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The CD45 tyrosine phosphatase regulates specific pools of antigen receptor-associated p59^{fyn} and CD4-associated p56^{lck} tyrosine kinases in human T-cells

Mark Biffen[1], Danielle McMichael-Phillips, Tammy Larson[2], Ashok Venkitaraman[2] and Denis Alexander[3]

Department of Immunology, AFRC Babraham Institute, Babraham, Cambridge CB2 4AT and [2]MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK
[1]Present address: Fisons plc, Department of Biochemistry, Bakewell Road, Loughborough, LE11 0RH, UK
[3]Corresponding author

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A newly isolated T-cell line (CB1) derived from a T-acute lymphoblastic leukaemia (T-ALL) patient contained cells (40% of total) which did not express the CD45 phosphotyrosine phosphatase. The cells were sorted into CD45$^-$ and CD45$^+$ populations and shown to be clonal in origin. T-cell receptor (TCR) cross-linking or co-ligation of the TCR with its CD4/CD8 co-receptors induced tyrosine phosphorylation and calcium signals in CD45$^+$ but not in CD45$^-$ cells. Unexpectedly, whole cell p56^{lck} and p59^{fyn} tyrosine kinase activities were not reduced in CD45$^-$ compared to CD45$^+$ cells. A novel technique was therefore developed to isolate specific pools of aggregated receptors expressed at the cell surface, together with their associated tyrosine kinases. Using this technique it was shown that cell surface CD4-p56^{lck} kinase activity was 78% lower in CD45$^-$ than in CD45$^+$ cells. Phosphorylation of TCR ζ- and γ-chains occurred in TCR immunocomplexes from CD45$^+$ but not CD45$^-$ cells, despite comparable levels of p59^{fyn} and TCR proteins. Furthermore, TCR-associated tyrosine kinase activity towards an exogenous substrate was 84% lower in CD45$^-$ than in CD45$^+$ cells. Addition of recombinant p59^{fyn} to TCR immunocomplexes isolated from CD45$^-$ cells restored the phosphorylation of the TCR ζ- and γ-chains. Our results demonstrate that CD45 selectively regulates the pools of p59^{fyn} and p56^{lck} kinases which are associated with the TCR and CD4 at the cell surface. Activation by CD45 of these receptor-associated kinase pools correlates with the ability of the TCR and its co-receptors to couple to intracellular signalling pathways.
Key words: CD45 phosphotyrosine phosphatase/p59^{fyn} tyrosine kinase/p56^{lck} tyrosine kinase/T-cell antigen receptor chain phosphorylation

Introduction

The T-cell antigen receptor (TCR) comprises an immunoglobulin-like $\alpha\beta$ heterodimer, the CD3 antigen comprising $\delta\epsilon$ and $\gamma\epsilon$ complexes and an associated $\zeta\zeta$ or $\zeta\eta$ subunit (reviewed in Ashwell and Klausner, 1990). Cross-linking the TCR induces a cascade of events initiated by a rapid increase in protein tyrosine phosphorylation (Hsi *et al.*, 1989) resulting in activation of PLCγ_1 (Park *et al.*, 1991; Secrist *et al.*, 1991; Weiss *et al.*, 1991), an increase in intracellular Ca^{2+} and activation of protein kinase C (reviewed in Izquierdo and Cantrell, 1992). The increase in protein tyrosine phosphorylation is mediated by tyrosine kinases associated with the TCR and other cell surface antigens. The tyrosine kinase p59^{fyn} associates with the TCR (Samelson *et al.*, 1990; Gassman *et al.*, 1991) by binding to specific components of the receptor, namely the ϵ-, γ- and ζ-chains, via its unique N-terminal region (Timson Gauen *et al.*, 1992). Binding of p59^{fyn} is to a 17 residue tyrosine- and leucine-based motif which is present in single copies in δ, ϵ, γ and in three repeat copies in ζ (Reth, 1989; Irving *et al.*, 1993). Evidence from transgenic mice shows that increased p59^{fyn} expression correlates with the hyper-responsiveness of thymocytes (Cooke *et al.*, 1991) whereas in mutant mice lacking expression of p59^{fyn} TCR-induced Ca^{2+} signals are reduced (Stein *et al.*, 1992), particularly in CD4$^+$ thymocytes (Appleby *et al.*, 1992). These findings point to a critical role for p59^{fyn} in mediating early signals generated via the TCR.

Activation of T-cells has been shown to promote recruitment of a further tyrosine kinase, ZAP-70, to the TCR (Chan *et al.*, 1992). This association depends on the tandem Src homology 2 (SH2) domains of ZAP-70 interacting with phosphotyrosine residues on the ϵ- and ζ-chains (Wange *et al.*, 1993). It has been suggested that the CD4/CD8 co-receptor-associated p56^{lck} tyrosine kinase is involved in this process (Straus and Weiss, 1993) and these co-receptors require p56^{lck} association in order to amplify TCR-mediated signals (Glaichenhaus *et al.*, 1991). In Jurkat T-cells signalling via the TCR has been shown to require active p56^{lck} (Straus and Weiss, 1992), although a kinase independent function of p56^{lck} in antigen-specific T-cell activation has also been proposed (Xu and Littman, 1993).

The CD45 transmembrane PTPase has a major role in regulating TCR signal transduction (reviewed in Alexander *et al.*, 1992a, 1993). In mutant cell lines and T-cell clones lacking CD45, TCR signalling is abolished, but is restored following transfection of CD45 cDNA (Pingel and Thomas, 1989; Koretzky *et al.*, 1990, 1991; Shiroo *et al.*, 1992). Both p56^{lck} and p59^{fyn} have regulatory C-terminal tyrosine residues which in their phosphorylated state bind to their own SH2 domains, so preventing substrate phosphorylation (Peri *et al.*, 1993; Sieh *et al.*, 1993). Chimeric proteins containing the cytoplasmic portion of CD45 also restore signalling, demonstrating the importance of the intracellular portion of CD45 with its associated PTPase activity (Desai *et al.*, 1993; Hovis *et al.*, 1993; Volarevic *et al.*, 1993). CD45 has been shown to regulate p59^{fyn} and p56^{lck} *in vitro* by dephosphorylating these C-terminal tyrosine residues (Mustelin *et al.*, 1989, 1992). A similar role for CD45 *in vivo* has been suggested by the observation that the C-terminal residues of p59^{fyn} and p56^{lck} are hyperphos-

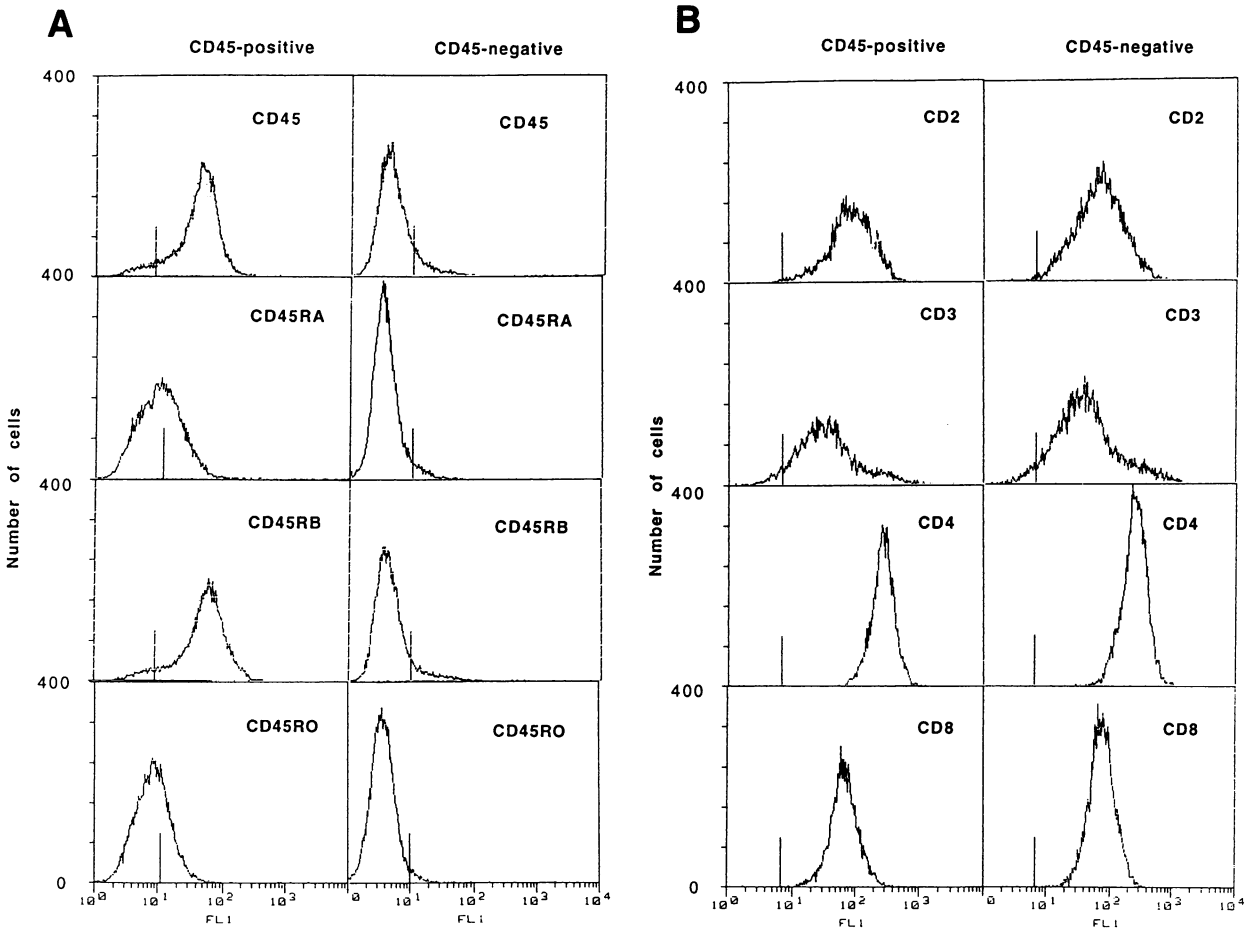

Fig. 1. Expression of cell surface markers and CD45 isoforms on CB1 T-ALL cells. Cells were stained with (**A**) CD45.2 (pan CD45), SN130 (CD45RA), PD7/26 (CD45RB), UCHL1 (CD45RO) or (**B**) OKT11 (CD2), OKT3 (CD3), QS4120 (CD4), OKT8 (CD8). The vertical line in each inset corresponds to the marker set at the upper limit of a sample of cells incubated with the second layer FITC goat anti-mouse only and represents the negative control.

phorylated in CD45$^-$ mutant murine T-lymphoma cell lines and T-cell clones (Ostergaard *et al.*, 1989; Cahir McFarland *et al.*, 1993; Hurley *et al.*, 1993), although in each of three leukaemic murine T-cell lines the level of phosphorylation varied considerably (Hurley *et al.*, 1993). We (Shiroo *et al.*, 1992) and others (Cahir McFarland *et al.*, 1993) have previously shown that expression of CD45 in T-cells correlates with increased p59^{fyn} kinase activity, although in HPB-ALL cells p56^{lck} activity was apparently not affected (Shiroo *et al.*, 1992; Alexander *et al.*, 1993).

These previous studies on CD45 have relied on long-established T-cell clones and cell lines which do not necessarily reflect the properties of T-cells *in situ*. We have recently identified a patient with T-acute lymphoblastic leukaemia (T-ALL) whose primary transformed CD3$^+$ CD4$^+$ CD8$^+$ T-cells comprised CD45$^-$ and CD45$^+$ populations. By establishing CD45$^-$ and CD45$^+$ subclones it was possible to demonstrate that CD45 regulates the activities of distinct pools of TCR-associated p59^{fyn} and CD4-associated p56^{lck} tyrosine kinases. Coupling of the TCR to intracellular signals was observed in the CD45$^+$ but not the CD45$^-$ subclones and this correlated with the ability of TCR-associated kinase(s) to phosphorylate TCR γ and ζ polypeptides on tyrosine residues. Our findings support the concept that the prime event in TCR signal transduction coupling is the phosphorylation of TCR components by CD45-activated TCR-associated p59^{fyn} and that this event can be amplified by CD45-activated CD4/CD8-associated p56^{lck}.

Results

CD45$^-$ and CD45$^+$ CB1 cells are derived from the same clone

A cell line (termed CB1) derived from a bone-marrow sample of a patient with T-ALL was established (see Materials and methods) and comprised a mixed population of CD45-expressing and non-expressing cells. This reflected the phenotype of the primary leukaemia which also contained CD45$^-$ and CD45$^+$ cells (data not shown). These were sorted with magnetic beads coated with CD45 monoclonal antibody (mAb) and after three rounds of depletion two subclones were established as CD45$^-$ (depleted) and CD45$^+$ (expressing) cells. The FACS profile of CD45 expression is shown in Figure 1A. CD45$^+$ cells express CD45RB-containing isoforms and lower levels of expression of the CD45RA and CD45RO isoforms. The CD45-depleted cells contained a residual proportion of cells (10%) expressing CD45 at very low levels (Figure 1A) and this population could not be removed by further magnetic bead panning or by cell sorting. Flow cytometry of the two subclones showed that the cell surface antigen expression

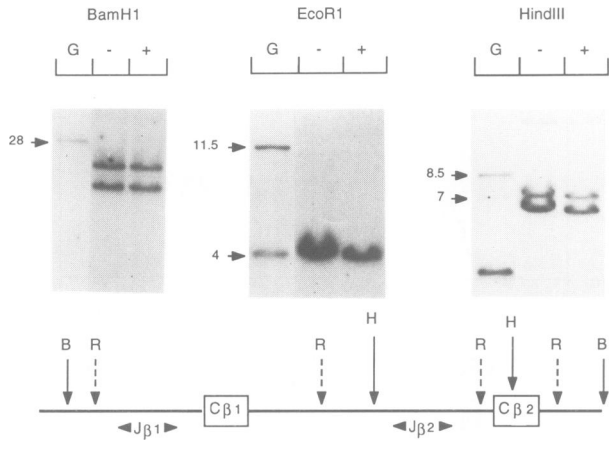

Fig. 2. Hybridization of TCR β-chain gene probes to DNA from CB1 CD45⁻ and CD45⁺ cells. DNA from DHLCL cells (G = germline), CD45⁻ (−) and CD45⁺ (+) cells was digested with the indicated restriction enzymes and separated in agarose gels before transferring to nitrocellulose filters. The filters were hybridized with a Cβ cDNA probe (M13B10BB1). Germline DNA was derived from DHLCL cells, a lymphoblastoid B cell line. *Bam*HI produces a single fragment of 28 kb in germline DNA containing both Cβ genes along with their respective J-clusters. Cutting with *Bam*HI produced a similar doublet in both subsets of CB1 cells showing that gene rearrangement had occurred on both chromosomes and that similar rearrangements are present in both subclones. To investigate the nature of the rearrangements the DNA was cut with *Eco*RI which produces two fragments which hybridize with the Cβ probe in germline DNA: one of 11.5 kb containing Cβ1 and its associated J-cluster and the other a 4 kb fragment containing only the Cβ2 gene. As *Eco*RI cleaves between Jβ2 and Cβ2, the restriction fragment is invariant unless deletion has occurred. The larger fragment produced by *Eco*RI digestion is absent in CD45⁻ and CD45⁺ CB1 cells, whereas the invariant 4 kb fragment is present. Hence Cβ1 has been deleted and Cβ2 is present in both subsets. To analyse rearrangements within the Cβ2 region further, the DNA was cut with *Hin*dIII which yields a 5′ 8.5 kb fragment and a 3′ 7 kb fragment in germline DNA. Digestion of CB1 DNA with *Hin*dIII produced the invariant 3′ fragment and showed that there were rearrangements in both copies of the Cβ2 gene. The identical patterns of DNA rearrangements and deletions in the Cβ genes of CD45⁻ and CD45⁺ CB1 cells provide substantial evidence that the cells are derived from the same clone.

of a number of T-cell markers was similar (Figure 1B) indicating that the cells might originate from the same clone. This was confirmed by examining the TCR genomic DNA Cβ genes (Rabbitts *et al.*, 1985) which revealed identical patterns of rearrangements and deletions in the CD45⁻ and CD45⁺ subclones (Figure 2).

Signal transduction pathways are blocked in CD45⁻ CB1 cells

TCR signal transduction in long-established T-cell lines has been shown to be dependent upon CD45 expression (Pingel and Thomas, 1989; Koretzky *et al.*, 1990, 1991; Shiroo *et al.*, 1992). We were interested to determine whether such a dependency existed in the recently isolated CB1 patient T-leukaemia cells. The effect of cross-linking CD3 alone, or with the CD4/CD8 co-receptors, was investigated with respect to calcium signalling (Figure 3). CD3 cross-linking induced a small rise (60 nM) in $[Ca^{2+}]_i$ in CD45⁺ CB1 cells, but had no effect on Ca^{2+} mobilisation in CD45⁻ cells consistent with observations in other CD45⁻ cells (Koretzky *et al.*, 1990; Shiroo *et al.*, 1992). The small rise in Ca^{2+} observed in CD45⁺ cells is consistent with the size

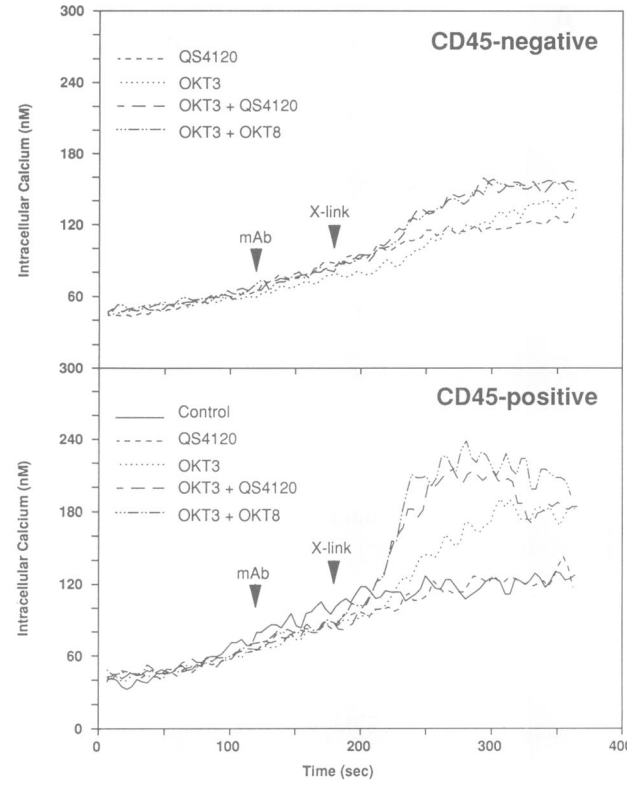

Fig. 3. Ca^{2+} mobilization in CD45⁻ and CD45⁺ CB1 subclones. Fura-2-loaded cells were incubated at 37°C before addition of mAb at 120 s (first arrow) followed 60 s later by sheep anti-mouse $F(ab')_2$ fragments (5 μg/ml). mAb concentrations were as follows: QS4120 (2 μg/ml), OKT3 (1 μg/ml), OKT8 (2 μg/ml). Determination of $[Ca^{2+}]_i$ is described in Materials and methods.

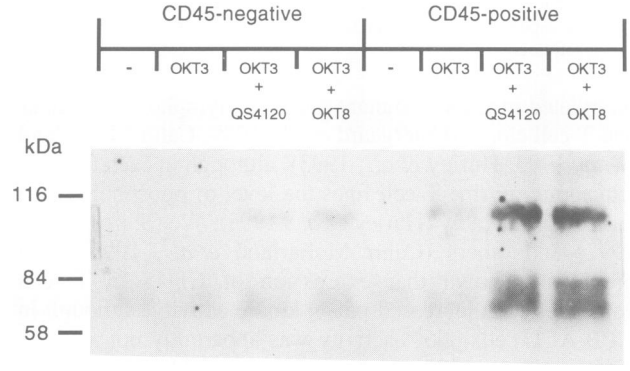

Fig. 4. Protein tyrosine phosphorylation in CB1 subclones. Cells were incubated with mAb for 2 min followed by addition of cross-linker before incubations were terminated with SDS sample buffer after a further 2 min. Where indicated added concentrations of mAb were OKT3 (1 μg/ml), OKT8 (2 μg/ml) and QS4120 (2 μg/ml). Proteins were separated on a 7.5% SDS−polyacrylamide gel under reducing conditions. Phosphotyrosine-containing proteins were probed with anti-phosphotyrosine antisera (4G10) and horseradish peroxidase conjugated anti-mouse IgG and detected by enhanced chemiluminescence. Comparable results were found in three separate experiments.

of Ca^{2+} signals observed in a number of patient bone-marrow derived T-ALL cells (Ledbetter *et al.*, 1991). Cross-linking CD3 with CD4 or CD8 produced a more rapid and larger (110 nM) mobilization of $[Ca^{2+}]_i$ in CD45⁺ CB1 cells. Similar effects of cross-linking CD3 with CD4 or CD8 have been observed in other CD4⁺ CD8⁺ T-cell lines

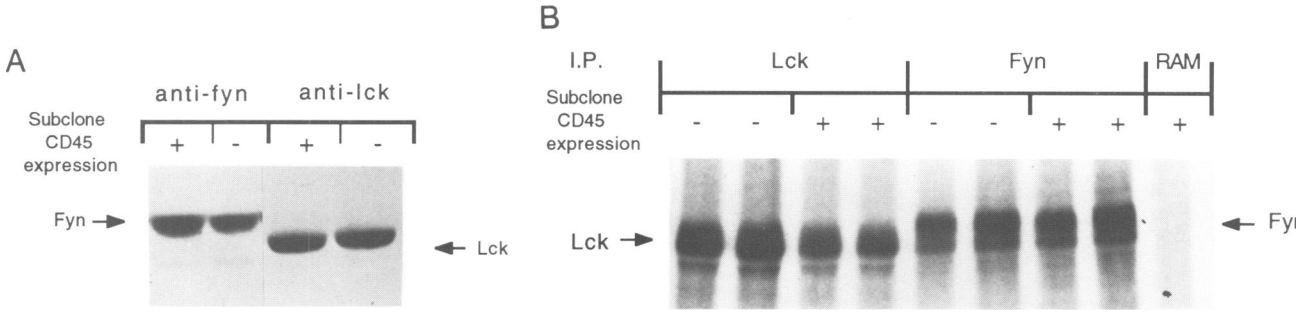

Fig. 5. Expression and activity of p59^{fyn} and p56^{lck} in CB1 subclones. **(A)** The level of expression of tyrosine kinases was determined from whole cell lysates (5 × 10^6 cells) resolved by 7.5% SDS–PAGE and transferred onto Immobilon-P membranes. p59^{fyn} and p56^{lck} were probed with specific antisera and detected with horseradish peroxidase-linked goat anti-rabbit IgG by enhanced chemiluminescence. **(B)** Activity of tyrosine kinases was determined following immunoprecipitation with antisera to p56^{lck} and p59^{fyn}. Protein A–Sepharose beads coated with rabbit anti-mouse IgG were used for a control immunoprecipitation. After kinase reactions the autophosphorylated proteins were separated on a 7.5% SDS–polyacrylamide gel and visualized by autoradiography. Comparable results were found in three separate experiments.

(Deans *et al.*, 1992; Shiroo *et al.*, 1992) and in patient T-ALL cells (Ledbetter *et al.*, 1991). However, whereas in CD45$^-$ HPB-ALL cells CD3 × CD4/CD8 co-ligation induces Ca^{2+} signals (Deans *et al.*, 1992; Shiroo *et al.*, 1992), in CD45$^-$ CB1 cells such a restoration of signalling was not observed. A small rise in Ca^{2+} (10–20 nM) was noted following co-ligation of CD3 with CD4/CD8 but this most likely reflects the 10% contaminating CD45$^+$ cells (Figure 1A).

Loss of CD45 has been associated with a defect in TCR-mediated protein tyrosine phosphorylation (Koretzky *et al.*, 1991; Shiroo *et al.*, 1992). In CB1 CD45$^+$ cells tyrosine phosphorylation of proteins of M_r 110, 80 and 70 kDa was observed following CD3 × CD4/CD8 cross-linking (Figure 4). CD3 cross-linking alone also increased the level of tyrosine phosphorylation of these proteins but to a much lesser extent. No increase in tyrosine phosphorylation was observed in CD45$^-$ CB1 cells following CD3 antigen cross-linking, neither were the phosphorylation signals restored following co-ligation of CD3 with CD4/CD8. These results suggest that in CD45$^-$ CB1 cells the inability to signal derives from an uncoupling of the TCR and its co-receptors from the actions of tyrosine kinases.

Level of expression and activity of whole cell p56^{lck} and p59^{fyn}

Loss of CD45 has been shown to result in increased phosphorylation of the C-terminal regulatory tyrosine of p56^{lck} and p59^{fyn} (Cahir McFarland *et al.*, 1993; Hurley *et al.*, 1993) which correlated with decreased activity of p59^{fyn} (Cahir McFarland *et al.*, 1993; Shiroo *et al.*, 1992). As these kinases have been shown to be important in mediating signals via the TCR, the level of their expression was determined in CB1 subclones (Figure 5A). Western blotting revealed that the level of p56^{lck} protein was similar in whole cell lysates from CD45$^+$ and CD45$^-$ cells, whereas the level of expression of p59^{fyn} appeared to be slightly higher in CD45$^+$ CB1 cells. Figure 10A shows that equivalent amounts of p59^{fyn} bound to the TCR in CD45$^+$ and CD45$^-$ CB1 cells, so the lower expression of total p59^{fyn} in the CD45$^-$ cells did not decrease the specific receptor-associated pool of this kinase.

As p59^{fyn} and p56^{lck} are expressed in CB1 cells, the inability to couple to intracellular signals in CD45$^-$ cells could result from a reduction in the activity of these kinases. Total cellular p59^{fyn} and p56^{lck} were immunoprecipitated from CB1 subclones and their activity determined by the level of autophosphorylation following *in vitro* kinase reactions. Surprisingly, no difference in total p59^{fyn} activity was apparent between the two subclones and total p56^{lck} activity was higher in immunoprecipitates from CD45$^-$ CB1 cells (Figure 5B). Since this result was opposite to that expected, we speculated that measurements of total kinase activity immunoprecipitated from cell lysates might not reflect the activity of specific intracellular enzyme pools. Further work was carried out to investigate this possibility.

Distribution and activity of p59^{fyn} and p56^{lck} in membranes from CB1 cells

Activities of p59^{fyn} and p56^{lck} were determined in a membrane fraction from CB1 CD45$^+$ cells (Figure 6). p59^{fyn} activity was the same whether immunoprecipitated from whole cell lsates or membranes from an equivalent number of cells (Figure 6A). In contrast, p56^{lck} activity in membranes was substantially reduced relative to the total activity in cells. The level of p59^{fyn} protein in membranes was comparable to that observed in the whole cell, whereas p56^{lck} levels were lower in membranes (Figure 6B). However, the decreased p56^{lck} level in membranes was not as striking as the marked decrease in p56^{lck} kinase activity in membranes when compared with whole cells (Figure 6A), suggesting that the cytoplasmic p56^{lck} pool may be more active than the membrane-bound pool. These results show that p59^{fyn} is principally membrane-associated in CB1 cells, whereas p56^{lck} is in both membrane and cytoplasmic pools, its kinase activity in these locations being differentially regulated.

Comparison of the activities of p59^{fyn} and p56^{lck} in the membrane fractions from CD45$^+$ and CD45$^-$ cells revealed a similar pattern of activities to those observed in immunoprecipitates from whole cells, namely, equivalent p59^{fyn} activities, but a higher p56^{lck} activity in the CD45$^-$ than in the CD45$^+$ subclones (Figure 6C). Confocal microscopy of fluorescently labelled p56^{lck} showed that much of the enzyme was in intracellular locations in CD45$^+$ CB1 cells (Figure 6D), a result previously observed in stimulated Jurkat cells (Marie-Cardine *et al.*, 1992). p56^{lck} labelling comparable to that shown in Figure 6D was also observed in CD45$^-$ cells (data not shown), indicating that within the limitations of the technique

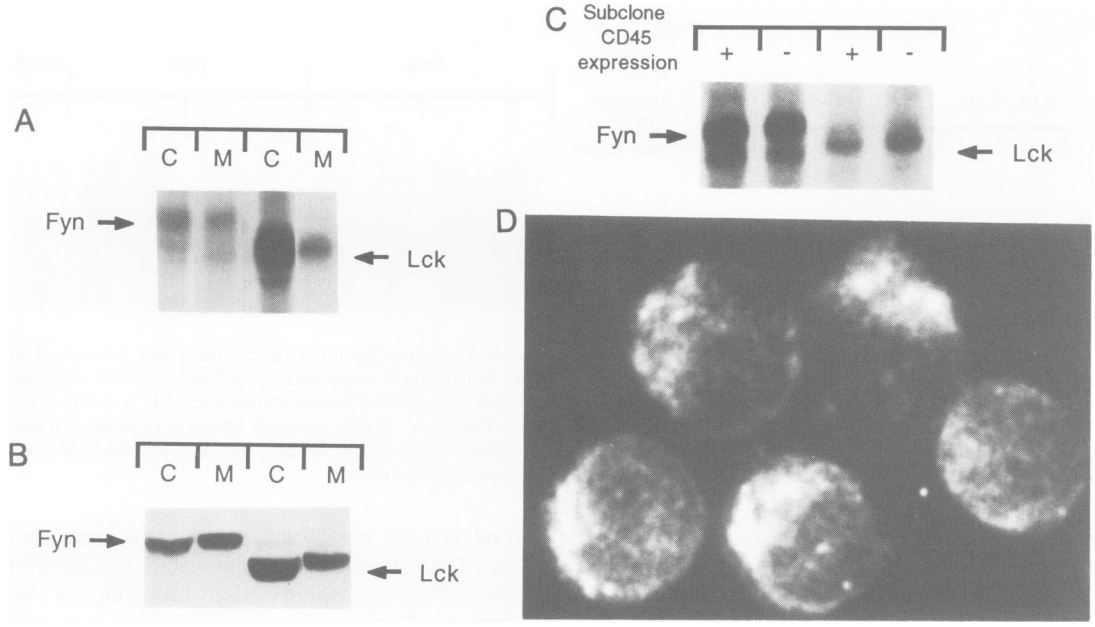

Fig. 6. Distribution and activity of tyrosine kinases in CB1 cells. A membrane fraction was prepared (see Materials and methods) from CD45$^+$ CB1 cells and the kinase activity (**A**) and protein levels (**B**) of p59^{fyn} and p56^{lck} were determined in whole cell lysates (C) and membranes (M) prepared from an equivalent cell number (5×10^6 cells). The whole cell lysates were made in 1% NP-40 lysis buffer (see Materials and methods) and the nuclear pellet removed before separating proteins on a 7.5% SDS–polyacrylamide gel. The activity of p56^{lck} and p59^{fyn} in membranes from CD45$^-$ and CD45$^+$ subclones of CB1 cells was determined (**C**) and the localization of p56^{lck} in CD45$^+$ CB1 cells determined by immunofluorescence and visualized in a whole cell projection by confocal microscopy (**D**). Comparable results were found in three separate experiments.

the distribution of p56^{lck} in the two subclones was comparable.

Determination of cell surface receptor associated pools of tyrosine kinases

A novel method was developed to isolate cross-linked cell surface receptors, together with their associated tyrosine kinases. The technique involved incubating cells with magnetic beads coated with cell surface receptor-specific mAbs. By harvesting the bead-bound cells with a magnet and then disrupting the cells with lysis buffer and extensively washing the beads, purified immunocomplexes were obtained comprising only those receptors which had been bound and aggregated by mAb on the surface of intact cells. Using this approach, CD4-associated p56^{lck} was immunoprecipitated and its activity determined by *in vitro* kinase assay. It was apparent that the autophosphorylation of p56^{lck} was reduced in CD45$^-$ relative to CD45$^+$ CB1 cells (Figure 7), precisely the opposite result to that obtained using p56^{lck} immunoprecipitates derived from whole cell lysates (Figure 5B) or from membranes (Figure 6C). The incorporation of ^{32}P into p56^{lck} in CD45$^+$ CB1 cells was 2143 ± 145 c.p.m. and in CD45$^-$ cells 1051 ± 7 c.p.m. for the average ± range of the bands shown in Figure 7. The average of four such determinations showed that p56^{lck} autophosphorylation was 53 ± 14% of that determined in CD45$^+$ cells. Phosphorylation of an exogenous substrate also revealed a striking reduction in p56^{lck} kinase activity associated with CD4 in CD45$^-$ CB1 cells. Phosphorylation of the peptide in magnetic bead–CD4–p56^{lck} immunocomplexes during a 15 min incubation amounted to 6343 ± 1343 and 28 735 ± 1714 for CD45$^-$ and CD45$^+$ cells respectively (n = 3, means ± SD). The reduction in p56^{lck} activity in CD45$^-$ cells was not the result of decreased

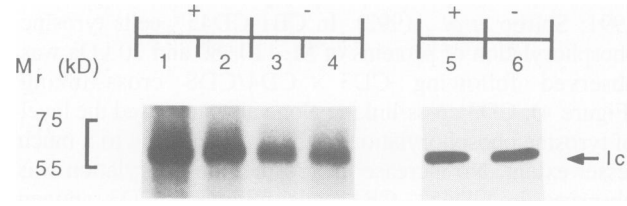

Fig. 7. Tyrosine kinases associated specifically with cell surface CD4 in CB1 subclones. Magnetic beads coated with mAb to CD4 (QS4120) were bound to cells at 4°C and the magnetic bead-associated cells harvested after 15 min. The cells were lysed in NP-40 lysis buffer and the beads harvested and washed twice more. *In vitro* kinase assays were performed on bead immunoprecipitates from CD45$^+$ (tracks 1 and 2) and CD45$^-$ (tracks 3 and 4). CB1 cells and the phosphorylated proteins were separated by SDS–PAGE and visualized by autoradiography, whereas the level of p56^{lck} associated with the beads (tracks 5 and 6) was determined by Western blotting.

p56^{lck} protein in the immunoprecipitates since the level of p56^{lck} immunoprecipitated was the same in both subclones (Figure 7, tracks 5 and 6).

A similar technique was used to immunoprecipitate specifically TCR-associated tyrosine kinases using mild detergent conditions which conserve TCR polypeptide associations (Figure 8). Cells were bound to OKT3 mAb-coated magnetic beads during incubation at 4°C and the cells either lysed immediately following this incubation or activated for 3 min at 37°C. Such a step has been shown to be important in recruiting the tyrosine kinase ZAP-70 to the TCR in Jurkat cells (Wange *et al.*, 1992). In CD45$^+$ CB1 cells immunoprecipitation of the TCR followed by *in vitro* kinase reactions resulted in the phosphorylation of a number of proteins (Figure 8, track 2), and the level of phosphorylation was enhanced following pre-incubation of the cells at 37°C for 3 min (Figure 8, tracks 5 and 6). In

contrast, very little phosphorylation was observed following immunoprecipitation of the TCR from CD45$^-$ cells (lane 1), and no enhancement was observed following activation of the cells (lanes 3 and 4). Phosphorylation of an exogenous peptide showed that the tyrosine kinase associated with the TCR in CD45$^-$ CB1 cells was 84 ± 11% less active than that in CD45$^+$ cells (n = 3 ± SD). These results show that in CD45$^-$ CB1 cells the specific pools of tyrosine kinase activities associated with CD4 and the TCR are greatly reduced.

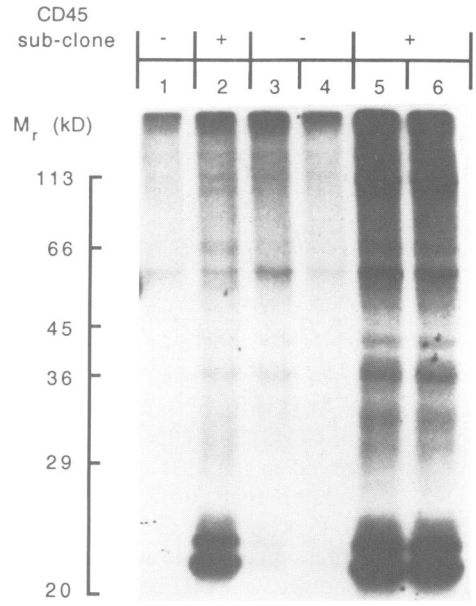

Fig. 8. Tyrosine kinase activity associated with the TCR in CB1 subclones. Magnetic beads coated with mAb to CD3 antigen (OKT3) were bound to cells during a 60 min incubation at 4°C. Cells were either harvested immediately with a magnet (tracks 1 and 2) or incubated for 3 min at 37°C (tracks 3–6) before harvesting. Cells were lysed in Brij-96 lysis buffer and the beads harvested and washed twice more. *In vitro* kinase assays were determined as described in Materials and methods and the phosphorylated proteins separated by SDS–PAGE and visualized by autoradiography.

Identification of phosphorylated components in TCR complexes

The most prominent proteins which were phosphorylated following *in vitro* kinase assays of CD45$^+$ CB1 cell TCR immunocomplexes were proteins of M$_r$ 21 and 23 kDa when separated on a gel under reducing conditions. To characterize these proteins further the phosphorylated immunoprecipitates were incubated in lysis buffer containing the detergent NP-40 to disrupt interactions between TCR components. Successful disruption was confirmed by harvesting the magnetic beads after this treatment and finding that nearly all the radioactivity resided in the supernatant (result not shown). The dissociated components of the TCR were then re-immunoprecipitated with specific antisera to the γ- and ζ-chains and when the proteins were separated by SDS–PAGE they co-migrated with the bands at 23 and 21 kDa respectively (Figure 9A, lanes 3 and 4). Confirmation that the 21 kDa protein was the ζ-chain was obtained by separating the proteins under non-reducing conditions which generated the expected ζζ homodimer of 42 kDa (Figure 9A, lane 1). The possibility existed that residues besides tyrosines were phosphorylated in the TCR immunocomplex. However, Figure 9B shows that treatment of TCR immunocomplexes with T-cell PTPase, which specifically removes phosphate from tyrosine (Cool *et al.*, 1990), completely removed the phosphate from the 42 and 23 kDa proteins. This dephosphorylation was inhibited by addition of the PTPase inhibitor, vanadate (Figure 9B), demonstrating that only tyrosine residues were phosphorylated. Western blotting revealed that the level of TCR components ζ and ε were identical in the immunocomplexes derived from the two subclones (Figure 9C) showing that the differences in phosphorylation observed in Figure 8 were not due to different amounts of receptor polypeptides in the complexes.

TCR-associated p59^{fyn} is active in CD45$^+$ but inactive in CD45$^-$ CB1 cells

p59^{fyn} has been shown to associate with the TCR (Samelson *et al.*, 1990; Timson Gauen *et al.*, 1992). Western blotting

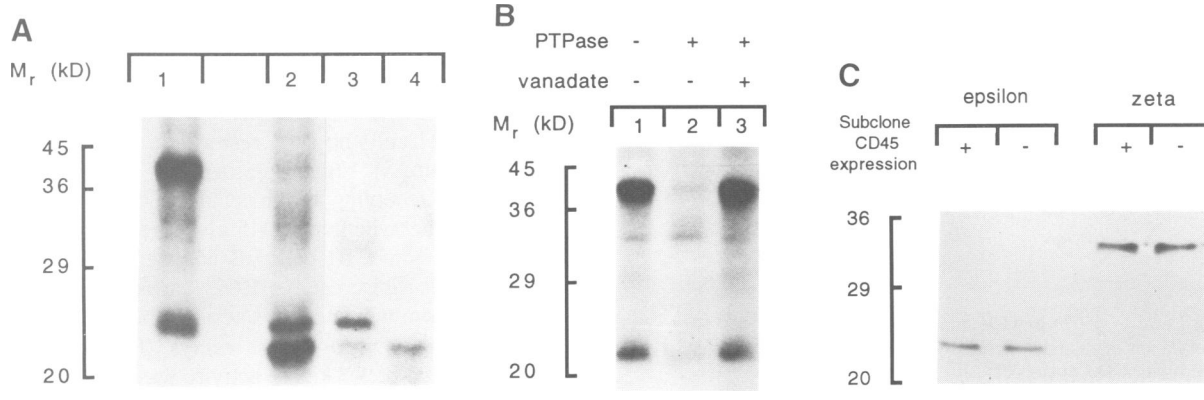

Fig. 9. Identification of TCR components tyrosine phosphorylated in *in vitro* kinase assays. (**A**) OKT3-coated magnetic beads were used to immunoprecipitate the TCR from CD45$^+$ CB1 cells. Following *in vitro* kinase assays the beads were washed three times in Brij-96 lysis buffer and the proteins separated by SDS–PAGE (11% cross-linking) under non-reducing (track 1) or reducing (track 2) conditions. Following the kinase reaction and washing, the TCR complex associated with the beads was dissociated in NP-40 lysis buffer (10 min on ice), the lysate removed and the γ-chain (track 3) and ζ-chain (track 4) immunoprecipitated with specific antisera. The proteins were separated under reducing conditions. (**B**) TCR-associated proteins phosphorylated during *in vitro* kinase reactions were further incubated with T-cell PTPase (0.3 U) (track 2) or with T-cell PTPase + vanadate (0.1 mM) (track 3) for 10 min at 37°C. Track 1 shows the extent of phosphorylation at t = 0. Proteins were separated by SDS–PAGE under non-reducing conditions. Comparable results were found in three separate experiments. (**C**) TCR immunocomplexes from CD45$^-$ and CD45$^+$ cells were separated by SDS–PAGE under non-reducing conditions, transferred to Immobilon-P and probed with antisera to ε and ζ.

of the magnetic bead-bound TCR immunocomplexes from CD45$^-$ and CD45$^+$ CB1 cells showed that equivalent levels of p59^{fyn} were immunoprecipitated (Figure 10A). To determine the activity of p59^{fyn} associated with the TCR, the immunoprecipitates from CD45$^-$ and CD45$^+$ cells were dissociated by addition of NP-40 lysis buffer. p59^{fyn} was then re-immunoprecipitated from this lysate and the ability to phosphorylate a peptide substrate was assessed. No substrate phosphorylation was observed with p59^{fyn} re-immunoprecipitated from CD45$^-$ CB1 cells, but an incorporation of 3931 ± 121 c.p.m. (n = 2 ± range) from p59^{fyn} derived from the TCR of CD45$^+$ CB1 cells was measured. These results demonstrate that p59^{fyn} was present in the TCR complexes of CD45$^-$ cells but that the kinase was inactive. Recombinant p59^{fyn} was added to magnetic bead–TCR immunocomplexes isolated from CD45$^-$ cells and, following an *in vitro* kinase assay, the complexes were washed in lysis buffer to remove any unassociated proteins present. Under these conditions phosphorylation of both the γ- and ζ-chains was observed, showing that these substrates can be phosphorylated by active recombinant p59^{fyn} in CD45$^-$ CB1 cells (Figure 10B). This confirmed that the inability of TCR-associated p59^{fyn} to phosphorylate TCR polypeptides in CD45$^-$ cells (Figure 8) was due to the inactivity of the kinase and not to abnormalities in its substrates.

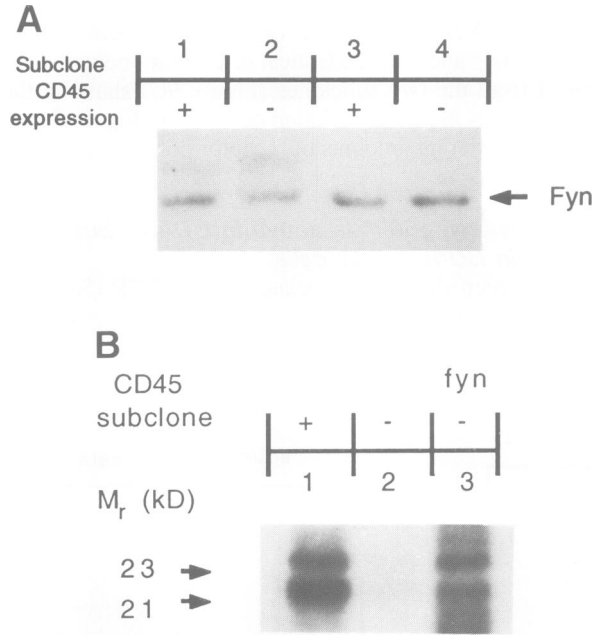

Fig. 10. Level of p59^{fyn} in TCR immunocomplexes and phosphorylation of ε- and ζ-chains by recombinant p59^{fyn}. (**A**) p59^{fyn} associated with CD3 mAb-coated magnetic bead immunocomplexes (lanes 3 and 4) or in equivalent loadings of cell lysates following removal of TCR immunocomplexes (lanes 1 and 2) was determined by Western blotting as described in Figure 5A. 5 × 10^6 cells were used as the starting material for each lane. Similar results were found in two separate experiments. (**B**) OKT3–magnetic bead immunoprecipitates of the TCR from CD45$^+$ (track 1) and CD45$^-$ (tracks 2 and 3) cells were phosphorylated *in vitro* under normal conditions (tracks 1 and 2) or with recombinant p59^{fyn} (track 3). Following the reaction the beads were washed three times in Brij-96 lysis buffer and the OKT3-associated phosphorylated proteins separated by SDS–PAGE under reducing conditions.

Discussion

An investigation into the role of CD45 in regulating the activity and actions of tyrosine kinase members of the *src* family was carried out using CD45$^-$ and CD45$^+$ mutant CB1 cells derived from a patient with T-ALL. In contrast to previous studies on human CD45$^-$ T-cells, which have depended on the use of long-established cell lines and clones (Pingel and Thomas, 1989; Koretzky *et al.*, 1990, 1991; Shiroo *et al.*, 1992), the CD45$^-$ phenotype of CB1 cells was observed within 24 h of obtaining the bone-marrow sample from which they were derived. This phenotype was maintained in culture (Figure 1) and it is therefore likely that the results we describe reflect the contrasting properties of CD45$^-$ and CD45$^+$ cells *in situ*.

The CD3^{lo} CD4^{hi} CD8^{hi} phenotype of CB1 cells (Figure 1) is characteristic of early transformed thymocytes. The clonal origin of the CD45$^-$ and CD45$^+$ subclones (Figure 2) indicates either that CD45 expression was lost from a T-cell clone *in situ* and transformation subsequently occurred, or that loss of CD45 was a consequence of transformation. Although our data do not distinguish between these possibilities, it is of interest that there have been previous reports of CD45$^-$ populations of leukaemia cells from patients (Behm *et al.*, 1992). In an analysis of 217 cases of T- and B-lineage ALL, 40% of T-ALL cases contained mixed populations of CD45$^-$ and CD45$^+$ blasts, similar to the expression observed in the CB1 cells (60% CD45$^+$; 40% CD45$^-$). Greater than 60% of B-ALL cases comprised mixed CD45 populations and in 32 out of the 191 cases no surface expression of CD45 was observed (Behm *et al.*, 1992). Our findings with CB1 cells therefore support a correlation between lymphocyte transformation and loss of CD45.

There are a number of reports that lack of CD45 expression on the cell surface causes ablation of signalling via the TCR (Pingel and Thomas, 1989; Koretzky *et al.*, 1990, 1991; Shiroo *et al.*, 1992). In CD45$^-$ CB1 cells this was similarly found to be the case when assaying tyrosine phosphorylation (Figure 4) or mobilization of $[Ca^{2+}]_i$ (Figure 3). In CD45$^-$ HPB-ALL cells signalling is restored when the TCR is co-ligated with either CD4 or CD8 (Deans *et al.*, 1992; Shiroo *et al.*, 1992). This is thought to be the result of p56^{lck} associated with these co-receptors compensating for the loss of p59^{fyn} activity (Shiroo *et al.*, 1992). When CD4 or CD8 were co-ligated with the TCR in CD45$^-$ CB1 cells no such restoration of signalling was observed (Figure 3), suggesting that p56^{lck} was incompetent to restore TCR-coupling to intracellular signals in these cells.

To determine whether CD45 regulated the activity of tyrosine kinases in CB1 cells, as is observed for p59^{fyn} in HPB-ALL cells (Shiroo *et al.*, 1992), kinases were immunoprecipitated from whole cell lysates and their kinase activity determined in *in vitro* kinase assays (Figure 5B). This showed that total p59^{fyn} kinase activity was equivalent in the two subclones, whereas the activity of p56^{lck} was actually higher in the CD45$^-$ CB1 cells. A similar elevation of p56^{lck} activity has been reported in immunoprecipitates from whole cell lysates of CD45$^-$ relative to CD45$^+$ Jurkat cells (Danielian *et al.*, 1992). It is difficult to reconcile the results obtained from total p56^{lck} and p59^{fyn} activities in CD45$^-$ CB1 cells with the lack of signalling in these cells, as enhanced levels of signalling might be expected to result from increased kinase activities.

Previous work using several T-cell lines revealed that the extent of C-terminal tyrosine phosphorylation of p56^{lck} and p59^{fyn} varies considerably as a result of loss of CD45 expression (Hurley *et al.*, 1993). This could imply either that p59^{fyn} and p56^{lck} are not solely regulated by CD45 or that distinct pools of these tyrosine kinases are regulated by CD45. It is clear that *in vitro* assays of tyrosine kinases in immunoprecipitates from whole cell lysates measure average kinase activities and do not necessarily reflect the activity of specific pools of enzymes associated with cell surface receptors. In an attempt to define these pools, p59^{fyn} and p56^{lck} kinase activities were measured in membrane fractions, but the results still reflected those observed from whole cell lysates (Figure 6). A novel technique was therefore devised using mAb-coated magnetic beads to isolate purified immunocomplexes containing aggregated cell surface receptors together with their associated kinases. Using this method it was apparent that in CD45$^-$ cells the CD4-associated p56^{lck} and TCR-associated kinase activities were substantially reduced (by 74−84% towards exogenous substrates) when compared with CD45$^+$ cells (Figures 7 and 8). This is the first demonstration that the actions of a PTPase can be regulated by its intracellular localization in relation to its physiologically relevant substrates *in situ*. Of particular interest was the observation that comparable levels of p59^{fyn} associated with the TCR in both CD45$^-$ and CD45$^+$ subclones (Figure 10A), although the activity of this specific TCR-associated kinase pool in CD45$^-$ cells was barely detectable (Figure 8). These results demonstrate that CD45 regulates the activity of p59^{fyn} and not its association with the TCR and are also consistent with the hypothesis that binding of p59^{fyn} to the TCR does not require tyrosine phosphorylation of TCR polypeptides. Co-expression of p59^{fyn} but not p56^{lck} with a homodimeric TCR $\beta\zeta$ fusion protein in COS cells, followed by cross-linking, was sufficient to enable activation of PLCγ_1 (Hall *et al.*, 1993), further supporting a pivotal role for p59^{fyn} in TCR-mediated signal transduction.

In CD45$^+$ cells activation of the cells resulted in increased tyrosine kinase activity (Figure 8). Activation of T-cells increases the level of tyrosine phosphorylation of all polypeptides comprising the CD3 antigen and also those of the associated $\zeta\zeta$ homodimer (Baniyash *et al.*, 1988; Qian *et al.*, 1993; Sancho *et al.*, 1993). Such an increase in tyrosine phosphorylation has been shown to mediate binding of ZAP-70 via its two SH2 domains to ϵ- and ζ-chains (Straus and Weiss, 1993; Wange *et al.*, 1993). This probably reflects recruitment of ZAP-70 kinase to the TCR (Chan *et al.*, 1992; Wange *et al.*, 1993). As such an enhancement was not observed in CD45$^-$ CB1 cells this suggests that in TCR-mediated signal transduction active p59^{fyn} is required for further recruitment of tyrosine kinases to the receptor. These results are consistent with a model in which the prime event in TCR signal transduction coupling is the phosphorylation of TCR components by CD45-activated, TCR-associated p59^{fyn}, resulting in the recruitment of a signal transduction complex by interaction of SH2 domain containing proteins with phosphorylated TCR polypeptide motifs. Thus the almost total lack of detectable tyrosine kinase activity in TCR immunocomplexes from CD45$^-$ CB1 cells (Figure 8) correlates with the uncoupling of the receptor in these cells from tyrosine phosphorylation and Ca^{2+} mobilization (Figures 3 and 4). The inability of CD4 or CD8 co-ligation with the TCR to restore signalling is also consistent with the 78% inhibition in CD4-associated p56^{lck} kinase activity, and provides support for the concept that the ability of CD4/CD8-TCR co-ligation to restore or amplify signal transduction coupling depends on p56^{lck} activity (Shiroo *et al.*, 1992). Thus, in our previous work on HPB-ALL T-cells in which p56^{lck} kinase activity was apparently comparable between CD45$^-$- and CD45-transfected subclones, CD4/CD8-TCR co-ligation did restore TCR-mediated signalling in CD45$^-$ cells (Shiroo *et al.*, 1992). The reason for the apparent inability of CD45 to regulate p56^{lck} activity in HPB-ALL cells is not known, but may be elucidated by further investigation of the specific increment of cell surface CD4/CD8-associated p56^{lck} in these cells.

Overall our results clearly show that in CB1 T-cells there is differential regulation of distinct pools of p56^{lck} and p59^{fyn} kinases in different intracellular localizations. CD45 appears to activate only those specific kinase pools which associate with cell surface receptors. In CB1 cells p59^{fyn} is membrane-associated whereas p56^{lck} is in both membrane and cytosolic fractions (Figure 6). Confocal microscopy revealed extensive distribution of p56^{lck} throughout the cytoplasm (Figure 6D), although the extent to which this pool of the enzyme is soluble and/or membrane-associated is unclear. Activation of Jurkat cells by cross-linking the CD2 antigen produces a redistribution of p56^{lck} from the plasma membrane to a vesicular fraction (Marie-Cardine *et al.*, 1992). This endocytosis of p56^{lck} also correlated with an increase in its activity, so the possibility arises that the overall increased activity of p56^{lck} in CD45$^-$ CB1 cells (Figure 5B) might result from its positive regulation at a site removed from the plasma membrane. The profound block in thymocyte development that occurs prior to CD4/CD8 expression in the absence of p56^{lck} (Molina *et al.*, 1992) is mimicked by the expression of a catalytically inactive form of the enzyme (Levin *et al.*, 1993) suggesting that a pool of p56^{lck} kinase plays a critical role in T-cell development prior to its association with the CD4/CD8 co-receptors. Since our present findings using CB1 cells indicate that CD45 may not be the only PTPase that activates p56^{lck}, the characterization of other PTPase(s) that regulate its kinase activity at specific stages of T-cell development is clearly of importance.

The striking correlation between lymphocyte transformation and loss of CD45 expression is intriguing and requires further elucidation. In CD4$^+$ CD8$^+$ thymocytes cross-linking of the TCR induces a rise in Ca^{2+} and apoptosis (McConkey *et al.*, 1989). Therefore, it is possible that in CD45$^-$ cells the inability to signal via the TCR and its co-receptors may prevent the induction of apoptosis by this pathway. This would result in increased cell life-spans, a situation which has marked similarities to those where the *bcl-2* gene product is over-expressed and increases cell survival (Vaux *et al.*, 1988). If loss of CD45 prevents the apoptosis of specific lymphocyte subclones, it is possible that this event leads to an inappropriate prolongation of cell proliferation, so increasing the likelihood of secondary transforming events. Although clonal deletion of super-antigen-reactive T-cells was observed in CD45 exon 6-deficient mice (Kishihara *et al.*, 1993) the report of CD45$^-$ populations in leukaemias (Behm *et al.*, 1992), together with our present observations on CB1 cells, indicates

that loss of CD45 may predispose a cell to the transformed state.

Materials and methods

CB1 cell line

A bone marrow sample (provided by Dr Valerie Broadbent and Sheila O'Connor, Addenbrooke's Hospital, Cambridge, UK) taken with consent from a patient with childhood T-ALL was purified by centrifuging the sample after layering onto Lymphoprep (Nycomed). The cells were washed and incubated in RPMI-1640 supplemented with 10% fetal calf serum (FCS). The cells were maintained in this medium and over 6 weeks expanded in number to give rise to the CB1 cell line which continued to grow in culture medium supplemented with 5% FCS.

mAbs

mAbs used were OKT3 (CD3, IgG2a), QS4120 (CD4, IgG1) and UCHL-1 (CD45RO, IgG2a) (from Prof. P.Beverley, Middlesex Hospital, London, UK); SN130 (CD45RA, IgG2a) (from Prof. G.Janossy, Royal Free Hospital, London, UK); PD7/26 (CD45RB, IgG1) (from Dr D.Mason, Department of Haematology, Oxford, UK); CD45.2 (pan-CD45, IgG1) (from Prof. S.Meuer, German Cancer Research Centre, Heidelberg, Germany) and OKT8 (CD8, IgG2a).

Flow cytometry

Cells were stained with mAbs specific for cell surface markers, washed, incubated with goat anti-mouse-FITC (fluorescein isothiocyanate) antisera and detected in a FACSCAN flow cytometer (Becton Dickinson).

DNA filter hybridizations

Genomic DNA was extracted by incubating the cells in Tris/EDTA pH 8.0, 150 mM NaCl, 0.2% SDS and digested overnight at room temperature with proteinase K (300 μg/ml). The sample was deproteinated by adding an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 by volume) and the supernatant washed with chloroform:isoamyl alcohol (24:1 v/v). The DNA was precipitated by addition of ethanol, washed in 70% ethanol and dissolved in Tris−NaOH pH 8.0, 1 mM EDTA. DNA was digested with restriction enzymes and separated in agarose gels before transferring to nitrocellulose filters. The filters were hybridized with a Cβ cDNA probe (M13B10BB1) in 3 × SSC with 0.1% SDS for 20 h at 65°C and visualized by autoradiography.

Calcium determinations

Cells (10^6/ml) were loaded with 1 μM Fura-2/AM and resuspended in HEPES-buffered Hanks' buffered salt solution pH 7.3 supplemented with 2% FCS and 0.1 mM sulfin pyrazone. Assays were performed on 4×10^6 cells in a Perkin-Elmer LS5B Luminescence Spectrophotometer using Fura-2 software provided by Perkin Elmer. $[Ca^{2+}]_i$ concentrations were calculated and plotted using Lotus software kindly provided by C.Naylor.

Protein tyrosine phosphorylation

Cells (5×10^6) were incubated with mAb for 2 min followed by addition of cross-linking sheep anti-mouse IgG F(ab')$_2$ fragments (5 μg/ml), before incubations were terminated with SDS sample buffer after a further 2 min. The proteins were separated by SDS−PAGE and the proteins transferred to Immobilon-P membranes. Phosphotyrosine-containing proteins were probed with anti-phosphotyrosine antisera (4G10) and horseradish peroxidase conjugated anti-mouse IgG and visualized by fluorography with enhanced chemiluminescence (Amersham).

Western blotting of $p56^{lck}$ and $p59^{fyn}$

Lysates from 5×10^6 cells were resolved on a 7.5% SDS−polyacrylamide gel and transferred to Immobilon-P membranes. $p59^{fyn}$ was probed using antisera supplied by Dr C.Rudd (Dana-Farber Institute, Boston, USA) and $p56^{lck}$ with antisera given by Dr L.Samelson (NIH, Bethesda, MD, USA). Detection was by ECL.

Immunoprecipitation of $p56^{lck}$ and $p59^{fyn}$

Cells (3×10^6) were lysed with 1% NP-40, 25 mM HEPES−NaOH pH 7.8, 150 mM NaCl, 10 mM EDTA, 1 mM EGTA, 0.1 mM sodium vanadate plus protease inhibitors. Protein A−Sepharose beads coated with $p56^{lck}$ antisera (supplied by Dr R.Abraham, Mayo Clinic, Rochester, NJ, USA) and $p59^{fyn}$ antisera (provided by Dr C.Rudd) were used to immunoprecipitate their respective tyrosine kinases from the post-nuclear supernatants.

In vitro kinase assays

Reactions were in 40 μl kinase assay buffer containing 50 mM HEPES pH 7.8, 10 mM $MgCl_2$, 25 μM ATP [γ-^{32}P]ATP (150 kBq), 0.1 mM vanadate at 37°C for 15 min. Reactions were terminated by addition of excess lysis buffer and the beads washed before separating the proteins on a 7.5% SDS−polyacrylamide gel. The autophosphorylated proteins were visualized by autoradiography. In some assays exogenous peptide substrates containing tyrosine residues (Src or ZAP peptides at 0.5 mM) were included to determine kinase activities. Reactions were terminated in the same way and the supernatants were applied to p81 phosphocellulose paper and washed in phosphoric acid (0.5%). Control values for kinase assay buffer alone were subtracted in each case. Peptide phosphorylation reactions were linear for at least 20 min.

CB1 membrane preparation

Cells in hypotonic buffer (10 mM HEPES−NaOH pH 7.4, 1 mM EGTA, 10 mM benzamidine, 0.1 mM vanadate) were disrupted by two rounds of freezing/thawing. The membranes were pelleted by centifugation at 15 000 *g* for 5 min and resuspended in NP-40 lysis buffer for subsequent immunoprecipitation of tyrosine kinases.

Immunoprecipitation of TCR and associated proteins

Magnetic beads covalently bound to sheep anti-mouse IgG were incubated overnight with mAb to CD3 antigen (OKT3). The beads were washed three times in PBS and added to aliquots of cells (1×10^7) in RPMI 1640/5% FCS and rotated at 4°C for 60 min. The cells were incubated for 3 min at 37°C and the reaction was stopped by placing the cells in a magnet followed by removal of cells not bound to beads and addition of ice-cold lysis buffer to cells bound to beads. Lysis buffer was as follows: 0.1% (w/v) Brij-96, 25 mM HEPES−NaOH pH 7.8, 150 mM NaCl, 10 mM EDTA, 1 mM EGTA, 10 mM benzamidine, 1 mM PMSF, 50 mM NaF, 0.1 mM vanadate. The beads were harvested and washed twice more with lysis buffer. The beads with their asssociated immunocomplexes were then incubated with the kinase reaction mixture and the incubations terminated after 15 min by addition of a 4-fold excess of lysis buffer. The beads were washed three times with lysis buffer and then heated at 105°C for 5 min in sample buffer. The phosphorylated proteins were separated by SDS−PAGE and visualized by autoradiography. TCR polypeptides were immunoprecipitated using γ-chain antisera previously described (Alexander *et al.*, 1992b) and ζ-chain antisera kindly provided by Dr D.Cantrell (Imperial Cancer Research Fund, London, UK). The recombinant T-cell PTPase was from Glaxo Ltd (London, UK).

Immunoprecipitation of CD4 antigen

The protocol was similar to that used for immunoprecipitation of the TCR except the beads were coated with anti-CD4 antigen mAb (QS4120) and the lysis buffer contained NP-40 in place of Brij-96.

Immunofluorescent staining of cells for $p56^{lck}$

Cells, cytospun onto glass slides coated with 3-aminopropyltriethoxysilane (3%) were fixed in methanol at −20°C for 7 min followed by acetone at 4°C for 5 min. After rinsing in PBS and blocking with goat serum the cells were incubated with anti-$p56^{lck}$ mAb (kindly given by Dr S.Fischer, Institut Cochin de Genetique Moleculaire, Paris, France). Following a further series of washes with PBS the cells were probed with FITC-conjugated goat anti-mouse IgG and, after washing and mounting in Citifluor aqueous mountant, examined by confocal microscopy.

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