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CD45 tyrosine phosphatase-activated p59^{fyn} couples the T cell antigen receptor to pathways of diacylglycerol production, protein kinase C activation and calcium influx

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The role of the CD45 phosphotyrosine phosphatase in coupling the T cell antigen receptor complex (TCR) to intracellular signals was investigated. CD45$^-$ HPB-ALL T cells were transfected with cDNA encoding the CD45RA$^+$B$^+$C$^-$ isoform. The tyrosine kinase activity of p59^{fyn} was found to be 65% less in CD45$^-$ cells than in CD45$^+$ cells, whereas p56^{lck} kinase activity was comparable in both sub-clones. In CD45$^-$ cells the TCR was uncoupled from protein tyrosine phosphorylation, phospholipase $C_{\gamma 1}$ regulation, inositol phosphate production, calcium signals, diacylglycerol production and protein kinase C activation. Restoration of TCR coupling to all these pathways correlated with the increased p59^{fyn} activity observed in CD45-transfected cells. Co-aggregation of CD4- or CD8-p56^{lck} kinase with the TCR in CD45$^-$ cells restored TCR-induced protein tyrosine phosphorylation, phospholipase $C_{\gamma 1}$ regulation and calcium signals. Receptor-mediated calcium signals were largely due (60-90%) to Ca^{2+} influx, and only a minor component (10-40%) was caused by Ca^{2+} release from intracellular stores. Maximal CD3-mediated Ca^{2+} influx occurred at CD3 mAb concentrations at which inositol phosphate production was non-detectable. These results indicate that CD45-regulated p59^{fyn} plays a critical role in coupling the TCR to specific intracellular signalling pathways and that CD4- or CD8-p56^{lck} can only restore signal transduction coupling in CD45$^-$ cells when brought into close association with the TCR.

Key words: CD45 phosphotyrosine phosphatase/diacylglycerol/p59^{fyn} tyrosine kinase/p56^{lck} tyrosine kinase/protein kinase C

Introduction

The T cell antigen receptor complex (TCR) mediates signalling pathways that can result in activation, anergy or apoptosis, the outcome depending on the T cell differentiation state and the nature of signals transmitted by co-receptors (reviewed by Weiss, 1991; Cohen *et al.*, 1992; Miller and Morahan, 1992). The TCR comprises a clonotypic $\alpha-\beta$ heterodimer, associated non-covalently with the invariant γ, δ and ϵ polypeptides of the CD3 antigen, and with two additional polypeptides, ζ and η (Samelson *et al.*, 1985; Baniyash *et al.*, 1988; Clevers *et al.*, 1988). The polypeptides that associate with the $\alpha-\beta$ heterodimer are involved in mediating T cell responses to antigen recognition (Irving and Weiss, 1991; Letourneur and Klausner, 1992; Wegener *et al.*, 1992). The biochemical consequences of antigen binding to the $\alpha-\beta$ heterodimer can be mimicked using mitogenic monoclonal antibodies (mAbs) that recognize epitopes on the CD3 antigen (Geppert *et al.*, 1990; Salmeron *et al.*, 1991). The earliest detectable TCR-induced biochemical event is an increase in tyrosine phosphorylation in specific proteins, indicating a role for tyrosine kinases in signal transduction coupling (June *et al.*, 1990; Klausner and Samelson, 1991). The importance of tyrosine kinases has also been suggested by the blockade of TCR-mediated signalling events observed using tyrosine kinase inhibitors (Mustelin *et al.*, 1990) and by the specific association of the p59^{fyn} tyrosine kinase member of the *src* family with the TCR (Samelson *et al.*, 1990). A critical role for p59^{fyn} is indicated by the amplified responses to CD3 mAb of T cells expressing an active form of p59^{fyn}, whereas thymocytes expressing a catalytically inactive form of p59^{fyn} are hyporesponsive (Cooke *et al.*, 1991; Davidson *et al.*, 1992). The ζ chains of the TCR appear to couple the receptor to pathway(s) of tyrosine phosphorylation different from those mediated by the CD3-ϵ chains (Letourneur and Klausner, 1992), and p59^{fyn} is not the only kinase to associate with the TCR (Chan *et al.*, 1991; Wange *et al.*, 1992), suggesting that more than one tyrosine kinase may be involved in receptor-mediated events.

The CD4 and CD8 co-receptors are involved in amplifying TCR-mediated mitogenic signal pathways (Janeway, 1992). This is presumed to occur *in vivo* when the appropriate CD4 or CD8 co-receptor and the TCR simultaneously bind the same MHC molecule, so transducing a coordinated activation signal (Janeway *et al.*, 1988). Co-receptor amplification of TCR-mediated signal pathways can be mimicked by cross-linking CD4 or CD8 with the CD3 antigen using mAbs (Ledbetter *et al.*, 1988). Another member of the *src* family of tyrosine kinases, p56^{lck}, specifically associates with the CD4 and CD8 antigens (Rudd *et al.*, 1988; Veillette *et al.*, 1988; Sefton, 1991) and mutations in CD4 or CD8 that abolish p56^{lck} binding inhibit amplification of TCR-induced mitogenic signals (Zamoyska *et al.*, 1989; Chalupny *et al.*, 1991; Glaichenhaus *et al.*, 1991). Thymocytes lacking p56^{lck} undergo a profound block in development, resulting in a major decrease in the CD4$^+$CD8$^+$ population and a lack of CD4$^+$ or CD8$^+$ mature thymocytes (Molina *et al.*, 1992), and the CD4 cytoplasmic tail, which binds p56^{lck}, is essential for delivering the signals to thymocytes that direct their differentiation into the CD4 lineage (Seong *et al.*, 1992).

An early consequence of antigen or mitogenic mAb binding to the TCR is an increase in tyrosine phosphorylation in phospholipase-$C_{\gamma 1}$ ($PLC_{\gamma 1}$), an enzyme that acts on phosphatidylinositol bisphosphate (PIP_2) to yield

diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3), which causes the release of calcium from intracellular stores (Imboden and Stobo, 1985; Secrist *et al.*, 1991; Dasgupta *et al.*, 1992). Evidence for the TCR-triggered influx of extracellular calcium into T cells via calcium channels has also been described (Premack and Gardner, 1991). An increase in DAG and intracellular calcium ($[Ca^{2+}]_i$) causes activation of protein kinase C (PKC) (Nishizuka, 1984), which leads to phosphorylation of specific cytosolic proteins, amongst them a 19 kDa protein (Gullberg *et al.*, 1990). In immature T cells, a rise in $[Ca^{2+}]_i$ and PKC activation have been implicated in pathways leading to apoptosis (Finkel *et al.*, 1989; Kizaki *et al.*, 1989). In mature T cells these same signals act synergistically to regulate the synthesis and translocation of transcription factors, thereby inducing expression of the IL2 and IL2R genes with consequent cell proliferation (Truneh *et al.*, 1985; Crabtree, 1989; McCrady *et al.*, 1988). Both PKC activation and increased $[Ca^{2+}]_i$ therefore play vital roles in the function of T cells of differing differentiation states.

The CD45 phosphotyrosine phosphatase (PTPase) is the prototype member of a recently described family of transmembrane PTPases thought to mediate signals by dephosphorylating tyrosine residues (reviewed by Thomas, 1989; Alexander, 1990). Murine T cell clones lacking CD45 on the cell surface do not secrete IL2 or proliferate normally in response to specific antigen (Pingel and Thomas, 1989; Weaver *et al.*, 1991). Furthermore, TCR-induced increases in tyrosine phosphorylation, phosphoinositide (PtdIns) turnover and $[Ca^{2+}]_i$ no longer occur in CD45-deficient mutant T leukaemia cell lines (Koretzky *et al.*, 1990, 1991). These findings point to a positive role for CD45 in maintaining the TCR in a primed state in which it is able to transduce signals (Alexander *et al.*, 1992).

We now show that CD45 regulates $p59^{fyn}$, but not $p56^{lck}$, tyrosine kinase activity in HPB-ALL T cells, and that activation of $p59^{fyn}$ correlates with the restoration of TCR-mediated signals, including Ca^{2+} influx, DAG production and PKC activation. Furthermore, we present evidence that CD4− and CD8−$p56^{lck}$ can mediate TCR-induced $PLC_{\gamma 1}$ phosphorylation and Ca^{2+} influx in $CD45^-$ cells in which $p59^{fyn}$ activity is low, providing that CD4− or CD8−$p56^{lck}$ is brought into close association with the TCR.

Results

CD45 regulates TCR-mediated protein tyrosine phosphorylation by activating $p59^{fyn}$ but not $p56^{lck}$

$CD45^-$ HPB-ALL human T leukaemia cells were obtained by fluorescent activated cell sorting of a parent cell line containing a mixture of $CD45^-$ and $CD45^+$ cells. Repeated analysis during the period of the experiments revealed no detectable contaminating $CD45^+$ cells in the $CD45^-$ sub-clone. Transfection of $CD45^-$ cells with cDNA encoding the $CD45RA^+B^+C^-$ isoform resulted in CD45 expression at the cell surface (Figure 1). Neither CD45 mRNA nor protein were detectable in the $CD45^-$ sub-clone by Northern and Western analysis, respectively, but CD45 mRNA and protein were clearly detectable in the transfected sub-clone (data not shown). PTPase activity was found in CD45 mAb immunoprecipitates from the transfected cells, but not from the $CD45^-$ cells (data not shown). The expression of CD3, CD4 and CD8 were comparable between the two sub-clones (Figure 1). The term '$CD45^+$ cells' used below refers to the transfected HPB-ALL sub-clone.

Figure 2 shows that triggering $CD45^-$ cells with CD3 mAb caused only minor increases in protein tyrosine phosphorylation (lane 8), but marked increases in at least seven proteins were noted in $CD45^+$ cells (lane 2). Interestingly, cross-linking of the CD4 or CD8 co-receptors with the TCR−CD3 in the $CD45^-$ cells restored induction of tyrosine phosphorylation to levels obtained using the CD3 mAb alone in $CD45^+$ cells and by the criterion of gel migration, the same set of proteins appeared to be phosphorylated in both cases (compare lanes 9 and 10 with lane 2). Cross-linking of CD4 or CD8 alone did not induce an increase in tyrosine phosphorylation (lanes 5, 6, 11 and 12). The same results were obtained using the 4G10 mAb to detect phosphotyrosine (not shown). These findings indicate that expression of CD45 on the cell surface is essential to couple the TCR to the actions of one or more tyrosine kinases, but that a CD4/CD8-associated tyrosine kinase that is active in $CD45^-$ cells can restore protein tyrosine phosphorylation, providing that it is brought into association with the TCR.

Since $p56^{lck}$ is the only tyrosine kinase known to be associated with CD4 or CD8 (Rudd *et al.*, 1988; Veillette

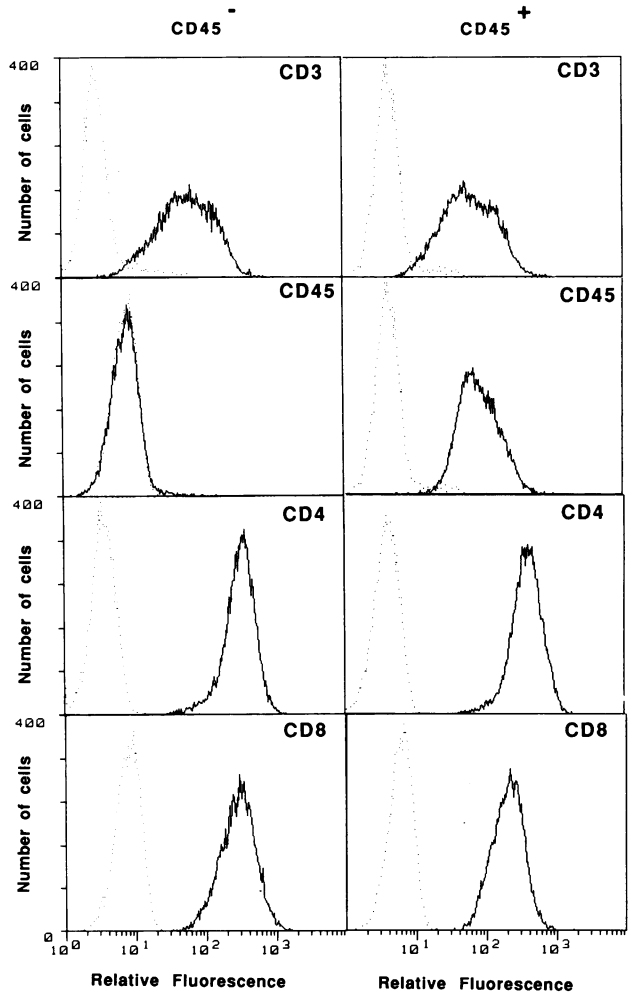

Fig. 1. Expression of cell surface antigens in $CD45^-$- and $CD45^-$-transfected HPB-ALL sub-clones. Cells were stained with OKT3 (CD3), PD7/26 (CD45RB), QS4120 (CD4) and OKT8 (CD8) mAbs and analysed by flow cytometry.

et al., 1988), we compared its activity and that of p59^{fyn}, in CD45$^-$ and CD45$^+$ cells. Figure 3A shows that the levels of p56^{lck} and p59^{fyn} protein were comparable in the two sub-clones. The p56^{lck} tyrosine kinase activities assayed in immunoprecipitates of the enzyme were also comparable in CD45$^-$ and CD45$^+$ cells, as shown by measuring p56^{lck} autophosphorylation, whereas p59^{fyn} activity was markedly lower in the CD45$^-$ cells (Figure 3B). Densitometric scanning of p56^{lck} and p59^{fyn} autophosphorylation bands on autoradiograms from 11 different experiments performed on different days showed that the kinase activity in CD45$^-$ cells was 102 ± 20% (range 72–133%) and 35 ± 16% (range 15–60%) of that determined in CD45$^+$ cells, respectively. Kinase assays using the RR-*src* peptide as substrate similarly revealed no marked differences in p56^{lck} activity between the two sub-clones, whereas p59^{fyn} activity in CD45$^-$ cells was about half that in CD45$^+$ cells (Table I). p56^{lck} autophosphorylation assays in CD4 or CD8 immunoprecipitates confirmed that there was no detectable difference in kinase activity in the p56^{lck} pool specifically associated with these molecules (Figure 3C, compare lanes 3 with 4 and 5 with 6). In contrast Figure 3C also shows that a TCR-associated kinase of identical M_r to p59^{fyn} had a reduced activity in CD45$^-$ cells (lane 1) compared with CD45$^+$ cells (lane 2). Confirmation that this kinase was p59^{fyn}, was obtained by dissociating the autophosphorylated kinase from TCR immunoprecipitates and re-precipitating with specific p59^{fyn} antibodies (data not shown). These results show that the differences in kinase activity noted in the total cellular pool of p59^{fyn} (Figure 3B) were reflected also in the specific pool of p59^{fyn} molecules that associate with the TCR (Figure 3C). No marked changes in either p56^{lck} or p59^{fyn} tyrosine kinase activity were detected in CD45$^-$ cells following CD4–CD3 cross-linking (Figure 3D) or CD3–CD3 cross-linking (data not shown). The same experiments in CD45$^+$ cells similarly revealed no apparent changes in kinase activities (data not shown). Incubation of p59^{fyn} immunoprecipitated from CD45$^-$ cells with purified CD45 PTPase *in vitro* increased the activity of the kinase 2- to 3-fold (data not shown) consistent with the findings of others (Mustelin *et al.*, 1992). These data therefore demonstrate that p59^{fyn} tyrosine kinase activity, but not p56^{lck} activity, is regulated by the CD45RA$^+$B$^+$C$^-$ isoform in HPB-ALL cells.

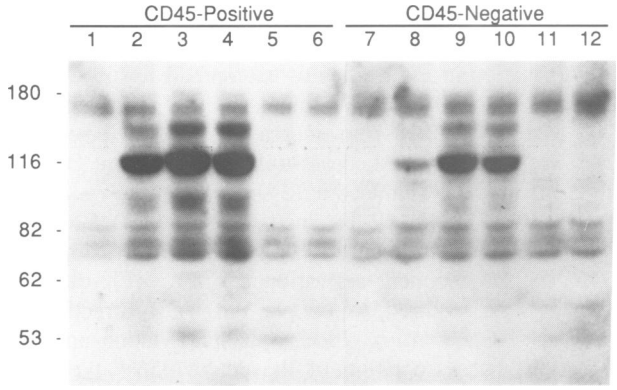

Fig. 2. Protein tyrosine phosphorylation in CD45$^-$ and CD45$^+$ HPB-ALL sub-clones. Tyrosine phosphoproteins were detected by immunoblotting in CD45$^+$ (lanes 1–6) and CD45$^-$ (lanes 7–12) cells after incubation for 2 min with medium alone (lanes 1 and 7) or with cross-linked OKT3 (lanes 2 and 8), OKT3 plus QS4120 (lanes 3 and 9), OKT3 plus OKT8 (lanes 4 and 10), QS4120 (lanes 5 and 11) and OKT8 mAbs (lanes 6 and 12).

CD45 regulates TCR – p59^{fyn}-induced calcium influx, PLC$_{\gamma 1}$ tyrosine phosphorylation and inositol phosphate production

It has been previously shown that transfection of CD45 cDNA into CD45$^-$ HPB-ALL cells restores TCR coupling to inositol phosphate (IP) production and a rise in $[Ca^{2+}]_i$

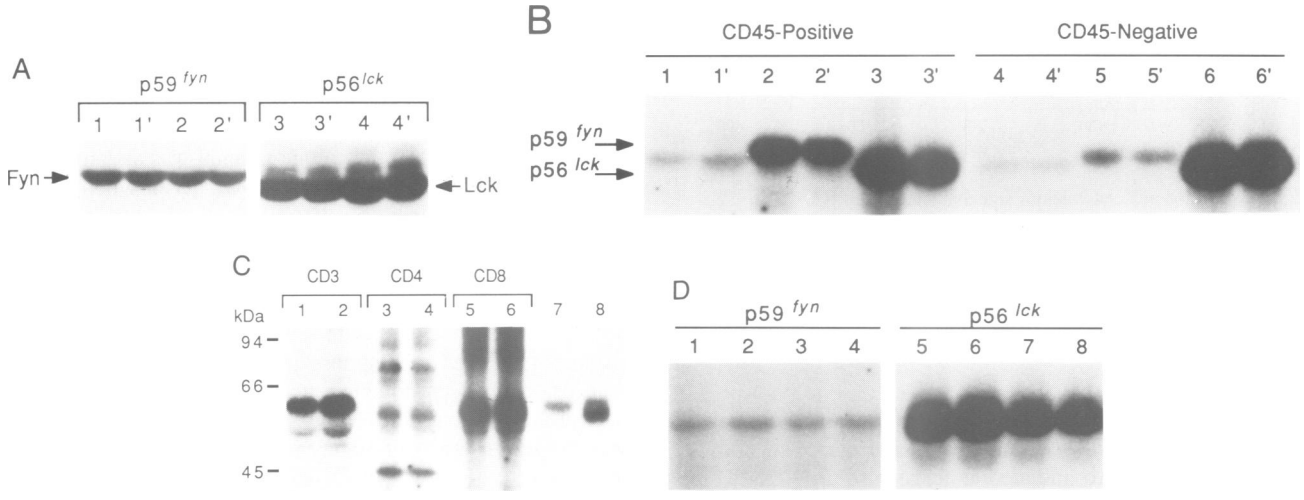

Fig. 3. A comparison of p56^{lck} and p59^{fyn} tyrosine kinases in CD45$^-$ and CD45$^+$ HPB-ALL cells. **A.** Immunoblotting of whole-cell lysates of CD45$^-$ (lanes 1 and 3) or CD45$^+$ (lanes 2 and 4) cells separated on duplicate lanes of a 7.5% SDS–PAGE gel followed by detection with p56^{lck} and p59^{fyn} antisera as described in Materials and methods. **B.** Autophosphorylation of p59^{fyn} and p56^{lck}. *In vitro* kinase assays were carried out on immunoprecipitates from CD45$^-$ or CD45$^+$ cells using control rabbit IgG antisera (lanes 1 and 4), p56^{fyn} antisera (lanes 2 and 5) or p56^{lck} antisera (lanes 3 and 6), followed by separation on duplicate lanes of a 7.5% SDS–PAGE gel. **C.** *In vitro* kinase assays in immunoprecipitates from CD45$^-$ (lanes 1,3,5) or CD45$^+$ (lanes 2, 4 and 6) cells using OKT3 mAb (lanes 1 and 2), QS4120 mAb (lanes 3 and 4) or OKT8 mAb (lanes 5 and 6). Lanes 7 and 8 show the positions of authentic autophosphorylated p56^{fyn} and p56^{lck}, respectively, identified with specific antisera. **D.** Time course of p56^{fyn} and p56^{lck} autophosphorylation in CD45$^-$ cells. *In vitro* kinase assays were performed in p56^{fyn} and p56^{lck} antisera immunoprecipitates following cross-linking of 1 μg/ml OKT3 and 5 μg/ml QS4120 with 10 μg/ml sheep anti-mouse F(ab')$_2$ fragments for 0 min (lanes 1 and 5), 0.5 min (lanes 2 and 6), 1 min (lanes 3 and 7) and 2 min (lanes 4 and 8).

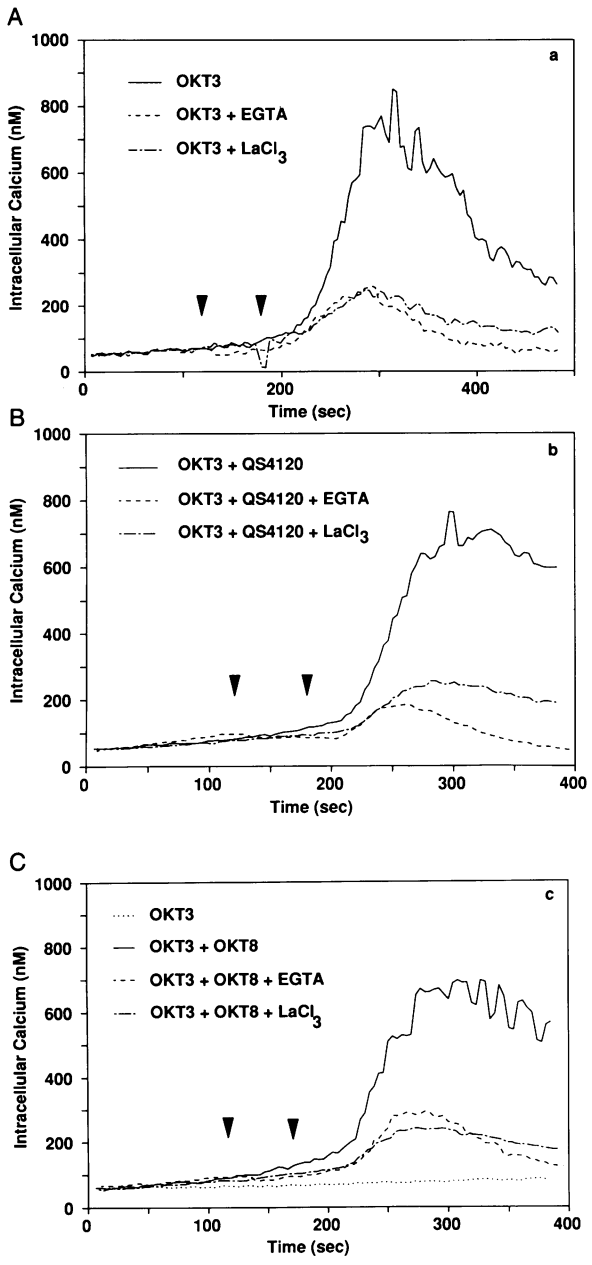

Fig. 4. Receptor-induced calcium signals. The rise in $[Ca^{2+}]_i$ in $CD45^+$ (**A**) or in $CD45^-$ (**B** and **C**) sub-clones was assayed in Fura2 loaded cells. The first arrow indicates addition of mAb and, where relevant, EGTA, the second arrow the addition of 8 μg/ml sheep-anti-mouse $F(ab')_2$ fragments. La^{3+} was added at time zero. Final concentrations of reagents used were 1 μg/ml anti-CD3 OKT3 mAb, 2 μg/ml anti-CD4 QS4120 mAb and anti-CD8 OKT8 mAb, 2.5 mM EGTA and 1 mM La^{3+}.

(Koretzky *et al.*, 1990). However, the basis for the observed rise in $[Ca^{2+}]_i$ has not been delineated. Figure 4A shows that the OKT3 mAb induced a large rise in $[Ca^{2+}]_i$ in $CD45^+$ cells that was inhibited 79% following the addition of lanthanum (La^{3+}) or by the chelation of extracellular Ca^{2+} with EGTA. Since EGTA prevents the influx of extracellular Ca^{2+}, an effect mimicked by the competitive actions of La^{3+} (Mela, 1968; Lehninger and Carafoli, 1971), these results suggest that CD45 regulates TCR coupling to a mechanism of Ca^{2+} influx. Cross-linking CD4 (Figure 4B) or CD8 (Figure 4C) with CD3 induced a rise in $[Ca^{2+}]_i$ in the $CD45^-$ cells, which was similarly inhibited by La^{3+} or EGTA. No CD3-induced calcium signal was observed in $CD45^-$ cells (Figure 4C) and cross-linking of CD4 or CD8 mAbs alone did not induce a rise in $[Ca^{2+}]_i$ in either the $CD45^-$ or $CD45^+$ sub-clones (data not shown).

To explore possible mechanisms underlying TCR-regulated Ca^{2+} influx, $PLC_{\gamma 1}$ tyrosine phosphorylation and PtdIns metabolism were investigated. Figure 5A shows that $PLC_{\gamma 1}$ protein levels were comparable in the $CD45^-$ and $CD45^+$ sub-clones (lanes 7 and 8). No CD3-induced $PLC_{\gamma 1}$ tyrosine phosphorylation was detectable in the $CD45^-$ cells (lane 5), but was clearly visible in the $CD45^+$ cells (lane 2). $PLC_{\gamma 1}$ phosphorylation was restored by CD3–CD8 cross-linking in the $CD45^-$ sub-clone (lane 6) and was markedly amplified in the $CD45^+$ sub-clone (lane 3). Assays of mAb-triggered total IP production in [^{3}H]*myo*-inositol labelled cells revealed, as expected, a similar pattern of response to the data shown for $PLC_{\gamma 1}$ phosphorylation in Figure 5. For example, 1 μg/ml OKT3 induced a 31 ± 10% (n = 4) increase in IP production in $CD45^+$ cells, whereas no increases were detected in $CD45^-$ cells; in contrast, cross-linking of 1 μg/ml OKT3 with 2 μg/ml OKT8 induced a 72 ± 16% and 68 ± 17% (n = 5) increase in IP production in the $CD45^-$ and $CD45^+$ sub-clones, respectively. Thus, expression of CD45, with consequent activation of $p59^{fyn}$ (Figure 3B, Table I), was required to couple the TCR directly to pathways of Ca^{2+} influx, $PLC_{\gamma 1}$ phosphorylation and IP production, but association of the active $p56^{lck}$ tyrosine kinase with the TCR in $CD45^-$ cells could restore coupling of the TCR to all these pathways.

Since neither La^{3+} nor EGTA completely blocked Ca^{2+} influx (Figure 4A–C), the component of $[Ca^{2+}]_i$ elevation resistant to inhibition was presumably caused by the IP_3-mediated release of Ca^{2+} from intracellular stores. However, in $CD45^+$ cells the OKT3-induced rise in $[Ca^{2+}]_i$ was found to be maximal at 250–500 ng/ml mAb, whereas at these concentrations of mAb there was no detectable rise in total IP production (Figure 5B). It is possible that the EGTA/La^{3+}-resistant component of this rise in $[Ca^{2+}]_i$ was due to IP_3 production at a level below the limits of detection of our assay, consistent with the observation that the EGTA-resistant component of the calcium signal was also maximal at 250–500 ng/ml OKT3 (Figure 5B). Even when OKT3 mAb was titrated up to 5–10 μg/ml, a concentration that induced maximal IP production (Figure 5B), total IPs were only increased 155% over background in the $CD45^+$ cells, compared with 6- to 10-fold increases in Jurkat T cells and normal T lymphocytes (data not shown). Separation of IP isomers produced in response to 10 μg/ml OKT3 showed that IP_3 and IP_4 production was barely detectable above background. The low level of OKT3-induced IP production was not due to any deficiency in the labelling of phosphoinositides with [^{3}H]*myo*-inositol, since $1.7-1.8\times10^5$ d.p.m./assay were routinely detected in the phospholipid fraction isolated from labelled cells. Separation of [^{3}H]phospholipids showed that 3% of the radioactivity was incorporated into the PI pool and 5% into the PIP_2 pool, so the cells were not deficient in PIP_2 production. Figure 5C provides further confirmation that the low level of CD3-induced IP production was not due to any defect in pathways of PtdIns metabolism, since either vanadate or GTPγS induced a 5- to 11-fold increase in IPs in

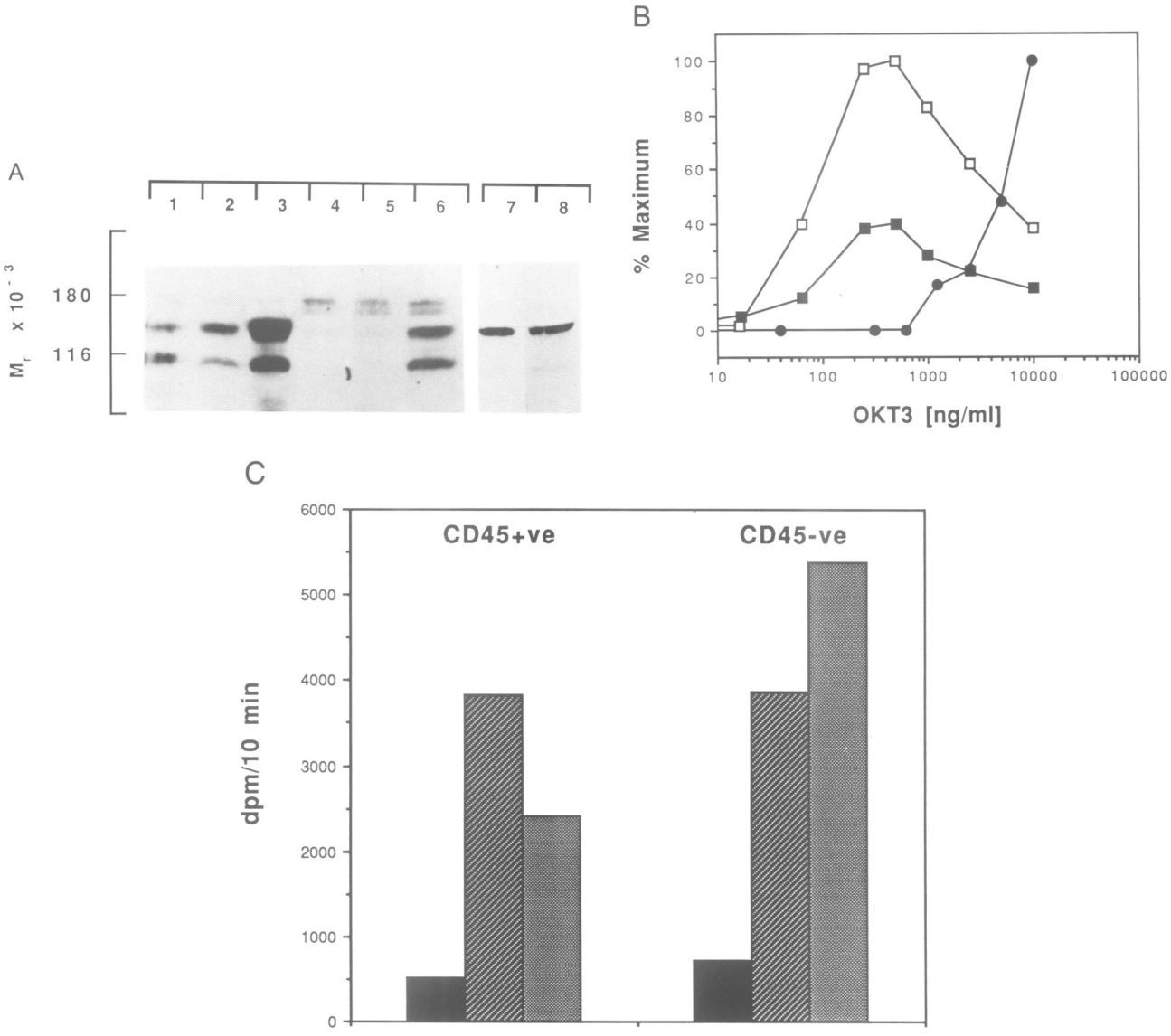

Fig. 5. PLC$_{\gamma 1}$ tyrosine phosphorylation, IP production and calcium signals in HPB-ALL cells. **A.** Tyrosine phosphorylation of proteins, including PLC$_{\gamma 1}$, in CD45$^+$ cells (lanes 1–3) and CD45$^-$ cells (lanes 4–6) stimulated with 5 μg/ml OKT3 (lanes 2 and 5), 1 μg/ml OKT3 plus 2 μg/ml OKT8 (lanes 3 and 6) or no addition (lanes 1 and 4). Tyrosine phosphorylated proteins were detected with anti-phosphotyrosine antisera. PLC$_{\gamma 1}$ in CD45$^+$ (lane 7) and CD45$^-$ cells (lane 8) was also detected with specific antisera. The positions of bound antibodies were revealed as described in Materials and methods. **B.** The dose–response of cross-linked OKT3-induced calcium signals (open squares), calcium signals in the presence of 2.5 mM EGTA (filled squares) and IP production (filled circles) in CD45$^+$ HPB-ALL cells. Calcium assays were carried out as in Figure 4, utilizing the rise in $[Ca^{2+}]_i$ above resting levels to calculate the % maximum values shown.The titration shown for IP values is representative of 3 separate experiments. **C.** IP production in permeabilized cells. The (^{3}H)*myo*-inositol labelled phospholipids in the CD45$^-$ cells contained 83 000 d.p.m. and in the CD45$^+$ cells 70 500 d.p.m. The results shown are representative of three separate experiments. No addition, fiiled bars; 10 μM GTPγS, hatched bars; 1 μM vanadate, stippled bars.

permeabilized HPB-ALL cells. Vanadate caused a striking increase in PLC$_{\gamma 1}$ tyrosine phosphorylation in these cells (not shown), whereas GTPγS presumably caused IP production via one or more G proteins, demonstrating that a marked increase in IP production can occur in the HPB-ALL sub-clone used in this work, mediated either by protein tyrosine phosphorylation or by G protein activation (Biffen *et al.*, 1992).

These results therefore indicate that the TCR–p59^{fyn}-induced rise in $[Ca^{2+}]_i$ in CD45$^+$ cells, or the CD3–CD4–p56^{lck}- or CD3–CD8–p56^{lck}-induced rise in CD45$^-$ cells, is due to two separate components: a smaller component (10–40%) presumably due to PLC$_{\gamma 1}$ tyrosine phosphorylation and the consequent IP$_3$-induced release of Ca^{2+} from intracellular stores, and a major component (60–90%) due to an increase in Ca^{2+} influx. The possible relation between these events is considered further in the Discussion.

CD45 regulates TCR–p59^{fyn}-induced DAG production and PKC activation

Activation of PKC in ^{32}P-labelled T cells has been shown by Gullberg *et al.* (1990) to result in the phosphorylation of a 19 kDa protein. Figure 6A shows that cross-linking of the CD3 antigen using the OKT3 mAb in CD45$^-$ HPB-ALL cells failed to induce phosphorylation of this immunoprecipitated 19 kDa protein (lane 2), whereas significant phosphorylation was observed in the CD45$^+$ cells (lane 5). Phorbol 12,13-dibutyrate (PdBu), a phorbol ester that directly activates PKC, induced comparable levels

Table I. Tyrosine kinase activities of $p59^{fyn}$ and $p56^{lck}$ in $CD45^-$ and $CD45^+$ HPB-ALL T cells

Experiment[a]	^{32}P-labelled RR-*src*[b] (c.p.m.)		Relative activity (%)
	$CD45^+$	$CD45^-$	$CD45^-/CD45^+$
$p59^{fyn}$			
1[c]	1723	436	25
2	2186	1487	68
3	1812	1168	64
Mean ± SD			52 ± 19
$p56^{lck}$			
1[c]	12058	14425	120
2	20233	18973	94
3	18352	18617	101
Mean ± SD			105 ± 11

[a]For each experiment *in vitro* assays were performed on duplicate immunoprecipitates of $p59^{fyn}$ or $p56^{lck}$. Estimates of ^{32}P incorporated into RR-*src* peptide were performed in triplicate for each assay as described in Materials and methods. The data shown (c.p.m.) represent the means of the six values obtained.
[b]Phosphorylation of RR-*src* was measured under linear conditions.
[c]Experiment 1 corresponds to Figure 3B.

of phosphorylation of the 19 kDa protein in both sub-clones (lanes 3 and 6), showing that the lack of 19 kDa phosphorylation in $CD45^-$ cells was not due to a deficiency of PKC or of 19 kDa protein.

To quantify differences in total DAG production and PKC activation between $CD45^-$ and $CD45^+$ cells, a sensitive assay system was used to determine both parameters in the same sample of activated cells (Alexander *et al.*, 1990; Shivnan *et al.*, 1992). Figure 6B shows that CD3 antigen cross-linking induced a 4-fold greater production of DAG in the $CD45^+$ sub-clone (lane 5) compared with $CD45^-$ cells (lane 2). Direct activation of PKC with PdBu caused a very minor increase in DAG production (lanes 3 and 6). Titration with the OKT3 mAb revealed no detectable CD3-induced PKC activation in the $CD45^-$ cells, whereas a marked restoration of PKC activation occurred in the $CD45^-$transfected cells (Figure 6C). Six separate assays of PKC activity induced by 5–10 μg/ml OKT3 carried out on different days in the $CD45^+$ sub-clone revealed a mean activation level of 173 ± 68 pmol/3×10^6 cells/5 min (range 115–289). However, these levels of activity were only ~5–10% those induced by 100 ng/ml PdBu (routinely >2000 pmol/3×10^6 cells/5 min), consistent with the contrasting levels of phosphorylation of the 19 kDa protein induced by OKT3 mAb and PdBu (Figure 6A, lanes 5 and 6). Figure 6C also shows that CD3-induced DAG production in the $CD45^-$ cells was 12–25% of that determined in $CD45^+$ cells. The absence of detectable PKC activity in the $CD45^-$ cells, despite this small increase in CD3-induced DAG production, is probably due to the inability of OKT3 mAb to trigger an elevation in $[Ca^{2+}]_i$ in the $CD45^-$ cells (Figure 4C). Since increases in both $[Ca^{2+}]_i$ and DAG are thought to be responsible for the translocation and activation of PKC (Nishizuka, 1984), it is unlikely that the activation of Ca^{2+}-dependent PKC isoforms would be detected in the assay system used in the present work in the absence of elevated $[Ca^{2+}]_i$, since PKC, which is not translocated rapidly, leaks from T cells following permeabilization with Streptolysin-O (Alexander *et al.*, 1989).

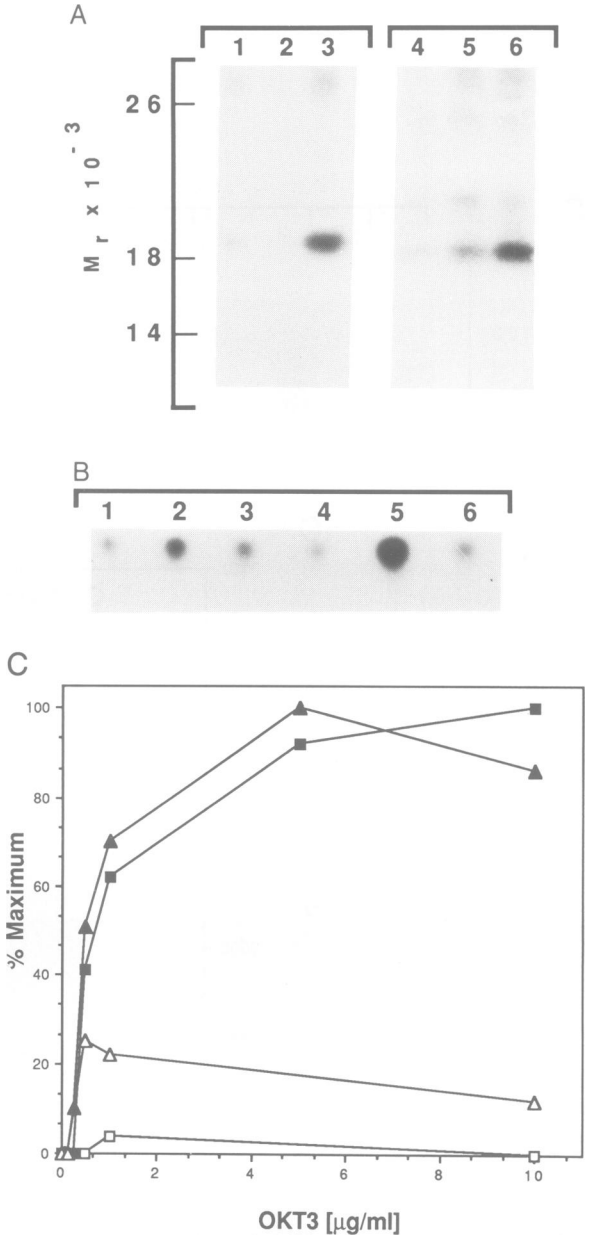

Fig. 6. CD3-induced DAG production and PKC activation in $CD45^-$ and $CD45^+$ HPB-ALL sub-clones. **A.** Phosphorylation of 19 kDa protein in ^{32}]P-labelled $CD45^-$ cells (lanes 1–3) and $CD45^+$ cells (lanes 4–6); lanes 1 and 4, no addition; lanes 2 and 5, +5 μg/ml OKT3 cross-linked with 8 μg/ml sheep-anti-mouse $F(ab')_2$ fragments; lanes 3 and 6, +100 ng/ml PdBu. Incubations were carried out at 37°C for 30 min before pelleting the cells and immunoprecipitating the 19 kDa protein with specific antisera from whole-cell lysates as described in Materials and methods. **B.** ^{32}P-labelled phosphatidic acid derived from DAG by the action of DAG kinase in permeabilized cells after incubation with [^{32}P]ATP. Lanes as in panel A. **C.** OKT3 mAb-induced DAG production and PKC activation in $CD45^-$ cells (open symbols) and $CD45^+$ cells (filled symbols). Triangles, ^{32}P-labelled phosphatidic acid; squares, PKC activity. Background values were comparable between the two sub-clones and have been subtracted. Percentage maximum is with reference to the maximal values obtained in $CD45^+$ cells.

These findings show that the increase in $p59^{fyn}$ tyrosine kinase activity that occurs in CD45-transfected cells correlates with the restoration of TCR coupling to one or more pathways leading to DAG production and PKC activation.

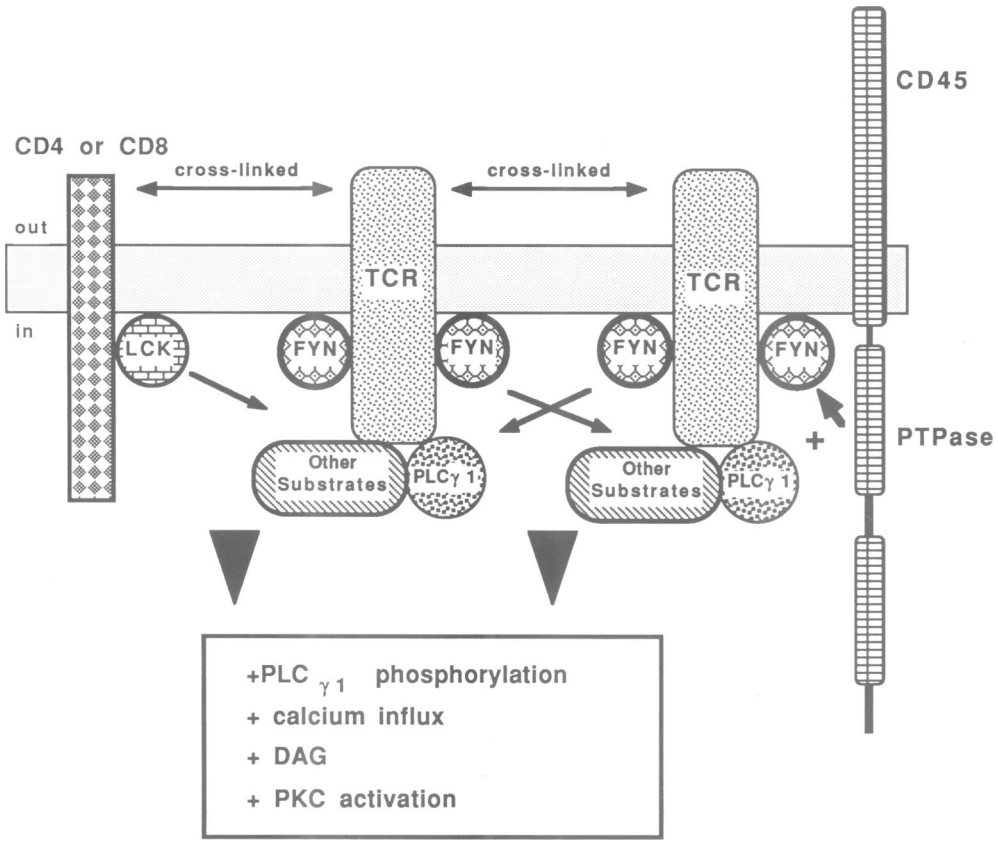

Fig. 7. Regulation of TCR-mediated signals by CD45 in HPB-ALL cells. The model illustrates the activation of the TCR-associated p59^{fyn} tyrosine kinase (FYN) by CD45 PTPase. Dimerization or oligomerization of TCRs in CD45$^+$ cells by cross-linking brings CD45-activated p59^{fyn} into association with its substrates, such as PLC$_{\gamma 1}$, so coupling the TCR to other intracellular signals, such as DAG production and PKC activation. In CD45$^-$ cells, in which p59^{fyn} tyrosine kinase activity is low, the TCR is uncoupled from signal pathways. Cross-linking of CD4 or CD8 with the TCR in CD45$^-$ cells brings active p56^{lck} (LCK) kinase into association with its substrates, such as PLC$_{\gamma 1}$, leading to PLC$_{\gamma 1}$ tyrosine phosphorylation, so restoring TCR-mediated IP production and elevation of $[Ca^{2+}]_i$. Receptor-associated molecules are shown to illustrate possible functional relationships only and no receptor-binding stoichiometries are implied.

Discussion

Our results have revealed three novel aspects of TCR signal transduction mechanisms in HPB-ALL T cells. First and most importantly, the CD45RA$^+$B$^+$C$^-$ isoform activates p59^{fyn}, but not p56^{lck}, tyrosine kinase activity (Figure 3B, Table I). Secondly, activation of p59^{fyn} by the CD45 PTPase *in situ* restores coupling of the TCR to PLC$_{\gamma 1}$ tyrosine phosphorylation, IP production, calcium influx, DAG production and PKC activation (Figures 4, 5 and 6). Thirdly, association of active CD4– and CD8–p56^{lck} with the TCR in CD45$^-$ cells couples the receptor to PLC$_{\gamma 1}$ tyrosine phosphorylation, IP production and calcium influx (Figures 4 and 5).

The model shown in Figure 7 is consistent with our findings. CD45-regulated p59^{fyn} (Figure 3C) and possibly another tyrosine kinase (Chan *et al.*, 1991), are associated with the TCR in HPB-ALL cells and couple the receptor to intracellular signals. It is envisaged that the initial event in TCR signal transduction is the dimerization (or oligomerization) of the receptor (Ledbetter *et al.*, 1986; Ratcliffe *et al.*, 1992), induced in the present work by means of CD3 mAb cross-linking, which brings active p59^{fyn} into contact with relevant substrates. By analogy with the PDGF and EGF receptors (Yarden and Ullrich, 1988), TCR aggregation presumably triggers recruitment of signalling molecules by means of interactions between phosphotyrosine residues and the SH2 domains contained in proteins such as p56^{lck} and p59^{fyn} (Koch *et al.*, 1991). In CD45$^-$ cells, the activity of p59^{fyn} is too low (Figure 3B and Table I) to mediate these events upon CD3–CD3 cross-linking, consistent with the uncoupling of the TCR from protein tyrosine phosphorylation (Figure 2). However, active CD4– or CD8–p56^{lck} restores tyrosine phosphorylation of PLC$_{\gamma 1}$ (Figure 5A) and other proteins (Figure 2), providing that it is brought into association with the TCR. The similar migration on an SDS–PAGE gel of proteins that had been phosphorylated on tyrosine residues after CD3–CD3 cross-linking in CD45$^+$ cells, compared with those phosphorylated after CD3–CD4 or CD3–CD8 cross-linking in CD45$^-$ cells (Figure 2) suggests, but does not prove, that some proteins are phosphorylated both by p59^{fyn} and by p56^{lck}. Our findings are therefore in agreement with those of others indicating that p56^{lck} amplifies TCR-mediated signals providing that the kinase is brought into intimate association with the TCR and its associated proteins (Abraham *et al.*, 1991; Haughn *et al.*, 1992). In light of the reported co-localization of CD4 and the TCR that occurs upon activating T cells with TCR mAbs (Mittler *et al.*, 1989; Dianzani *et al.*, 1992), it is striking that in the present work CD3 cross-linking failed to restore signals in CD45$^-$ cells, despite the presence of abundant CD4–/CD8–p56^{lck} tyrosine kinase activity (Figure 3B, Table I). An intriguing

possibility is that CD3 mAbs, such as OKT3, can only induce association of CD4− or CD8−p56^{lck} with the TCR-associated signal transduction complex when *both* p56^{lck} and p59^{fyn} are enzymatically active. This speculation is consistent with the findings of Collins *et al.* (1992), which show that p56^{lck} association with CD4 is required for the interaction between CD4 molecules and the TCR. The failure of OKT3 mAb to induce association of CD4 or CD8 with the TCR in CD45$^-$ cells would explain why CD4−CD3 or CD8−CD3 cross-linking with F(ab')$_2$ fragments was necessary to restore signal transduction coupling in CD45$^-$ cells (Figures 2, 4 and 5). Further work is necessary to elucidate such a possibility.

It has been previously shown that mutation of Tyr505 on p56^{lck} to a non-phosphorylatable residue reveals the transforming activity of the enzyme (Marth *et al.*, 1988) and it has been suggested that CD45 regulates p56^{lck} tyrosine kinase activity *in situ* by dephosphorylating Tyr505 (Ostergaard *et al.*, 1989). It is clear that p56^{lck} is a substrate for the CD45 PTPase *in vitro* and that p56^{lck} dephosphorylation results in an increase in its tyrosine kinase activity (Mustelin and Altman, 1990). Furthermore, a CD45$^-$ mutant murine lymphoma cell line was reported to contain increased phosphate incorporation at Tyr505 compared with the CD45$^+$ parent cell line (Ostergaard *et al.*, 1989). However, consistent with the present work (Figure 3B and Table I), the p56^{lck} tyrosine kinase activities in immunoprecipitates isolated from these CD45$^-$ and CD45$^+$ cell lines were comparable (Ostergaard and Trowbridge, 1990), suggesting that although CD45 may regulate the dephosphorylation of p56^{lck} tyrosine residues, other factors are involved in the regulation of p56^{lck} kinase activity *in situ*. After completion of the present work, comparable p56^{lck} tyrosine kinase activities were also reported in CD45$^-$ and CD45$^+$ HPB-ALL cells derived by cell sorting, although the CD45 isoforms expressed in the CD45$^+$ cells were not described (Deans *et al.*, 1992). The presence of active p56^{lck} in CD45$^-$ HPB-ALL cells is consistent with the marked increase in tyrosine phosphorylation observed following CD3−CD4− or CD3−CD8−p56^{lck} cross-linking (Figure 2). Thus, as yet, there is no convincing evidence that CD45 regulates p56^{lck} kinase activity *in situ*, although this does not exclude a role for CD45 in modulating other aspects of p56^{lck} actions, which depend on several highly specific structural requirements (Abraham *et al.*, 1991; Caron *et al.*, 1992). Furthermore, it is possible that the selective activation of *src* family tyrosine kinases by the CD45 PTPase is regulated by the association of specific CD45 isoforms with the cell surface receptors, such as CD4/CD8 or the TCR, with which the kinases associate (Alexander *et al.*, 1992). In the present work the CD45RA$^+$B$^+$C$^-$ isoform activated p59^{fyn}, but it has yet to be determined whether the expression of other CD45 isoforms on the HPB-ALL cell surface may selectively regulate kinases such as p59^{fyn} and p56^{lck}.

Our finding that CD3−CD3 or CD3−CD4 cross-linking did not stimulate p59^{fyn} kinase activity (Figure 3D) is consistent with previous work (Samelson *et al.*, 1990; Davidson *et al.*, 1992). However, the observation that cross-linking of CD4 or CD8 alone did not induce increased protein tyrosine phosphorylation (Figure 2) or detectable changes in p56^{lck} kinase activity measured in subsequent *in vitro* assays (M.Shiroo, unpublished data), is at variance with the findings of others in murine T cells in which CD4 cross-linking induced increases in p56^{lck} tyrosine phosphorylation and kinase activity (Veillette *et al.*, 1989; Luo and Sefton, 1990). Nevertheless, CD4 cross-linking did not increase protein tyrosine phosphorylation in human T lymphocytes (June *et al.*, 1990), so it is possible that such differences are due to the species-origin of the cells and/or the CD4 mAbs used. As in the present work (Figure 3B), marked p56^{lck} tyrosine kinase activity has been consistently noted in all T cells expressing p56^{lck} protein, whether the cells have been activated or not (Veillette *et al.*, 1989; June *et al.*, 1990; Luo and Sefton, 1990), suggesting that it may be the relocation of the kinase in relation to its substrates, rather than its activation *per se*, which is important during TCR-mediated signalling (Figure 7), a possibility discussed by Sefton (1991).

The similar increases in PLC$_{\gamma 1}$ tyrosine phosphorylation mediated by TCR−p59^{fyn} or by CD8−p56^{lck} in CD45$^+$ or CD45$^-$ cells, respectively (Figure 5A), suggest that PLC$_{\gamma 1}$ may be a substrate for both p56^{lck} (Caron *et al.*, 1992) and p59^{fyn}. This is consistent with the more marked PLC$_{\gamma 1}$ tyrosine phosphorylation induced by CD8−CD3 cross-linking in CD45$^+$ cells in which both p56^{lck} and p59^{fyn} are active (Figure 5A) as suggested by our model (Figure 7). The phosphorylation of PLC$_{\gamma 1}$ (Figure 5A) or other proteins (Figure 2) by TCR−p59^{fyn} in CD45$^+$ cells, or by TCR-associated CD4/CD8−p56^{lck} in CD45$^-$ cells, were closely paralleled by the increases in $[Ca^{2+}]_i$ observed (Figure 4). Irrespective of whether the rise in $[Ca^{2+}]_i$ was induced by CD3−CD3 or CD4/CD8−CD3 cross-linking, the signal appeared to comprise the same components and to be largely due to Ca^{2+} influx (Figure 4). Similar conclusions were originally reported for HPB-ALL cells by Oettgen *et al.* (1985), although these workers found that in the sub-clone used in their work effectively all the CD3-induced rise in $[Ca^{2+}]_i$ was due to increased Ca^{2+} influx. Furthermore, Brattsand *et al.* (1990) were unable to detect any CD3-induced IP production in their HPB-ALL sub-clone, although the expression of CD45 in these cells was not described. Our own data can be interpreted in two ways. First, the EGTA/La^{3+}-resistant signal was due to the release of Ca^{2+} from intracellular stores, possibly by IP_3 production below the level of detection (Figure 5B), and the depletion of these stores then stimulated Ca^{2+} influx by a mechanism that may or may not involve the continued actions of IP_3 and/or IP_4 (Imboden and Weiss, 1987; Berridge and Irvine, 1989; Irvine, 1990; Bird *et al.*, 1991; Peppelenbosch *et al.*, 1991; Hoth and Penner, 1992). The minor intracellular release of Ca^{2+} may have been sufficient to cause the marked rise in Ca^{2+} influx observed (600−800 nM Ca^{2+}) by a 'store-dependent' mechanism since, for example, the treatment of thymic lymphocytes with reagents that deplete intracellular Ca^{2+} pools have been reported to cause marked increases in Ca^{2+} influx (Mason *et al.*, 1991). The dose−response curves of the total OKT3-induced increase in $[Ca^{2+}]_i$ compared with the EGTA-resistant component, were very similar (Figure 5B), consistent with a close relationship between these components. Nevertheless, the kinetics of the EGTA/La^{3+} inhibited and resistant components of the calcium signal were identical (Figure 4), making theories that depend on cause and effect relationships between these components problematic. The second interpretation of these data, therefore, is that the major component of the calcium signal (80−90%) was caused by direct coupling between the TCR and a Ca^{2+} influx

mechanism. Non-voltage gated Ca^{2+} channels have been described in T cells (Gardner *et al.*, 1989), though the mechanism(s) whereby the TCR regulates these channels remain controversial (reviewed by Premack and Gardner, 1991). Our findings suggest that TCR – p59^{fyn} and CD4/CD8 – p56^{lck} not only mediate $PLC_{\gamma 1}$ tyrosine phosphorylation, but could also regulate the tyrosine phosphorylation of another protein, yet to be identified that may be involved in the regulation of Ca^{2+} channel opening. In this respect it is of interest that introduction of the v-*src* tyrosine kinase into a T cell hybridoma enhanced TCR-induced Ca^{2+} influx in the absence of any increase in PtdIns hydrolysis (Niklinska *et al.*, 1992). The striking reduction in calcium signals at higher OKT3 mAb concentrations in the present work (Figure 5B) appears to be due to PKC activation, which inhibits Ca^{2+} influx, but not CD3-induced IP production (D.Alexander and M.Biffen, in preparation), consistent with the presence in HPB-ALL cells of protein(s) that regulate Ca^{2+} influx independently of PtdIns metabolism.

It is not yet clear whether CD45-activated p59^{fyn} restores TCR coupling to all the PKC isoforms that may potentially be activated in HPB-ALL cells, since to the best of our knowledge, the PKC assays used in this work do not distinguish between the activities of specific isoforms (Figure 6A and C). HPB-ALL cells have been reported to express α and β PKC isoforms (Lucas *et al.*, 1990; Sawamura *et al.*, 1989) and it is possible that specific DAG species contribute to the activation of specific isoforms, which probably play distinct roles in T cell signal transduction pathways (Alexander and Cantrell, 1989). Further work will be necessary to determine whether PtdIns are the only phospholipid source of the DAG that activates PKC in the $CD45^+$ HPB-ALL cells.

The differential regulation by TCR – p59^{fyn} and CD4/CD8 – p56^{lck} of enzymes such as $PLC_{\gamma 1}$ and PKC in the $CD45^-$ and $CD45^+$ HPB-ALL sub-clones, may provide a useful model for investigating the functional significance of specific signalling pathways. In possessing the $CD4^+CD8^+CD3^{lo}$ phenotype, HPB-ALL cells are transformed cells characteristic of early thymocytes (Kisielow *et al.*, 1984). Most thymocytes possessing this phenotype die in the thymus, either because they have not been positively selected or because they have been negatively selected as a consequence of TCR-mediated signals stimulated by self antigens expressed in the thymus (Finkel *et al.*, 1989, 1991; Nakayama *et al.*, 1991; Cohen *et al.*, 1992). Elevation of $[Ca^{2+}]_i$, PKC activation and the actions of p56^{lck} as well as p59^{fyn} have all been implicated in the signalling pathways presumed to mediate thymocyte selection events (Kizaki *et al.*, 1989; Rothenberg, 1990; Finkel *et al.*, 1991; Nakayama *et al.*, 1991; Molina *et al.*, 1992; Sancho *et al.*, 1992). Our findings emphasize the importance in these transformed cells of the CD45 PTPase in regulating TCR coupling to specific signal pathways. It is expected that equivalent coupling processes in thymocytes mediate pathways that result in positive and negative selection events, understanding of which should elucidate mechanisms of T cell differentiation and transformation.

Materials and methods

Materials

PdBu and other biochemicals were obtained from Sigma Chemical Co., Streptolysin-O was from Burroughs Wellcome, protein A – Sepharose from Pharmacia, Fura 2-AM was from Calbiochem and [γ-^{32}P]ATP from Amersham International. Mouse mAbs used were: OKT3 (CD3, IgG2a); QS4120 (CD4, IgG1) (from Professor P.Beverley, Middlesex Hospital, London); SN130 (CD45RA, IgG2a)(from Professor 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 Professor S.Meuer, German Cancer Research Centre, Heidelberg, Germany) and OKT8 (CD8, IgG2a). mAbs were purified from hybridoma supernatants by affinity chromatography on protein A – Sepharose (Pharmacia). mAbs used in permeabilized cell assays were further purified by FPLC (Mono Q column). Cross-linking of mAbs was performed using purified $F(ab')_2$ fragments of sheep anti-mouse IgG antisera (Sigma). Rabbit antisera to p56^{lck} was from Dr R.Abraham (Department of Immunology, Mayo Clinic, Rochester, USA) and Dr L.Samelson (National Institute of Health, Maryland, USA). Rabbit antisera to p59^{fyn} was from Dr J.Bolen (Bristol-Myers Squibb Research Institute, New Jersey, USA), Dr R.Abraham and Dr C.Rudd (Dana-Farber Institute, Boston, USA). Rabbit polyclonal antisera against recombinant 19 kDa protein was from Dr M.Gullberg (University of Umea, Sweden) and against $PLC_{\gamma 1}$ from Dr P.Parker (Imperial Cancer Research Fund, London, UK). D67 phosphotyrosine antisera was made as described by Kamps and Sefton (1988).

Mutant cell isolation and transfection

HPB-ALL T leukaemia cells were cultured in RPMI-1640 medium containing penicillin (100 units/ml), streptomycin (50 μg/ml) and 3% fetal calf serum. The parent cell line was 65% $CD45^-$ and 35% $CD45^+$. A $CD45^-$ sub-clone was obtained by cell sorting with a FACS Star Plus flow cytometer (Becton Dickinson). Human CD45 cDNA clone LCA.338 encoding full length CD45 cDNA containing exons A and B in its extracellular domain was provided by Dr M.Streuli (Streuli *et al.*, 1988) in a retroviral vector pZipNeoSV(X). The $CD45^-$ HPB-ALL sub-clone was transfected with the cDNA construct by electroporation at 960 μF and 350 V using a Gene Pulser (Bio-Rad). Geneticin-resistant transfectants were screened for expression of CD45RA and CD45RB with SN130 and PD7/26 mAbs, respectively. Positive clones were selected using the flow cytometer by staining human antigens with mouse mAbs detected with a FITC-conjugated second Ab (Dako).

Western blotting

For detection of $PLC_{\gamma 1}$ protein, HPB-ALL cells (3×10^6) were lysed in 1% Triton X-100, 10 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM EGTA, 10% glycerol and protease inhibitors. Lysates were resolved on a 7.5% SDS – PAGE gel and transferred onto Immobilon-P membranes (Millipore). $PLC_{\gamma 1}$ was detected with a specific rabbit antisera and horseradish peroxidase-conjugated anti-rabbit IgG Ab (Dako) using the ECL system (Amersham). Identification of proteins containing phosphotyrosine was performed by the same procedure, except that 1 mM Na_3VO_4 was added to the lysis buffer and D67 rabbit anti-phosphotyrosine antisera and [^{125}I]protein A (ICN) were used as a detection system.

Tyrosine kinase assays

HPB-ALL cells (1×10^7) were lysed with 1% Brij 96 in 10 mM triethanolamine buffer (pH 7.8) containing 150 mM NaCl, 2 mM Na_3VO_4, 50 mM EDTA, 1 mM EGTA and protease inhibitors. Procedures for immunoprecipitation and *in vitro* kinase reactions were as described (Hurley *et al.*, 1989). Kinase reactions were performed in 20 μl of kinase assay buffer containing 20 mM HEPES (pH 7.5), 100 mM NaCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$ and 10 μM ATP (2 μCi [γ-^{32}P]ATP) at 25°C for 10 min. SDS-solubilized samples were subjected to SDS – PAGE and autoradiography. Tyrosine kinase activity in kinase immunoprecipitates was also measured in the kinase assay buffer supplemented with 1 mM RR-*src* peptide (RRLIEDAEYAARG, Gibco BRL). At the end of the reactions 50 mM EDTA was added. Ab-bound beads were removed by centrifugation and supernatants were applied to P81 cellulose phosphate paper (Whatman). After washing the paper with 0.5% phosphoric acid solution, radioactivity in RR-*src* was estimated.

Immunoprecipitation of 19 kDa protein

^{32}P-labelled cells (2×10^7 per lane) were lysed on ice in 1 ml of 1% (v/v) NP-40, 10 mM Tris – HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 50 mM NaF and 20 mM sodium pyrophosphate plus protease inhibitors. Cell lysates were pre-cleared with *Staphylococcus aureus* protein A for 15 mins and the 19 kDa protein then immunoprecipitated by rotating lysates at 4°C with specific antisera pre-bound to protein A – Sepharose beads (50 μl beads/lane). Immunoprecipitates were washed twice with 200 μl lysis buffer before heating for 5 min in 100 μl sample buffer and separating the eluted proteins on 12.5% polyacrylamide gels.

Calcium assay

Cells were loaded with 1 mM Fura 2/AM at 10^6/ml and resuspended in 2% HBSS with FCS−0.1 mM sulfin pyrazone. Assays were performed on 4×10^6 cells at 37°C in a Perkin−Elmer LS5B Luminescence Spectrometer using Fura 2 software provided by Perkin−Elmer. $[Ca^{2+}]_i$ was calculated and plotted using Lotus software kindly provided by Craig Naylor.

PKC assay

Cells were washed in PBS, resuspended in 2×10^6 cell aliquots in 100 μl PBS at 37°C and mAbs added with rapid mixing. After 5 min cells were pelleted within 15 s and permeabilized with streptolysin-O in an 'intracellular buffer' supplemented with [γ-^{32}P]ATP and a specific PKC substrate, peptide GS, as described by Alexander *et al.* (1990). Reactions were stopped after 5 min by the addition of 100 μl of 25% (w/v) trichloroacetic acid−2 M acetic acid and the incorporation of ^{32}P into peptide GS was estimated.

DAG assay

Relative determinations of DAG mass were made by removing 4×10^5 permeabilized cells from PKC assays immediately prior to stopping reactions and extracting phospholipids with 600 μl portions of acidified chloroform:methanol (1:2). ^{32}P-labelled phospholipids were separated on Silica Gel-60 TLC plates developed with chloroform:methanol:acetone:acetic acid:water (80:26:30:24:16), identified using authentic standards (kindly provided by Dr Robin Irvine) and quantified by densitometric scanning following exposure to Kodak XS film. The incorporation of ^{32}P from [γ-^{32}P]ATP into phosphatidic acid by the action of endogenous DAG kinase gave a direct estimate of DAG mass, this being the sole pathway for synthesis of [^{32}P]phosphatidic acid under the conditions of this assay.

Inositol phosphate determination

Cells were labelled overnight at 37°C with [^{3}H]*myo*-inositol (1 μCi/ml) at a cell density of 10^6 cells/ml in *myo*-inositol-deficient RPMI-1640/2% FCS. Labelled cells were then pelleted and incubated in complete RPMI-1640/2% FCS for a further 1 h before recovering the cells and incubating aliquots of 2×10^6 cells in 20 mM HEPES-buffered HBSS, pH 7.4 at 37°C for 20 min prior to addition of agonist. For IP determination in permeabilized cells, the washed cells were incubated in PBS (15 min) before permeabilizing with streptolysin-O, 0.5 mM ATP and 10 mM LiCl in 'intracellular buffer' as described for PKC assays. Extraction and separation of IPs were as described by Berridge *et al.* (1983). Total IPs were applied to Dowex AG1×8 resin columns, formate form (Bio-Rad) and [^{3}H]*myo*-inositol and [^{3}H]glycerophosphoinositol run to waste before collecting the remaining IPs as a single fraction. Samples were solubilized in Optiphase HiSafe 3 (Pharmacia) prior to determination of radioactivity. The values shown are the means of duplicate assays.

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