHI-231 B lymphoma cells depends on the duration of signaling before the G1 phase restriction point. *Mol. Cell. Biol.* 10:3003.

24. Ostergaard, H. L., D. A. Shackelford, T. R. Hurley, P. Johnson, R. Hyman, B. M. Sefton, and I. S. Trowbridge. 1989. Expression of CD45 alters phosphorylation of the lck-encoded tyrosine protein kinase in murine lymphoma T-cell lines. *Proc. Natl. Acad. Sci. U. S. A.* 86:8959.

25. Arion, D., L. Meijer, L. Brizuela, and D. Beach. 1988. cdc2 is a component of the M phase-specific histone H1 kinase: evidence for identity with MPF. *Cell.* 55:371.

26. Labbe, J. C., M. G. Lee, P. Nurse, A. Picard, and M. Doree. 1988. Activation at M-phase of a protein kinase encoded by a starfish homologue of the cell cycle control gene cdc2+. *Nature.* 335:251.

27. Hoffmann, I., P. R. Clarke, M. J. Marcote, E. Karsenti, and G. Draetta. 1993. Phosphorylation and activation of human cdc25-C by cdc2--cyclin B and its involvement in the self-amplification of MPF at mitosis. *Embo. J.* 12:53.

28. Clarke, P. R., I. Hoffmann, G. Draetta, and E. Karsenti. 1993. Dephosphorylation of cdc25-C by a type-2A protein phosphatase: specific regulation during the cell cycle in Xenopus egg extracts. *Mol. Biol. Cell.* 4:397.

29. Millar, J. B., P. Russell, J. E. Dixon, and K. L. Guan. 1992. Negative regulation of mitosis by two functionally overlapping PTPases in fission yeast. *Embo. J.* 11:4943.

30. Doree, M. 1990. Control of M-phase by maturation-promoting factor. *Curr. Opin. Cell. Biol.* 2:269.

31. Coleman, T. R., D. J. Fishkind, M. S. Mooseker, and J. S. Morrow. 1989. Functional diversity among spectrin isoforms. *Cell. Motil. Cytoskeleton.* 12:225.

32. Bennett, V. 1990. Spectrin-based membrane skeleton: a multipotential adaptor between plasma membrane and cytoplasm. *Physiol. Rev.* 70:1029.

33. Stover, D. R., H. Charbonneau, N. K. Tonks, and K. A. Walsh. 1991. Protein-tyrosine-phosphatase CD45 is phosphorylated transiently on tyrosine upon activation of Jurkat T cells. *Proc. Natl. Acad. Sci. U. S. A.* 88:7704.

34. Autero, M., and C. G. Gahmberg. 1987. Phorbol diesters increase the phosphorylation of the leukocyte common antigen CD45 in human T cells. *Eur. J. Immunol.* 17:1503.

35. Tonks, N. K., C. D. Diltz, and E. H. Fischer. 1990. CD45, an integral membrane protein tyrosine phosphatase. Characterization of enzyme activity. *J. Biol. Chem.* 265:10674.

36. Valentine, M. A., M. B. Widmer, J. A. Ledbetter, F. Pinault, R. Voice, E. A. Clark, B. Gallis, and D. L. Brautigan. 1991. Interleukin 2 stimulates serine phosphorylation of CD45 in CTLL-2.4 cells. *Eur. J. Immunol.* 21:913.

37. Cool, D. E., P. R. Andreassen, N. K. Tonks, E. G. Krebs, E. H. Fischer, and R. L. Margolis. 1992. Cytokinetic failure and asynchronous nuclear division in BHK cells overexpressing a truncated protein-tyrosine-phosphatase. *Proc. Natl. Acad. Sci. U. S. A.* 89:5422.

CHAPTER THREE

CELL CYCLE-RELATED EXPRESSION OF A CD45-ASSOCIATED SERINE-THREONINE KINASE[1]

FOOTNOTES

[1] This work was supported by NIH grant GM35774.

[2] Abbreviations used in this paper: PTPase, protein tyrosine phosphatase; PTK, protein tyrosine kinase; PAO, phenylarsine oxide; SH2, src homology region 2; PI, propidium iodide; DTBP, dimethyl 3,3'-dithiobispropionimidate; PVDF, polyvinylidene difluoride; ECL, Enhanced ChemiLuminescence; PHA, phytohemagglutinin; LPAP, lymphocyte phosphatase-associated phosphoprotein.

ABSTRACT

The protein tyrosine phosphatase (PTPase) CD45 is essential to couple antigen receptor stimulation to second messenger signalling pathways in T and B cells. In this role, CD45 appears to regulate src family kinases, and the use of mild detergents has led to the identification of complexes of CD45 with $p56^{lck}$ in T cells and $p53^{lyn}$ in B cells. Although it has been hypothesized that CD45 also has a role in mitosis, the accessory proteins in this role have not been identified. In this paper, we report that a serine-threonine kinase is associated with CD45 throughout the cell cycle in CTLL-2 cells. Centrifugal elutriation was used to obtain subpopulations of cells enriched for the various stages of the cell cycle. Two approaches, chemical cross-linking and lysis with a mild detergent, were used to identify accessory proteins which coprecipitate with CD45. Proteins of 60 to 70 kD were found in CD45 immunoprecipitates arising from both procedures. In *in vitro* phosphorylation reactions, these bands were labelled on serine and, to a lesser extent, threonine residues. The *in vitro* phosphorylation by the serine-threonine kinase in the CD45 immunoprecipitates was elevated in late G_2+M-enriched samples, which correspond to the time in the cell cycle when the PTPase activity of CD45 is elevated.

INTRODUCTION

The leukocyte common antigen, CD45, is a family of heterogeneous transmembrane proteins which have been identified in numerous mammals, as well as in chickens and a shark species (reviewed in 1, 2). The extracellular region of CD45 varies as a result of cell lineage- and activation state-determined exon usage (1). In contrast to the variation seen in the extracellular region of CD45, the intracellular region of CD45 does not vary among leukocytes within an organism and is highly conserved among mammals. The intracellular region of CD45 contains two active PTPase[2] catalytic domains (3-6). CD45 is abundant on leukocyte membranes and may constitute up to 10% of leukocyte surface glycoproteins (7, 8). Both the abundance and conservation of CD45 in the hematopoietic system suggest that it is involved in one or more basic roles critical to leukocyte function.

Indeed, CD45 is essential for activation of B and T lymphocytes through their antigen and accessory receptors. CD45-deficient $CD4^+$ and $CD8^+$ T cell lines fail to proliferate in response to TCR/CD3 stimulation, and, in addition, CD45-deficient $CD8^+$ cells have diminished capacity to produce cytokines and lyse cells after stimulation (9, 10). CD45 deficiency uncouples TCR and CD2 stimulation from both the tyrosine phosphorylation and phosphatidyl inositol second messenger pathways, which are the initial responses detected after TCR or CD2 stimulation (11-14). CD45

expression is also required to couple the phosphatidyl inositol pathway to stimulation through the membrane IgM antigen receptor on B cells (15). Normal tyrosine phosphorylation and Ca^{2+} flux responses to antigen receptor stimulation are restored to $CD45^-$ T and B cells when the intracellular region of CD45 is expressed in the cells, even when the external CD45 region is absent or replaced by MHC class I or epidermal growth factor receptor extracellular sequences (15-19).

In addition to its role in the activation of lymphocytes, CD45 appears to have a role in mitosis (20). Using centrifugal elutriation to obtain subpopulations of asynchronous B and T cell cultures which were enriched for cells in various stages of the cell cycle, it was determined that CD45 PTPase activity is elevated 2- to 5-fold in G_2+M when compared to its activity during other stages of the cell cycle.

In the activation of T cells, CD45 may regulate the activity of both $p56^{lck}$ and $p59^{fyn}$, two src family PTKs which are involved in the response to TCR/CD3 stimulation (16, 21-28). Analyses of CD45 immunoprecipitates from digitonin or Brij 96 lysates of human peripheral blood T lymphocytes and cultured human and murine T cells have shown that CD45 associates with $p56^{lck}$ and a group of phosphoproteins of variable molecular weights, pp29-34 (29-33). Likewise, $p53^{lyn}$, a B cell PTK implicated in the response to antigen, has been coprecipitated with CD45 from B cells (34).

Phosphotyrosine-containing proteins of 55-62 kD have also been found in CD45 immunoprecipitates from Triton X-100 lysates of human peripheral blood T lymphocytes after treatment of the cells with PAO, a PTPase inhibitor (35). When $p56^{lck}$ was immunoprecipitated from PAO-treated, periodate-NaB^3H_4 surface-labelled

cells, tritiated cell-surface glycoproteins of 180-205 kD were found in the $p56^{lck}$ immunoprecipitates. Autero *et al.* (35) proposed that these data indicate that $p56^{lck}$ and tyrosine-phosphorylated CD45 form a stable complex in cells. *In vitro* studies using purified CD45 and recombinant $p56^{lck}$ supported the theory that $p56^{lck}$ binds to tyrosine-phosphorylated CD45 via the SH2 domain of the PTK.

CD45 also interacts with cytoskeletal elements during its capping on B cells and during capping of either CD45 or Thy-1 on a thymocyte cell line (36-38). Moreover, binding of fodrin to CD45 increases its PTPase activity 7.5-fold (39).

The present study was undertaken to determine what proteins interact with CD45 in the progression through the cell cycle. In particular, we wished to determine which proteins interact with CD45 when it reaches peak activity in the G_2+M phases of the cell cycle. Centrifugal elutriation was used to obtain cell cycle stage-enriched subpopulations of CTLL-2 cells, and proteins which coprecipitated with CD45 were examined.

MATERIALS AND METHODS

Cell lines and antibodies

Cell lines were obtained from the American Type Culture Collection (Rockville, MD). The cytolytic T cell line CTLL-2 was maintained at 37°C in a humidified environment with 7.5% CO_2 in RPMI 1640 (GIBCO/BRL, Gaithersburg, MD) with 10% FBS (GIBCO/BRL), 15 mM HEPES (Boehringer Mannheim Biochemicals, Indianapolis, IN), 50 μM 2-ME (Sigma Chemical Co., St. Louis, MO), and 7 U/ml recombinant IL-2 (Cetus Corporation, Emeryville, CA) (complete medium). Anti-CD45 mAb was prepared from the M1/9.3.4 hybridoma line as were the control mAbs: 33D1, an anti-dendritic-cell receptor mAb, and 53-6.72, an anti-CD8α mAb. Anti-fyn, anti-lck and anti-src polyclonal antibodies, which are specific for the respective mouse as well as human proteins, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Counterflow centrifugal elutriation

Approximately 4 x 10^8 exponentially-growing cells were loaded into the standard elutriation chamber of a JE-6B rotor in a J2-21M/E centrifuge (Beckman Instruments, Palo Alto, CA) at 2250 RPM and 12°C with an initial buffer flow rate of 4.5 ml/min produced by a Masterflex peristaltic pump (Cole Palmer Instruments, Chicago, IL). Fractions were elutriated in Hank's balanced salt solution (GIBCO/BRL) with 1

mg/ml bovine serum albumin (Sigma). Fractions were collected for 5 min at each increment of flow rate, which was typically 1 to 2 ml/min.

In some experiments, cells collected aseptically during the elutriation were resuspended at 5 x 10^6 cells/ml in complete medium and recultured in standard temperature and CO_2 conditions for 4 to 4.5 hrs, at which time the cells were harvested and treated as were the samples collected directly from the elutriator.

Cell cycle analysis

For each sample, 1 x 10^6 cells were fixed by adding 1.2 ml ice-cold 70% ethanol to cells suspended in 0.4 ml 50% PBS (8 mM Na_2HPO_4, 1 mM KH_2PO_4, 137 mM NaCl, 3 mM KCl, pH 7.3), 50% FBS and storing the suspension for approximately 24 hr at -20°C. To stain the DNA of the fixed cells, the cells were washed twice in PBS with 5% (v/v) FCS and suspended in PI staining solution (PBS with 0.1% [v/v] Triton X-100 [Boehringer Mannheim], 0.1 mM EDTA [Sigma], 50 μg/ml RNase A [Boehringer Mannheim], and 50 μg/ml PI [Sigma]) for 1 hr at room temperature. Fluorescence of the singlet population was analyzed on a Becton Dickinson Vantage FACS (San Jose, CA). Cell cycle analysis was performed using PC-Lysys version 1.0 software (Becton Dickinson).

Cross-linking and cytoskeletal preparations

Cells were washed twice with PBS and suspended at 6 x 10^6 cells/ml in PBS, pH 8, with 5 mM $MgCl_2$ and 1 mM Na_3VO_4 (Sigma), and with or without 3 mM DTBP (Sigma) (40). The suspension was gently shaken every 5 min for 30 min at room temperature. Cross-linking was quenched by the addition of Tris, pH 7.4, to a final concentration of 50 mM, and the cells were washed twice with PBS. Cells were

lysed at 1 x 10^8 cells/ml in a hypotonic, alkaline buffer (10 mM Tris, pH 8.9, 1 mM EGTA, 1 mM PMSF) and rocked for 30 min at 4°C before the insoluble cytoskeletal lattice was pelleted by centrifugation at 10,000 g for 10 min at 4°C (40). After the supernatant was transferred to a new tube, its pH was neutralized by the addition of 20 μl 1 M Tris, pH 7.0, per ml. Proteins were extracted from the insoluble cytoskeletal pellet for 30 min at 4°C on a rocker platform in Triton X-100 extraction buffer (50 mM Tris, pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, 0.5% Triton X-100, 1 mM PMSF). The suspension was centrifuged at 10,000 g for 10 min at 4°C, and the supernatant was transferred to a new tube. Proteins were further extracted from the remaining insoluble pellet for 30 min at 4°C on the rocker platform in Triton X-100/sarcosyl extraction buffer (50 mM Tris, pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, 0.5% Triton X-100, 0.5% N-lauroylsarcosine [Sigma], 1 mM PMSF). After 10 min centrifugation at 10,000 g and 4°C, the supernatant was transferred to a new tube. In the extraction steps, 1 ml of Triton X-100 extraction buffer was used per 10^8 cells, and 1 ml of Triton X-100/sarcosyl extraction buffer was used per 2 x 10^8 cells. Bradford protein microassays were performed using 5 μl aliquots of the extracts (41).

For immunoprecipitation, each extract was divided into two aliquots, and either CD45 or CD8α was immunoprecipitated from each aliquot for 1 hr at 4°C using mAbs M1/9.3.4 or 53-6.72, respectively, precoupled to protein G-agarose (Pharmacia, Piscataway, NJ). Immunoprecipitate pellets were washed once with PBS then 0.5 M LiCl, pH 7.4, and twice with 50 mM Tris, pH 8.

Coprecipitation of proteins with CD45 from digitonin lysates

A stock of 10% digitonin was prepared as described by Schraven *et al.* (29). Digitonin (Sigma) was dissolved in boiling H_2O for 2 min with stirring. After the suspension was stored at room temperature for at least 3 days, the insoluble material was removed by filtration through a 0.4 μm filter.

Cells were washed twice with PBS and lysed at 2.5 x 10^7 cells/ml in digitonin lysis buffer (20 mM Tris, pH 7.4, 140 mM NaCl, 1% digitonin, 1 mM PMSF, 1 μg/ml aprotinin, 0.7 μg/ml pepstatin A, 10 mM NaF, 1 mM Na_3VO_4, 10 mM $Na_4P_2O_7$). Postnuclear lysates were precleared with 100 μl of packed protein G-agarose and 100 μl of packed protein G-agarose precoupled to 33D1 per ml lysate. Bradford protein assays were performed on 5 μl aliquots of the lysates. Immunoprecipitation was done for 1 hr at 4°C using M1/9.3.4 mAb precoupled to protein G-agarose. Immunoprecipitate pellets were washed once with digitonin lysis buffer, twice with 0.5 M LiCl, pH 7.4, and twice with 50 mM Tris, pH 8. Pellets were stored at -20 or 4°C in an equal volume of 20 mM Tris, pH 7.4, 1 mM PMSF, 1 μg/ml aprotinin, 0.7 μg/ml pepstatin A, and 2 mM Na_3VO_4.

In vitro *phosphorylation reactions*

To normalize the amount of immunoprecipitate used in *in vitro* phosphorylation reactions for a given elutriation, the average protein concentration for digitonin lysates from the elutriation was calculated, and this was taken to be the protein amount which is obtained from 1.25 x 10^5 cells, *i.e.* the number of cells expected to be in each 5 μl aliquot used in the Bradford assay. For each sample, then, the

amount of immunoprecipitate which would have resulted from 1 x 10^6 cells, based on the average protein concentration, was used in the phosphorylation reaction.

In each 20 μl reaction, the amount of immunoprecipitate representing 1 x 10^6 cells was combined with 1 μCi [γ-^{32}P]-ATP (Dupont NEN, Boston, MA) in 20 mM Tris, pH 7.4, 10 mM $MnCl_2$, 1 mM Na_3VO_4 and incubated at 30°C for 45 min. The reaction was stopped by the addition of SDS-PAGE sample buffer and boiling for 2 min.

SDS-PAGE and Western blotting

For SDS-PAGE 4-15% gradient gels were used. For silver staining, gels were first stained with Coomassie blue then destained. Gels were equilibrated to H_2O and treated with 5 μg/ml DTT in H_2O for 30 min then with 1 mg/ml $AgNO_3$ for 30 min. The stain was developed with 3% (w/v) Na_2CO_3, 0.05% (v/v) HCHO and developing was stopped by the addition of 10% (v/v) CH_3COOH (method modified from 42).

For Western blotting, gels were equilibrated to 25 mM Tris base, 192 mM glycine, 20% (v/v) methanol and blotted onto PVDF membrane (Bio-Rad Laboratories Inc., Melville, NY, or Millipore, Bedford, MA) for 30 min at 200 mAmps followed by 4.5 hrs at 400 mAmps, 4°C. To detect ^{32}P-labelled phosphorylation reaction products, blots were air dried and used to expose Hyperfilm (Amersham, Arlington Heights, IL). For anti-fyn, anti-lck and anti-src probing of Western blots, blots were blocked with 2% (v/v) normal goat serum (GIBCO/BRL) in TBS (10 mM Tris, pH 8, 150 mM NaCl) and probed with the primary antibody diluted to 0.8 μg/ml in TBST (TBS with 0.1% [v/v] Tween 20 [Bio-Rad]) followed

by goat anti-rabbit IgG-peroxidase (Boehringer Mannheim) in TBST. ECL reagents (Amersham) and Hyperfilm were used to detect the bound antibodies.

Phosphoamino acid analysis

The protein bands for phosphoamino acid analysis were cut from the PVDF blots. The blot pieces were wetted in methanol and equilibrated to H_2O and then treated with 5.7 N HCl at 100°C for 2 hr. The digest products were dried then dissolved in 5 or 10 μl phosphoamino acid standards (1 μg/μl each phosphoserine, phosphothreonine, and phosphotyrosine). Samples were spotted onto thin layer cellulose plates (Kodak, Rochester, NY) and electrophoresed at 500 to 1000 V in pH 2.5 buffer: 5.9% (v/v) acetic acid, 0.83% (v/v) formic acid, 0.33% (v/v) pyridine, and 0.33 mM EDTA (43). Air-dried plates were sprayed with ninhydrin (Sigma) to identify the positions of the phosphoamino acid standards, and the positions of the ^{32}P-residues from the phosphorylation reaction products were determined by autoradiography using Hyperfilm or by employing a Beta Scope 603 blot analyzer (Betagen Corporation, Waltham, MA).

RESULTS

Centrifugal elutriation of asynchronous cells produces fractions of cells enriched for various stages of the cell cycle

In preparation for elutriation studies, CTLL-2 cells from unseparated, asynchronous cultures were stained with PI (a fluorescent DNA stain), and the fluorescence of the cells was detected by flow cytometry. When DNA fluorescence was plotted as a histogram, the predominant peak from the asynchronous cells represents cells which are in G_1 (Fig. 1*A*). As cells progress through S, DNA is duplicated leading to increased PI fluorescence. Cells in G_2+M, having double the DNA material of cells in G_1, have approximately 2-fold higher PI fluorescence. Centrifugal elutriation was then used to separate cells from the asynchronous culture into fractions enriched for the various stages of the cell cycle with minimal perturbation to the cells (44, 45). Flow cytometry of PI-stained CTLL-2 cells obtained from elutriation fractions indicated that the fractions were sequentially enriched for the stages of the cell cycle starting with G_1 and progressing through S then G_2+M (Fig. 1, *B-Q*).

Proteins of 60-70 kD are associated with CD45 in S and G_2+M stages of the cell cycle

It has been shown that the activity of the PTPase CD45 is elevated during G_2+M (20). In order to identify proteins which interact with active CD45 in G_2+M,

Figure 1. FACS analysis of the DNA content of elutriated CTLL-2 cells. Histograms of the PI fluorescence of cell populations are shown for (*A*) an exponentially-growing asynchronous starting culture and (*B-Q*) its elutriation fractions 1-16. The DNA content based on PI staining is indicated. (*A*) In asynchronous cultures, the predominant peak represents cells in G_1; as cells progress through S, their DNA content and, thus, PI fluorescence increases; when cells are in G_2+M, their DNA content is double that of cells in G_1, and hence their PI fluorescence is approximately double that of G_1 cells. The DNA staining patterns were interpreted as follows: *B-J*, fractions 1-9, G_1 and S phases; *K-M*, fractions 10-12, S phase; and *N-Q*, fractions 13-16, G_2+M phases.

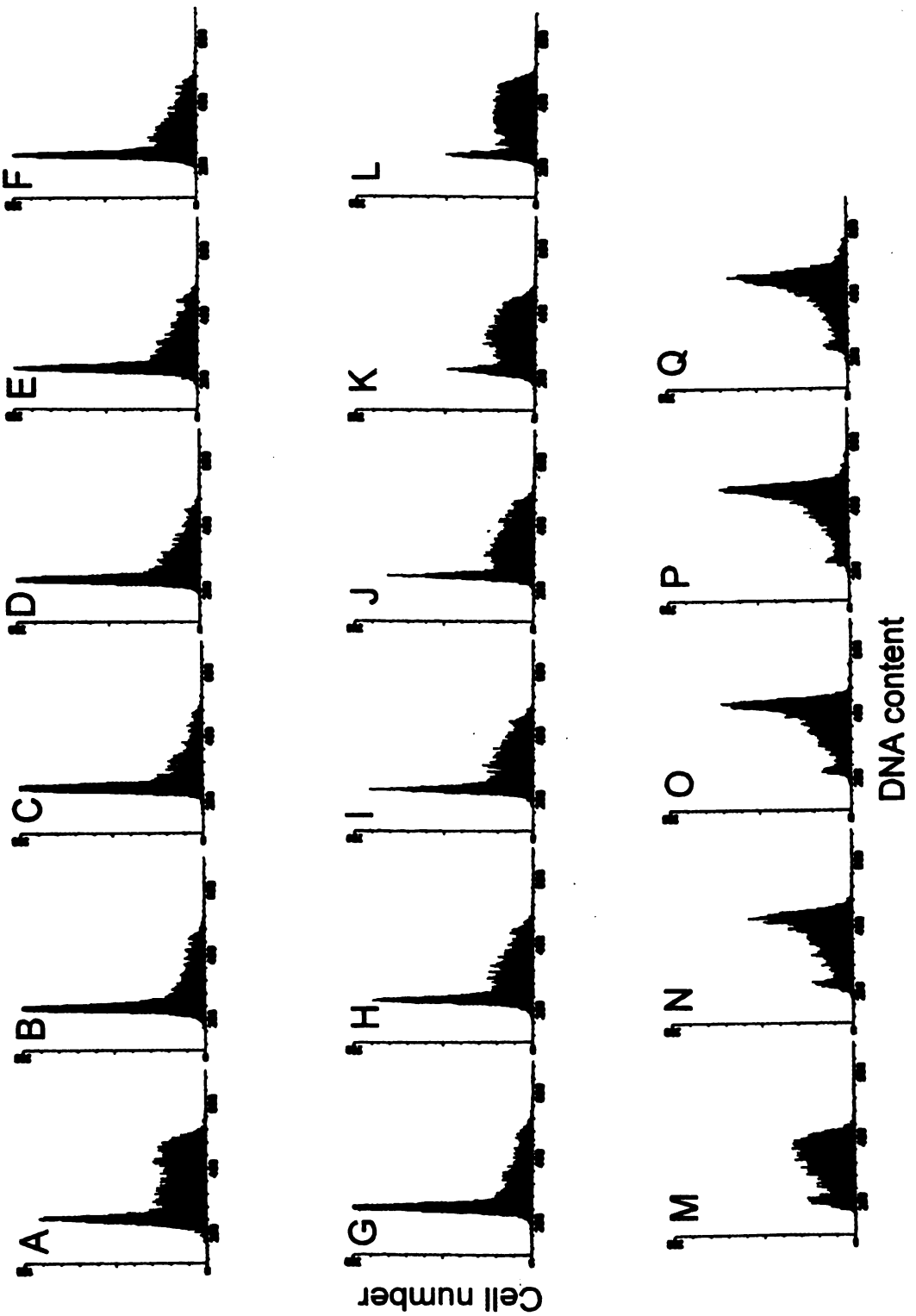

Figure 1.

cross-linking studies were undertaken. When asynchronous cells were treated with the cross-linker DTBP and then cellular proteins were extracted sequentially with hypotonic, alkaline buffer, Triton X-100 buffer, and Triton X-100/sarcosyl buffer, CD45 was primarily found in the Triton X-100 extract while CD8α was found in the hypotonic, alkaline extract (Fig. 2, *lanes* 1 and 6). CD45 was located in the Triton X-100 extract even when the cross-linker was not used (Fig. 2, *lane* 5). Two protein bands in the 50 to 60 kD range coprecipitated with CD8α: these may be forms of $p56^{lck}$, which interacts with the cytoplasmic tail of CD8α (46, 47). Two proteins of 60 to 70 kD were precipitated along with CD45 from the Triton X-100 extract from DTBP-treated cells (Fig. 2, *lane* 6).

When CD45 was immunoprecipitated from the Triton X-100 extracts from fractions enriched for cells from the various stages of the cell cycle, *i.e.* from elutriation fractions, protein bands in the 60 to 70 kD range were observed in CD45 immunoprecipitates from S through G_2+M (Figs. 3 and 4*A*). The bands were difficult to detect in gels run specifically for silver staining, but when gels were stained after electroblotting, the bands could be readily seen above the immunoglobulin heavy chain band (Fig. 4*A*). Other bands which appeared between the immunoglobulin heavy and light chains were observed in many immunoprecipitates, including those using other mAbs, and thus were not considered to be specific to CD45 immunoprecipitates.

Western blot analysis showed that neither anti-fyn nor anti-lck antibodies interacted with these bands, but the anti-src polyclonal antibody reacted with one or two of the bands weakly (Fig 4*B* and data not shown). The bands seen as a result of

Figure 2. CD8α and CD45 immunoprecipitates from DTBP-treated CTLL-2 cells. Samples of 5 x 10^6 asynchronous CTLL-2 cells were treated with the chemical cross-linker DTBP (*lanes* 1, 3-4, 6-7 and 9) or, as a control, DTBP treatment was omitted (*lanes* 2, 5, and 8). Proteins were then extracted from the cells initially with a hypotonic, alkaline buffer (*lanes* 1-3), secondly with a Triton X-100 buffer (*lanes* 4-6), and finally with a Triton X-100/sarcosyl buffer (*lanes* 7-9). Either CD8α (*lanes* 1, 4, and 7) or CD45 (*lanes* 2-3, 5-6, and 8-9) was then immunoprecipitated from the extracts.

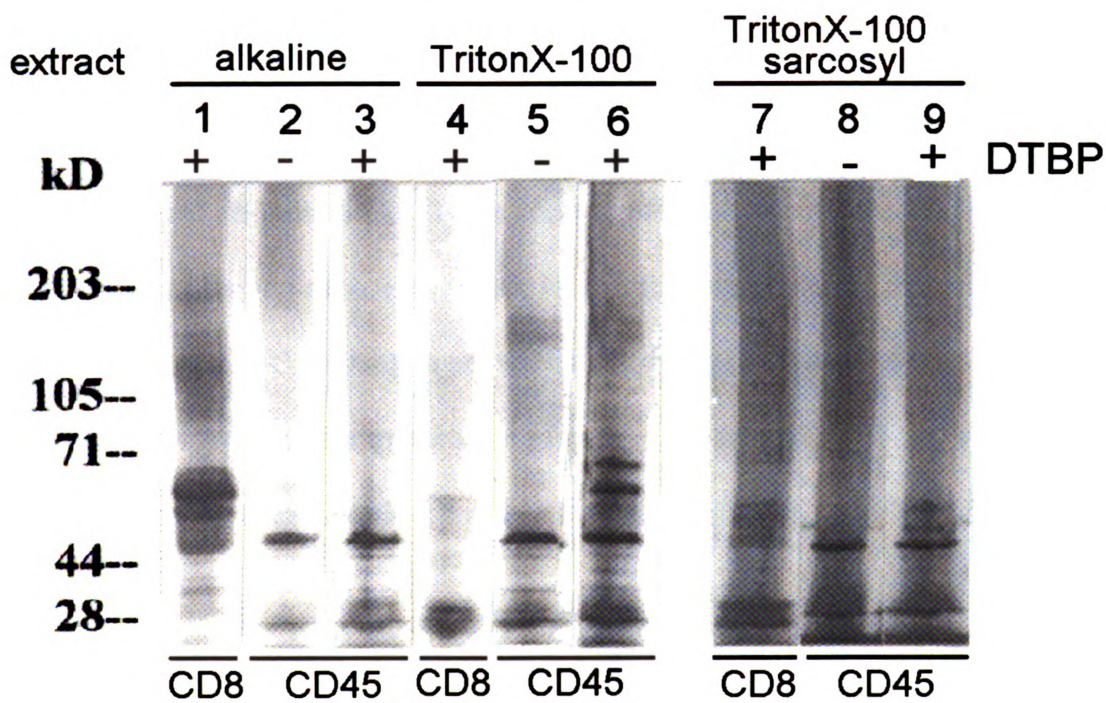
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Figure 2.

Figure 3. FACS analysis of the DNA content of CTLL-2 elutriation fractions analyzed in Fig. 4. (*A*) Cell cycle profile of the asynchronous culture used in this experiment. (*B-F*) DNA content histograms for the elutriation fractions 1-5 analyzed further in Fig. 4. The DNA staining patterns were interpreted as follows: (*B*) fraction 1, G_1 and S; (*C* and *D*) fractions 2 and 3, S; (*E*) fraction 4, S and G_2; and (*F*) fraction 5, G_2+M.

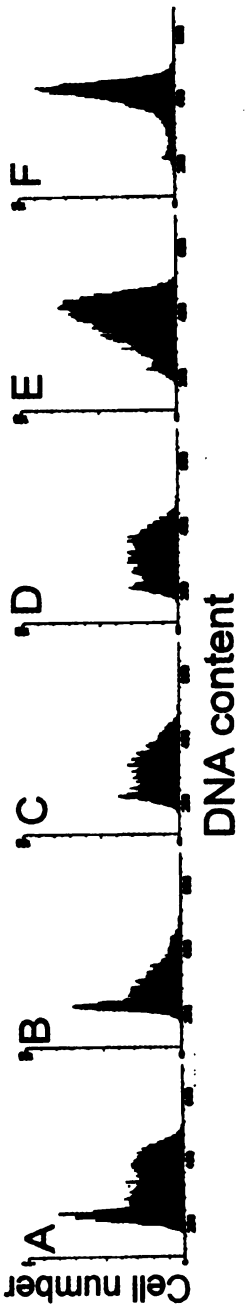

Figure 3.

Figure 4. CD45 immunoprecipitates from DTBP-treated CTLL-2 elutriation fractions. Samples of 5 x 10^6 cells from the elutriation fractions analyzed in Fig. 3 were treated with DTBP and then lysed in hypotonic, alkaline buffer. Proteins were extracted from the insoluble cytoskeletal matrix with Triton X-100 buffer, and CD45 was immunoprecipitated from this extract. Immunoprecipitates were subjected to SDS-PAGE followed by Western blotting. Samples in *lanes* 1-5 are from elutriation fractions 1-5, respectively, of Fig. 3 (*panels B-F*). (*A*) Silver-stained SDS-PAGE gels. The coprecipitating 60 to 70 kD proteins were most readily seen in SDS-PAGE gels after the majority of the proteins from the immunoprecipitates had been blotted onto PVDF membrane. HC and LC: heavy and light chain, respectively, of M1/9.3.4. (*B*) Anti-src probing of the Western blot of CD45 immunoprecipitates from the elutriation fractions (*lanes* 1-5) and of a Western blot of whole cell lysate of 5 x 10^6 CTLL-2 cells (*lane* 6). The arrow indicates the position of $p60^{src}$.

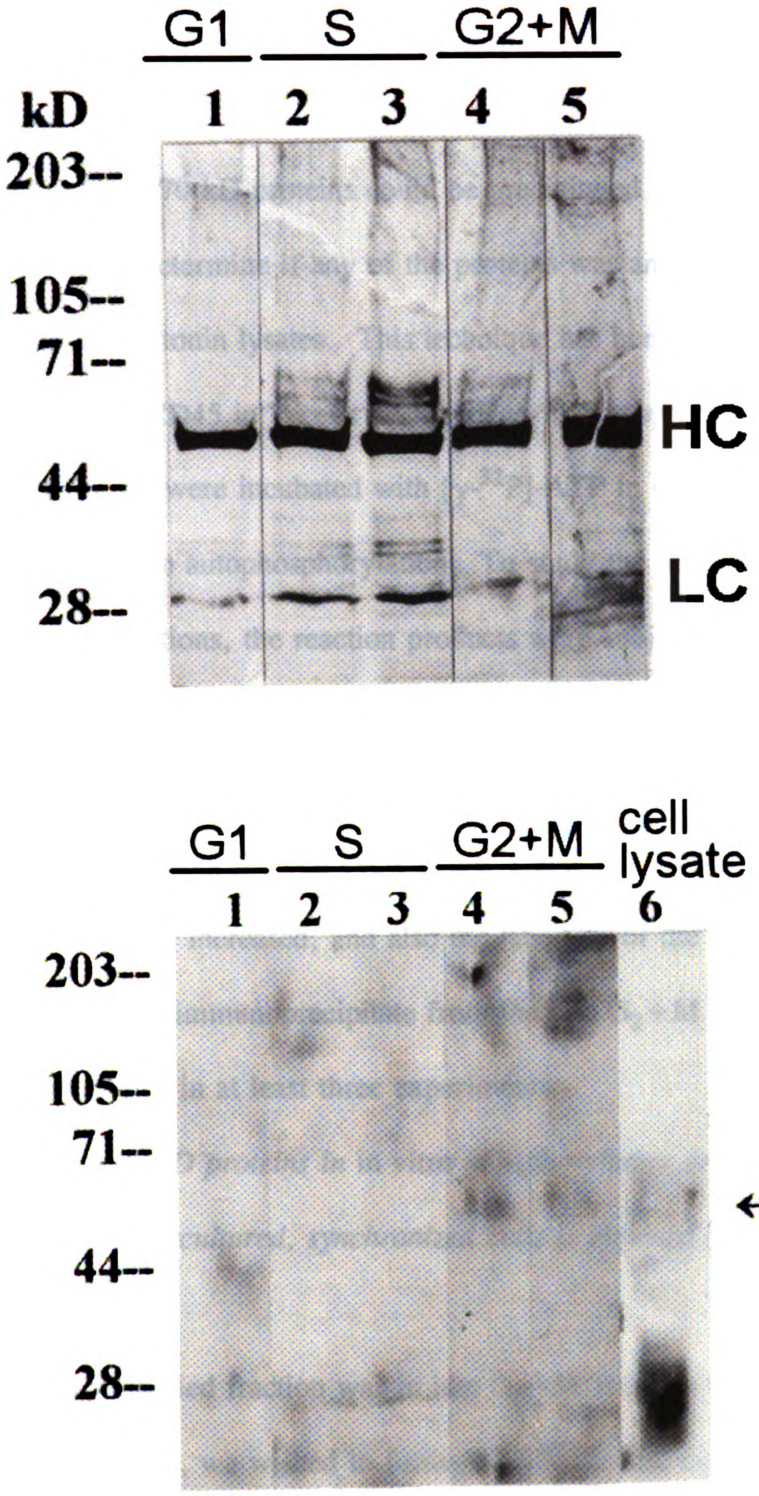
G1
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Figure 4.

the anti-src probing of the elutriation fractions were located above the position of the immunoglobulin heavy chain band.

Sixty to 70 kD proteins are labelled in in vitro *phosphorylation reactions of CD45 immunoprecipitates*

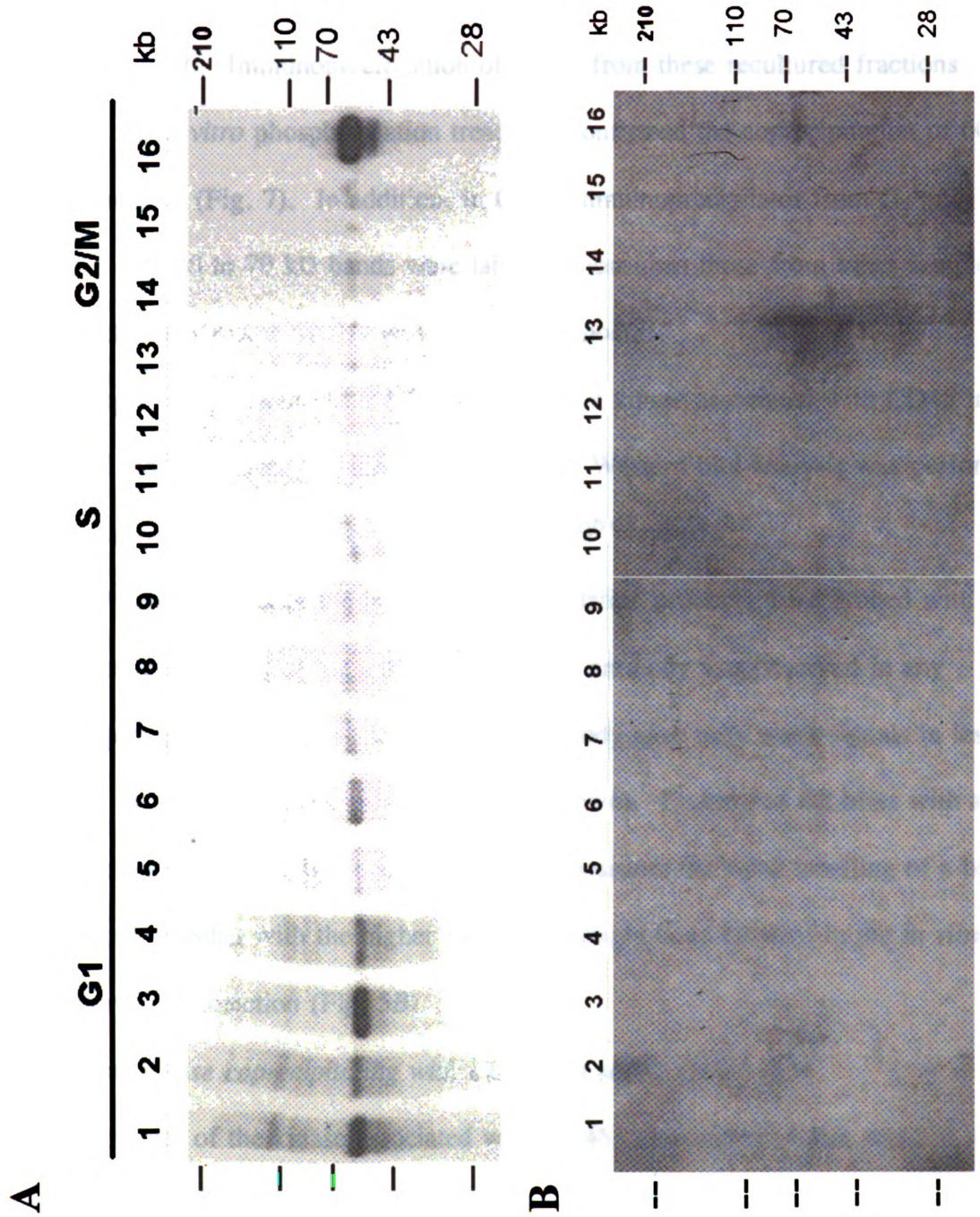
To determine if the 60 to 70 kD proteins could be precipitated with CD45 by a second technique and to determine if any of the proteins was an active kinase, CD45 was precipitated from digitonin lysates. This technique has been used to show that $p56^{lck}$ is associated with CD45 in T cells (29). CD45 immunoprecipitates representing 1 x 10^6 cells were incubated with [γ-^{32}P]-ATP in conditions in which src family kinases can undergo autophosphorylation. To study the products from the *in vitro* phosphorylation reactions, the reaction products were subjected to SDS-PAGE followed by electroblotting.

Two ^{32}P-labelled 60 to 70 kD proteins were found in the CD45 immunoprecipitates throughout the cell cycle (Figs. 1 and 5*A*). The ^{32}P-labelling of the larger band was greatly increased, and also the labelling of the smaller band to a lesser extent, in the CD45 immunoprecipitate from the late G_2+M fraction taken directly from the elutriator in at least three experiments.

Labelling of the 60 to 70 kD proteins in in vitro *phosphorylation reactions of CD45 immunoprecipitates from recultured, synchronized cells is elevated in the mitotic fractions*

Since the most highly-labelled fraction was in late G_2+M and this fraction was one of the last elutriation fractions, we wished to further verify the cell cycle position of the peak kinase activity by reculturing cells. This was performed by first obtaining

Figure 5. *In vitro* phosphorylation of proteins coprecipitated with CD45. CD45 was immunoprecipitated from digitonin lysates of elutriation fractions, and the amount of CD45 immunoprecipitate representing 1 x 10^6 cells from each fraction was incubated with [γ-^{32}P]-ATP in *in vitro* phosphorylation reactions and then subjected to SDS-PAGE and Western blotting. Samples in *lanes* 1-16 are from elutriation fractions 1-16, respectively, from Fig. 1 (*panels B-Q*). (*A*) *In vitro*-phosphorylated proteins were visualized by autoradiography. (*B*) The Western blots were probed with anti-src polyclonal antibody and the appropriate secondary antibody.


A
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kb
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28
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
kb
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Figure 5.

synchronized cell populations by elutriation. The cells were then cultured for 4.25 hr to allow them to progress through the cell cycle so that G_2+M fractions were situated between S and G_1 fractions. The final cell cycle analysis of the recultured samples is shown in Fig. 6. Immunoprecipitation of CD45 from these recultured fractions followed by *in vitro* phosphorylation treatment confirmed the coprecipitation of 60 to 70 kD proteins (Fig. 7). In addition, in CD45 immunoprecipitates from G_2+M fractions, the 60 to 70 kD bands were labelled more than those from other samples.

The 70 kD band reacts weakly with anti-src antibodies

These experiments demonstrated the presence of a kinase associated with CD45 in all stages of the cell cycle. To identify this kinase, Western blot analysis was performed with antisera to the src family PTKs, $p60^{src}$, $p59^{fyn}$ and $p56^{lck}$.

When the blots of the *in vitro* phosphorylation products were probed with antibodies, no binding of the anti-fyn polyclonal antibody was observed in any immunoprecipitate, and anti-lck polyclonal antibody gave very weak signals in less than 7% of the immunoprecipitates (data not shown). Probing of the blots with anti-src polyclonal antibody, however, resulted in consistent yet weak labelling of a band which corresponded with the higher molecular weight band labelled in the *in vitro* phosphorylation reaction (Fig. 5*B*).

The active kinase coprecipitating with CD45 is a serine kinase

The specificity of the kinase associated with CD45 immunoprecipitates was determined by phosphoamino acid analysis of the ^{32}P-labelled products obtained from the previous experiments. When the 60 to 70 kD ^{32}P-labelled protein bands were excised from blots and analyzed for phosphoamino acid content, approximately two

Figure 6. FACS analysis of the DNA content of recultured CTLL-2 subpopulations. Samples of cells from elutriation fractions were recultured for 4.25 hrs prior to analysis. (*A-I*) Cell cycle profiles of elutriation fractions 1-9 after reculturing. The DNA staining patterns were interpreted as follows: (*A-C*) fractions 1-3, S; (*D*) fraction 4, S and G_2; (*E-G*) fractions 5-7, M; (*H-I*) fractions 8 and 9, G_1 and S (The G_2 peak is no longer decreasing so these cells do not appear to be progressing through mitosis.).

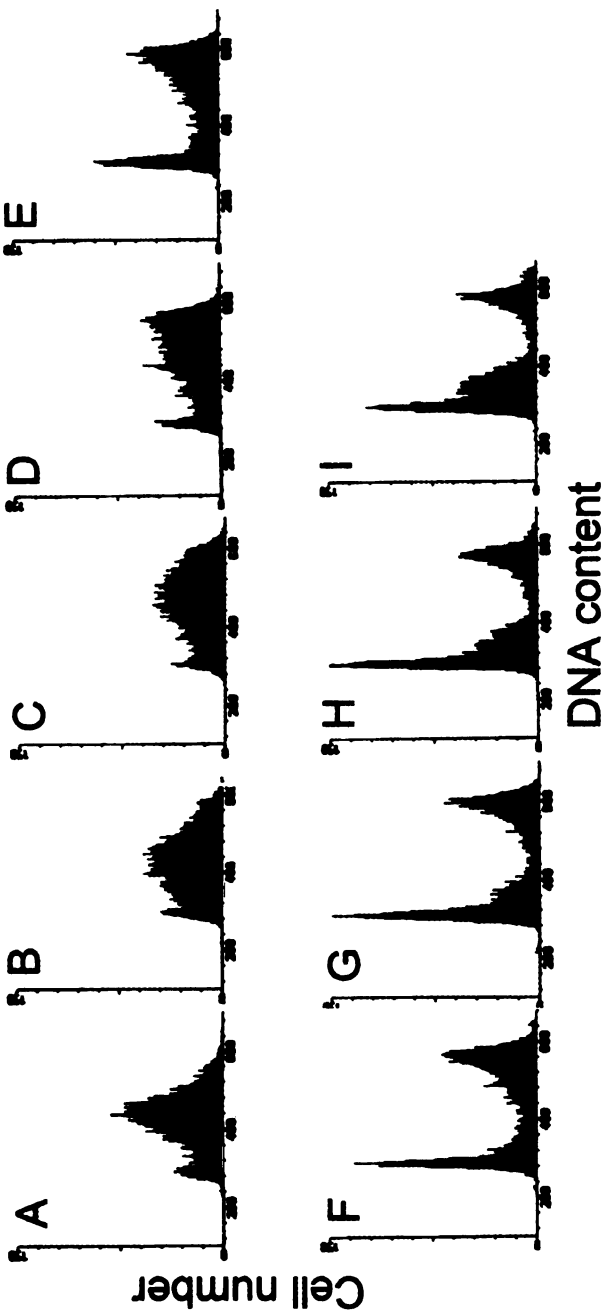

Figure 6.

Figure 7. ***In vitro*** phosphorylation of proteins coprecipitated with CD45. CD45 was immunoprecipitated from digitonin lysates of recultured CTLL-2 synchronized subpopulations, and the amount of CD45 immunoprecipitate representing 1 x 10^6 cells from each fraction was incubated with [γ-^{32}P]-ATP in *in vitro* phosphorylation reactions and then subjected to SDS-PAGE and electroblotting. Phosphorylated products were visualized by autoradiography. Samples in *lanes* 1-9 are from recultured fractions 1-9, respectively, depicted in Fig. 6 (*panels A-I*).

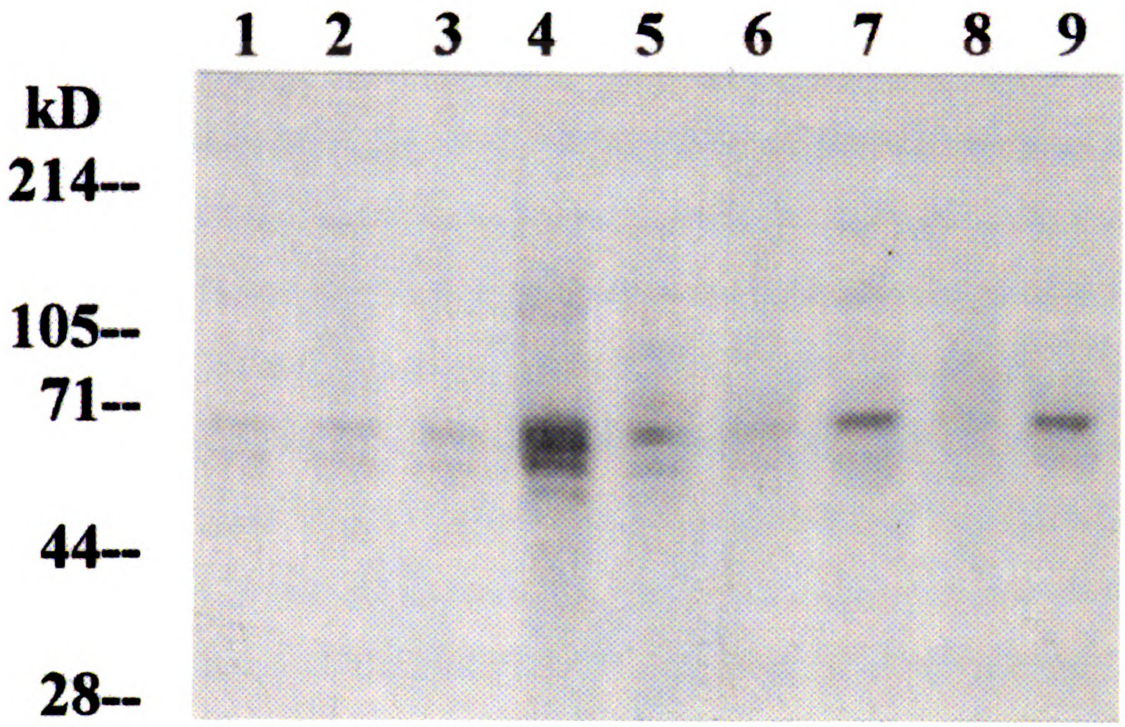

Figure 7.

thirds of the label was found in phosphoserine and one third in phosphothreonine (Fig. 8 and data not shown). The phosphorylation pattern of the upper band was the same as that of the lower band in each sample tested (data not shown). Labelling of phosphotyrosine was barely detectable in these samples. Thus we concluded that the primary activity associated with CD45 immunoprecipitates was that of a serine threonine kinase.

Figure 8. Phosphoamino acid analysis of *in vitro* phosphorylation products. ^{32}P-labelled bands were excised from the Western blots of kinase reaction products, and the phosphoamino acids were released from the blot pieces by digestion with 5.7 N HCl. One-dimensional electrophoresis on thin-layer cellulose plates was used to resolve the phosphoamino acids. The ^{32}P-labelled phosphoamino acids were visualized with the Beta Scope analyzer. *Lanes* 1 and 2 show the phosphoamino acid analysis of the lower band in *lanes* 4 and 5, respectively, of Fig. 7. *Lane* 3 shows the analysis of a band from the fraction with peak kinase activity from an experiment not depicted elsewhere in this paper. *Lane* 4 shows the analysis for the upper band from *lane* 16 of Fig. 5. *Lane* 5 shows the position of the phosphotyrosine standard while in *lane* 6 the positions of all three phosphoamino acid standards are shown.

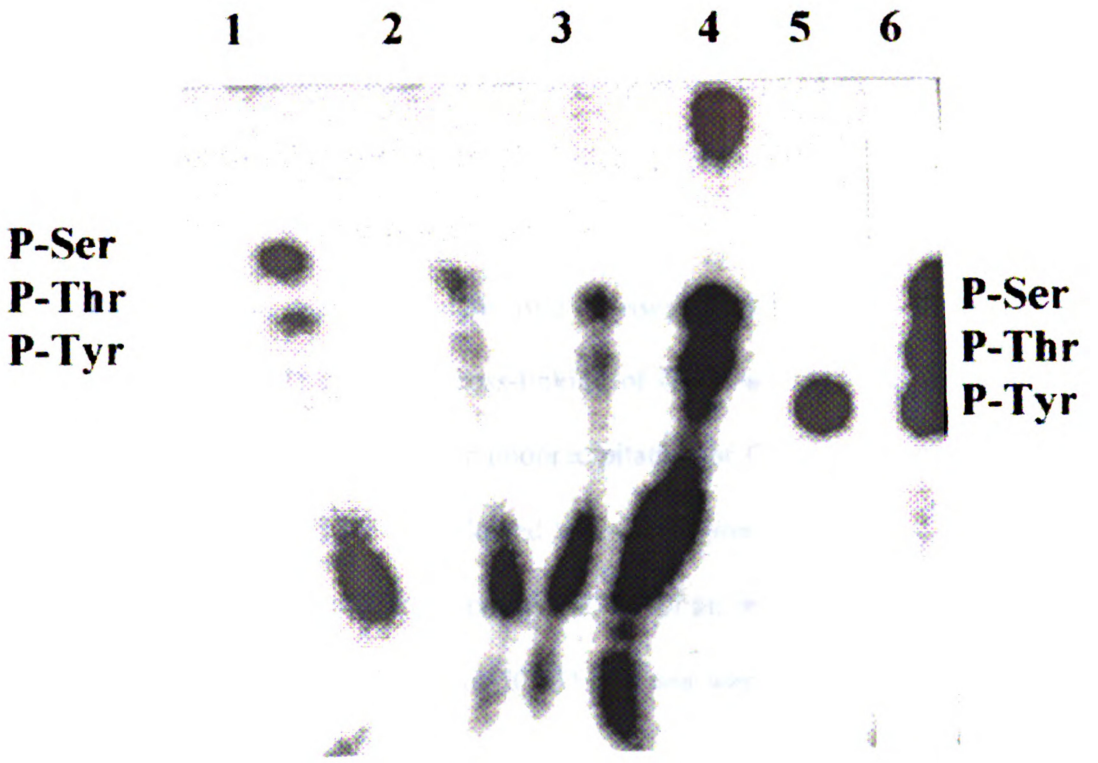

Figure 8.

DISCUSSION

The current study was undertaken to determine if accessory proteins associate with CD45 when it exhibits peak PTPase activity in the G_2+M stages of the cell cycle (20). Centrifugal elutriation of CTLL-2 T lymphocytes was used to obtain subpopulations which were enriched for cells in each of the various stages of the cell cycle. Two different approaches were used to assess the association of accessory proteins with CD45: 1) chemical cross-linking of CD45 and putative accessory proteins with DTBP followed by immunoprecipitation of CD45 and 2) lysis of cells with the mild detergent digitonin followed by immunoprecipitation of CD45 and analysis for the presence of kinase activity by use of an *in vitro* phosphorylation assay. Using these approaches, 60 to 70 kD proteins were found throughout the cell cycle in CD45 immunoprecipitates.

When the CD45 immunoprecipitates from digitonin lysates were incubated with [γ-^{32}P]-ATP using conditions in which src family kinases undergo autophosphorylation, the 60 to 70 kD proteins did indeed become phosphorylated. The *in vitro* phosphorylation of these bands was higher in the CD45 immunoprecipitates from G_2+M-enriched samples than from G_1 or S samples. Phosphoamino acid analysis of the labelled products determined that serine and threonine residues were being phosphorylated in the *in vitro* reactions. These data

demonstrate that an active serine-threonine kinase is associated with CD45 in CTLL-2 cells throughout the cell cycle, and that its activity or its association with CD45 is increased during G_2+M.

When Western blots of the *in vitro* phosphorylation products were analyzed for reactivity with anti-fyn, anti-lck, or anti-src antibodies, only the anti-src antibody consistently, albeit weakly, reacted with a protein on the blots. The anti-fyn and anti-lck antibodies did identify proteins of the appropriate molecular weights when blots of whole cell lysates from 1 x 10^6 CTLL-2 cells were tested with the antibodies (data not shown). Thus, the antibodies were capable of detecting their respective epitopes on Western blots of CTLL-2 proteins.

In several previous studies of CD45 immunoprecipitates from digitonin lysates of T cells, $p56^{lck}$ has been found associated with CD45 (29, 32, 33). In the previous studies, the CD45 immunoprecipitate from 1 to 5 x 10^7 cell equivalents was loaded per lane on the Western blots used to detect $p56^{lck}$, amounts which were 10 to 50-fold higher than the amounts used in the present study. When CD45 immunoprecipitates from PHA-activated CTLL-2 cells were tested to determine the minimum cell number necessary to detect $p56^{lck}$ in the immunoprecipitates on Western blots, a signal was obtained from 4 x 10^6 cells, but not from fewer cells (data not shown). Thus, to further test the CD45 immunoprecipitates from digitonin lysates of elutriation fractions, Western blots of 5 x 10^6 cell equivalents were probed with the antibodies for the src family kinases. The data obtained from these blots, however, did not differ from those obtained from the *in vitro* phosphorylation product blots (data not shown).

In previous studies, a group of proteins in the 29 to 34 kD range have been identified in immunoprecipitates of CD45 when mild detergents, *i.e.* digitonin, Brij 58, or Brij 96, were used to lyse cells (29-33). Homologous genes encoding proteins of about 30 kD which bind to CD45 have been cloned from both murine and human genomes (48, 49). This protein has been named the lymphocyte phosphatase-associated phosphoprotein (LPAP) (49). The multiple bands seen in CD45 immunoprecipitates from human T cells appear to arise from differential phosphorylation of LPAP (49). In several of the previous studies involving *in vitro* phosphorylation of CD45 immunoprecipitates, LPAP was the most predominant labelled product (29, 30, 33). In contrast, almost no labelling of proteins in the 23 to 45 kD range was observed in our current study. There are several potential reasons for this including: 1) CTLL-2 cells do not express as much LPAP as do the cell types used in the other studies, 2) LPAP cannot be detected from 1 x 10^6 cells, or 3) LPAP is present in the CD45 immunoprecipitates in the current study, but it is not phosphorylated in the *in vitro* phosphorylation reactions. LPAP is in fact phosphorylated *in vivo* on serine residues (29, 30, 32, 33). Phosphoamino acid analysis of the 60 to 70 kD proteins which were labelled in the current study revealed that the active kinase coprecipitating with CD45 is a serine-threonine kinase. Thus, it is possible that *in vivo* phosphorylation of LPAP prevented subsequent phosphorylation in *in vitro* reactions.

While one study reported the exclusive labelling of tyrosine residues in LPAP in *in vitro* phosphorylation treatments of CD45 immunoprecipitates (29), a second reported that both serine and, to a lesser extent, tyrosine were labelled (32).

Phosphoamino acid analyses of the ^{32}P-labelled 50 to 60 kD proteins were not reported in these studies although Schraven and his co-workers (29) noted that autophosphorylation of p56^{lck}, which was readily observed in lck immunoprecipitates from Triton X-100 lysates, was barely detected when lck was immunoprecipitated from digitonin lysates. The *in vitro* labelling of both serine and tyrosine residues suggests that multiple kinases may coprecipitate with CD45.

In the current study, although some p60^{src} appears to coprecipitate with CD45 from digitonin lysates of CTLL-2 cells, the predominant kinase activity in the immunoprecipitates was clearly that of a serine-threonine kinase. The activity of the serine-threonine kinase which coprecipitated with CD45 throughout the cell cycle was increased in late G_2+M-enriched fractions, a time in the cell cycle when the PTPase activity of CD45 is elevated (20). In *in vitro* studies, Stover and Walsh (50) determined that sequential phosphorylation of CD45, first on tyrosine residues then on serine residues, enhanced the PTPase activity of CD45 toward one artificial substrate but not another. In addition, the activity of p60^{src} increases during mitosis coincident with increases in threonine and possibly serine phosphorylation (51). Thus, it is possible that the serine-threonine kinase which coprecipitates with CD45 could be acting on CD45 itself or on other proteins associated with CD45 such as p60^{src}.

Data on the *in vivo* phosphorylation state of CD45 would seem to support the hypothesis that the serine-threonine kinase is acting on CD45. Early studies of the phosphorylation of CD45 *in vivo* typically found serine residues were heavily phosphorylated, and threonine and possibly tyrosine were phosphorylated weakly or not at all (52-54). These studies were done without the use of the potent PTPase

inhibitor PAO, which allows the detection of phosphotyrosine in *in vivo*-labelled CD45 (35, 55). In the present study, after *in vitro* phosphorylation treatments, ^{32}P-labelled protein bands in the appropriate range for CD45 were observed in a few samples, but they were only weakly labelled (Figs. 5*A* and 7). Thus, the serine-threonine kinase which coprecipitates with CD45 does not phosphorylate CD45, at least in the conditions used in the present study.

The identity of the serine-threonine kinase responsible for the *in vitro* phosphorylation observed in our studies is as yet unknown. One candidate kinase being considered is Raf-1. Raf-1 is a p70-74 serine-threonine kinase which is activated after stimulation of T cells via the TCR/CD3 complex or a number of other receptors (56-58). Raf-1 is capable of undergoing autophosphorylation in conditions similar to those used in the *in vitro* phosphorylation reactions in the current study with one notable difference: *in vitro* kinase treatments of immunoprecipitated Raf-1 have included the sulfhydryl-reducing compound DTT (59, 60). Interestingly, in a study designed to determine the regulation of Raf-1 activity by tyrosine phosphorylation, it was shown that *in vitro* treatment of Raf-1 with CD45 reduced the activity of this serine-threonine kinase (61).

Future studies are planned to identify the serine-threonine kinase which coprecipitates with CD45 from CTLL-2 cells. In addition, further studies will be done to ascertain if $p60^{src}$ is indeed in the CD45 immunoprecipitates giving rise to the weak signal which is detected after anti-src probing of the Western blots. Finally, studies to determine who is acting upon whom in the CD45/$p60^{src}$/serine-threonine kinase complex are planned.

ACKNOWLEDGMENTS

I thank Dr. S. Conrad (Michigan State University, East Lansing, MI) for use of the JE-6B elutriation system and Dr. L. King (Michigan State University, East Lansing, MI) for technical support and assistance with the FACS.

REFERENCES

1. Thomas, M. L. 1989. The leukocyte common antigen family. *Annu. Rev. Immunol.* 7:339.

2. Trowbridge, I. S., H. L. Ostergaard, and P. Johnson. 1991. CD45: a leukocyte-specific member of the protein tyrosine phosphatase family. *Biochim. Biophys. Acta.* 1095:46.

3. Charbonneau, H., N. K. Tonks, K. A. Walsh, and E. H. Fischer. 1988. The leukocyte common antigen (CD45): a putative receptor-linked protein tyrosine phosphatase. *Proc. Natl. Acad. Sci. U. S. A.* 85:7182.

4. Tonks, N. K., H. Charbonneau, C. D. Diltz, E. H. Fischer, and K. A. Walsh. 1988. Demonstration that the leukocyte common antigen CD45 is a protein tyrosine phosphatase. *Biochemistry.* 27:8695.

5. Streuli, M., N. X. Krueger, A. Y. Tsai, and H. Saito. 1989. A family of receptor-linked protein tyrosine phosphatases in humans and Drosophila. *Proc. Natl. Acad. Sci. U. S. A.* 86:8698.

6. Tan, X., D. R. Stover, and K. A. Walsh. 1993. Demonstration of protein tyrosine phosphatase activity in the second of two homologous domains of CD45. *J. Biol. Chem.* 268:6835.

7. Trowbridge, I. S. 1978. Interspecies spleen-myeloma hybrid producing monoclonal antibodies against mouse lymphocyte surface glycoprotein, T200. *J. Exp. Med.* 148:313.

8. Williams, A. F., and A. N. Barclay. 1986. Glycoprotein antigens of the lymphocyte surface and their purification by antibody affinity chromatography. In *Handbook of Experimental Immunology in Four Volumes, 4th ed., vol. 1.* D. M. Weir, ed. Blackwell Scientific Publications, Oxford, p. 22.1.

9. Pingel, J. T., and M. L. Thomas. 1989. Evidence that the leukocyte-common antigen is required for antigen-induced T lymphocyte proliferation. *Cell.* 58:1055.

10. Weaver, C. T., J. T. Pingel, J. O. Nelson, and M. L. Thomas. 1991. CD8+ T-cell clones deficient in the expression of the CD45 protein tyrosine phosphatase have impaired responses to T-cell receptor stimuli. *Mol. Cell. Biol.* 11:4415.

11. June, C. H., M. C. Fletcher, J. A. Ledbetter, and L. E. Samelson. 1990. Increases in tyrosine phosphorylation are detectable before phospholipase C activation after T cell receptor stimulation. *J. Immunol.* 144:1591.

12. Koretzky, G. A., J. Picus, M. L. Thomas, and A. Weiss. 1990. Tyrosine phosphatase CD45 is essential for coupling T-cell antigen receptor to the phosphatidyl inositol pathway. *Nature.* 346:66.

13. Koretzky, G. A., J. Picus, T. Schultz, and A. Weiss. 1991. Tyrosine phosphatase CD45 is required for T-cell antigen receptor and CD2-mediated activation of a protein tyrosine kinase and interleukin 2 production. *Proc. Natl. Acad. Sci. U. S. A.* 88:2037.

14. Volarevic, S., B. B. Niklinska, C. M. Burns, H. Yamada, C. H. June, F. J. Dumont, and J. D. Ashwell. 1992. The CD45 tyrosine phosphatase regulates phosphotyrosine homeostasis and its loss reveals a novel pattern of late T cell receptor-induced Ca2+ oscillations. *J. Exp. Med.* 176:835.

15. Justement, L. B., K. S. Campbell, N. C. Chien, and J. C. Cambier. 1991. Regulation of B cell antigen receptor signal transduction and phosphorylation by CD45. *Science.* 252:1839.

16. Shiroo, M., L. Goff, M. Biffen, E. Shivnan, and D. Alexander. 1992. CD45 tyrosine phosphatase-activated p59fyn couples the T cell antigen receptor to pathways of diacylglycerol production, protein kinase C activation and calcium influx. *Embo. J.* 11:4887.

17. Volarevic, S., B. B. Niklinska, C. M. Burns, C. H. June, A. M. Weissman, and J. D. Ashwell. 1993. Regulation of TCR signaling by CD45 lacking transmembrane and extracellular domains. *Science.* 260:541.

18. Hovis, R. R., J. A. Donovan, M. A. Musci, D. G. Motto, F. D. Goldman, S. E. Ross, and G. A. Koretzky. 1993. Rescue of signaling by a chimeric protein containing the cytoplasmic domain of CD45. *Science.* 260:544.

19. Desai, D. M., J. Sap, J. Schlessinger, and A. Weiss. 1993. Ligand-mediated negative regulation of a chimeric transmembrane receptor tyrosine phosphatase. *Cell.* 73:541.

20. Melkerson-Watson, L. J., M. E. Waldmann, A. D. Gunter, M. H. Zaroukian, and W. J. Esselman. 1994. Elevation of lymphocyte CD45 protein tyrosine phosphatase activity during mitosis. *J. Immunol.* 153:2004.

21. Ostergaard, H. L., and I. S. Trowbridge. 1990. Coclustering CD45 with CD4 or CD8 alters the phosphorylation and kinase activity of p56lck. *J. Exp. Med.* 172:347.

22. Ostergaard, H. L., D. A. Shackelford, T. R. Hurley, P. Johnson, R. Hyman, B. M. Sefton, and I. S. Trowbridge. 1989. Expression of CD45 alters phosphorylation of the lck-encoded tyrosine protein kinase in murine lymphoma T-cell lines. *Proc. Natl. Acad. Sci. U. S. A.* 86:8959.

23. Mustelin, T., K. M. Coggeshall, and A. Altman. 1989. Rapid activation of the T-cell tyrosine protein kinase pp56lck by the CD45 phosphotyrosine phosphatase. *Proc. Natl. Acad. Sci. U. S. A.* 86:6302.

24. Mustelin, T., and A. Altman. 1990. Dephosphorylation and activation of the T cell tyrosine kinase pp56lck by the leukocyte common antigen (CD45). *Oncogene.* 5:809.

25. Mustelin, T., T. Pessa-Morikawa, M. Autero, M. Gassmann, L. C. Andersson, C. G. Gahmberg, and P. Burn. 1992. Regulation of the p59fyn protein tyrosine kinase by the CD45 phosphotyrosine phosphatase. *Eur. J. Immunol.* 22:1173.

26. McFarland, E. D., T. R. Hurley, J. T. Pingel, B. M. Sefton, A. Shaw, and M. L. Thomas. 1993. Correlation between Src family member regulation by the protein-tyrosine-phosphatase CD45 and transmembrane signaling through the T-cell receptor. *Proc. Natl. Acad. Sci. U. S. A.* 90:1402.

27. Burns, C. M., K. Sakaguchi, E. Appella, and J. D. Ashwell. 1994. CD45 regulation of tyrosine phosphorylation and enzyme activity of src family kinases. *J. Biol. Chem.* 269:13594.

28. Biffen, M., D. McMichael-Phillips, T. Larson, A. Venkitaraman, and D. Alexander. 1994. The CD45 tyrosine phosphatase regulates specific pools of antigen receptor-associated p59fyn and CD4-associated p56lck tyrosine in human T-cells. *Embo. J.* 13:1920.

29. Schraven, B., H. Kirchgessner, B. Gaber, Y. Samstag, and S. Meuer. 1991. A functional complex is formed in human T lymphocytes between the protein tyrosine phosphatase CD45, the protein tyrosine kinase p56lck and pp32, a possible common substrate. *Eur. J. Immunol.* 21:2469.

30. Schraven, B., A. Schirren, H. Kirchgessner, B. Siebert, and S. C. Meuer. 1992. Four CD45/P56lck-associated phosphorproteins (pp29-pp32) undergo alterations in human T cell activation. *Eur. J. Immunol.* 22:1857.

31. Takeda, A., J. J. Wu, and A. L. Maizel. 1992. Evidence for monomeric and dimeric forms of CD45 associated with a 30-kDa phosphorylated protein. *J. Biol. Chem.* 267:16651.

32. Koretzky, G. A., M. Kohmetscher, and S. Ross. 1993. CD45-associated kinase activity requires lck but not T cell receptor expression in the Jurkat T cell line. *J. Biol. Chem.* 268:8958.

33. Ross, S. E., B. Schraven, F. D. Goldman, J. Crabtree, and G. A. Koretzky. 1994. The association between CD45 and lck does not require CD4 or CD8 and is independent of T cell receptor stimulation. *Biochem. Biophys. Res. Commun.* 198:88.

34. Brown, V. K., E. W. Ogle, A. L. Burkhardt, R. B. Rowley, J. B. Bolen, and L. B. Justement. 1994. Multiple components of the B cell antigen receptor complex associate with the protein tyrosine phosphatase, CD45. *J. Biol. Chem.* 269:17238.

35. Autero, M., J. Saharinen, T. Pessa-Morikawa, M. Soula-Rothhut, C. Oetken, M. Gassmann, M. Bergman, K. Alitalo, P. Burn, C. G. Gahmberg, et al. 1994. Tyrosine phosphorylation of CD45 phosphotyrosine phosphatase by p50csk kinase creates a binding site for p56lck tyrosine kinase and activates the phosphatase. *Mol. Cell. Biol.* 14:1308.

36. Lin, J., V. K. Brown, and L. B. Justement. 1992. Regulation of basal tyrosine phosphorylation of the B cell antigen receptor complex by the protein tyrosine phosphatase, CD45. *J. Immunol.* 149:3182.

37. Bourguignon, L. Y., S. J. Suchard, M. L. Nagpal, and J. R. Glenney Jr. 1985. A T-lymphoma transmembrane glycoprotein (gp180) is linked to the cytoskeletal protein, fodrin. *J. Cell. Biol.* 101:477.

38. Suchard, S. J., and L. Y. Bourguignon. 1987. Further characterization of a fodrin-containing transmembrane complex from mouse T-lymphoma cells. *Biochim. Biophys. Acta.* 896:35.

39. Lokeshwar, V. B., and L. Y. Bourguignon. 1992. Tyrosine phosphatase activity of lymphoma CD45 (GP180) is regulated by a direct interaction with the cytoskeleton. *J. Biol. Chem.* 267:21551.

40. Belin, M. T., and P. Boulanger. 1985. Cytoskeletal proteins associated with intracytoplasmic human adenovirus at an early stage of infection. *Exp. Cell. Res.* 160:356.

41. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248.

42. Gorg, A., W. Postel, J. Weser, H. W. Schiwara, and W. M. Boesken. 1985. Horizontal SDS electrophoresis in ultrathin pore-gradient gels for the analysis of urinary proteins. *Science Tools (Sweden)* 32:5.

43. Jelinek, T., and M. J. Weber. 1993. Optimization of the resolution of phosphoamino acids by one-dimensional thin-layer electrophoresis. *BioTechniques* 15(4):628.

44. Feder, J. N., Y. G. Assaraf, L. C. Seamer, and R. T. Schimke. 1989. The pattern of dihydrofolate reductase expression through the cell cycle in rodent and human cultured cells. *J. Biol. Chem.* 264:20583.

45. Page, D. M., and A. L. DeFranco. 1990. Antigen receptor-induced cell cycle arrest in WEHI-231 B lymphoma cells depends on the duration of signaling before the G1 phase restriction point. *Mol. Cell. Biol.* 10:3003.

46. Veillette, A., M. A. Bookman, E. M. Horak, and J. B. Bolen. 1988. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase p56lck. *Cell.* 55:301.

47. Veillette, A., and D. Davidson. 1992. Src-related protein tyrosine kinases and T-cell receptor signalling. *Trends Genet.* 8:61.

48. Takeda, A., A. L. Maizel, K. Kitamura, T. Ohta, and S. Kimura. 1994. Molecular cloning of the CD45-associated 30-kDa protein. *J. Biol. Chem.* 269:2357.

49. Schraven, B., D. Schoenhaut, E. Bruyns, G. Koretzky, C. Eckerskorn, R. Wallich, H. Kirchgessner, P. Sakorafas, B. Labkovsky, S. Ratnofsky and S. Meuer. 1994. LPAP, a novel 32-kDa phophoprotein that interacts with CD45 in human lymphocytes. *J. Biol. Chem.* 269(46):29102.

50. Stover, D. R., and K. A. Walsh. 1994. Protein-tyrosine phosphatase activity of CD45 is activated by sequential phosphorylation by two kinases. *Mol. Cell. Biol.* 14:5523.

51. Chackalaparampil, I., and D. Shalloway. 1988. Altered phosphorylation and activation of pp60c-src during fibroblast mitosis. *Cell.* 52:801.

52. Omary, M. B., and I. S. Trowbridge. 1980. Disposition of T200 glycoprotein in the plasma membrane of a murine lymphoma cell line. *J. Biol. Chem.* 255:1662.

53. Watson, A. J. 1982. Ly-5 (T200) structural polymorphism: the identification of intramolecular sites of phosphorylation. *Immunogenetics.* 16:485.

54. Valentine, M. A., M. B. Widmer, J. A. Ledbetter, F. Pinault, R. Voice, E. A. Clark, B. Gallis, and D. L. Brautigan. 1991. Interleukin 2 stimulates serine phosphorylation of CD45 in CTLL-2.4 cells. *Eur. J. Immunol.* 21:913.

55. Stover, D. R., H. Charbonneau, N. K. Tonks, and K. A. Walsh. 1991. Protein-tyrosine-phosphatase CD45 is phosphorylated transiently on tyrosine upon activation of Jurkat T cells. *Proc. Natl. Acad. Sci. U. S. A.* 88:7704.

56. Siegel, J. N., C. H. June, H. Yamada, U. R. Rapp, and L. E. Samelson. 1993. Rapid activation of C-Raf-1 after stimulation of the T-cell receptor or the muscarinic receptor type 1 in resting T cells. *J. Immunol.* 151:4116.

57. Siegel, J. N., R. D. Klausner, U. R. Rapp, and L. E. Samelson. 1990. T cell antigen receptor engagement stimulates c-raf phosphorylation and induces c-raf-associated kinase activity via a protein kinase C-dependent pathway. *J. Biol. Chem.* 265:18472.

58. Turner, B., U. Rapp, H. App, M. Greene, K. Dobashi, and J. Reed. 1991. Interleukin 2 induces tyrosine phosphorylation and activation of p72-74 Raf-1 kinase in a T-cell line. *Proc. Natl. Acad. Sci. U. S. A.* 88:1227.

59. Williams, N. G., T. M. Roberts, and P. Li. 1992. Both p21ras and pp60v-src are required, but neither alone is sufficient, to activate the Raf-1 kinase. *Proc. Natl. Acad. Sci. U. S. A.* 89:2922.

60. Morrison, D. K., G. Heidecker, U. R. Rapp, and T. D. Copeland. 1993. Identification of the major phosphorylation sites of the Raf-1 kinase. *J. Biol. Chem.* 268:17309.

61. Turner, B. C., N. K. Tonks, U. R. Rapp, and J. C. Reed. 1993. Interleukin 2 regulates Raf-1 kinase activity through a tyrosine phosphorylation-dependent mechanism in a T-cell line. *Proc. Natl. Acad. Sci. U. S. A.* 90:5544.