# CD45 and RPTPα display different protein tyrosine phosphatase activities in T lymphocytes

David H. W. NG*, Mojgan D. JABALI*, Arpita MAITI*, Peter BORODCHAK*, Kenneth W. HARDER†, Thomas BROCKER‡, Bernard MALISSEN‡, Frank R. JIRIK† and Pauline JOHNSON*[1]

*Department of Microbiology and Immunology, 300-6174 University Boulevard, University of British Columbia, Vancouver, B.C., Canada V6T 1Z3, †Biomedical Research Centre, 2222 Health Sciences Mall, University of British Columbia, Vancouver, B.C., Canada V6T 1Z3, and ‡Centre d'immunologie, INSERM-CNRS de Marseille-Luminy, F-15288 Marseille 9, France

To examine the substrate specificity and function of two receptor protein tyrosine phosphatases, CD45 and RPTPα, RPTPα was expressed in a CD45$^-$, T-cell receptor (TCR)$^+$, BW5147 T-lymphoma cell. High levels of expression of RPTPα did not fully restore either proximal or distal TCR-mediated signalling events. RPTPα was unable to reconstitute the phosphorylation of CD3ζ and did not increase the expression of the activation marker, CD69, on stimulation with TCR/CD3. RPTPα did not significantly alter the phosphorylation state or kinase activity of two CD45 substrates, p56$^{lck}$ or p59$^{fyn}$, suggesting that RPTPα does not have the same specificity or function as CD45 in T-cells. Further comparison of the two phosphatases indicated that immunoprecipitated RPTPα was approx. one-seventh to one-tenth as active as CD45 when tested against artificial substrates. This difference in activity was also observed *in vitro* with purified recombinant enzymes at physiological pH. Additional analysis with Src family phosphopeptides and recombinant p56$^{lck}$ as substrates indicated that CD45 was consistently more active than RPTPα, having both higher $V_{max}$ and lower $K_m$ values. Thus CD45 is intrinsically a much more active phosphatase than RPTPα, which provides one reason why RPTPα cannot effectively dephosphorylate p56$^{lck}$ and substitute for CD45 in T-cells. This work establishes that these two related protein tyrosine phosphatases are not interchangeable in T-cells and that this is due, at least in part, to quantitative differences in phosphatase activity.

## INTRODUCTION

Protein tyrosine phosphatases (PTPs), together with protein tyrosine kinases, are responsible for controlling the level of tyrosine phosphorylation within cells. Tyrosine phosphorylation is a reversible, post-translational modification that acts to propagate cellular responses to external stimuli and to regulate several essential cellular processes such as cell signalling, division, differentiation, survival and cell death (reviewed in [1,2]). Since the purification, characterization and amino acid sequence determination of the first PTP [3–5], over 40 PTPs have been identified. It is likely that this growing family will soon approach protein tyrosine kinases in terms of numbers, complexity and structural diversity. Despite the identification of a large number of these enzymes, relatively little is known about their substrates, specificity or regulation in cells.

PTPs, like protein tyrosine kinases, can exist as soluble, single-domain phosphatases or as transmembrane, receptor-like phosphatases, which generally contain two cytoplasmic PTP domains (reviewed in [6–8]). In the two-domain transmembrane phosphatases such as CD45, LAR and RPTPα, the catalytic cysteine resides in PTP domain I, yet PTP domain II seems to be required for optimum catalytic activity ([9–11], and K. W. Harder and F. R. Jirik, unpublished work). Whether PTP domain II has its own unique substrate specificity, as suggested for CD45 and RPTPα [12,13], or whether it has a distinct regulatory function is still a controversial issue. Many PTPs have been shown to be catalytically active on small artificial substrates such as phosphotyrosine, *p*-nitrophenyl phosphate (PNPP), 7–13-mer phosphopeptides and on tyrosine-phosphorylated proteins such as myelin basic protein and reduced carboxyamidomethylated and maleylated lysozyme, indicating that PTPs can act on a wide range of substrates *in vitro*. However, results suggest that PTPs do have a much more restricted substrate specificity in the cell, although little is understood about how this is achieved.

CD45 is currently one of the most studied transmembrane PTPs. It is a leucocyte-specific molecule and a major component of the lymphocyte cell surface [14,15]. CD45 is required for effective signal transduction by the T-cell and B-cell antigen receptor complex and is essential for both T-cell development and antigen-induced T-cell and B-cell activation (reviewed in [15]). T-cells lacking CD45 are defective in the earliest detectable event occurring on T-cell receptor (TCR)/CD3-mediated stimulation, the induction of tyrosine-phosphorylated proteins [16]. The Src family tyrosine kinases, p56$^{lck}$ [17,18] and, to a smaller extent, p59$^{fyn}$ [19,20], have been identified as T-cell substrates for CD45 *in vitro* and *in vivo*. Phosphorylated CD3ζ and ZAP-70 have also been suggested as potential substrates for CD45 in T-cells [21,22]. In T-cells CD45 is required for the dephosphorylation of the negative regulatory tyrosine of p56$^{lck}$ (Tyr-505) and, in some T-cells, also for the dephosphorylation of p59$^{fyn}$ (Tyr-531) [17,23,24]. CD45 might thus be required to dephosphorylate p56$^{lck}$ and p59$^{fyn}$, which in turn is required for their active and effective participation in TCR/CD3 signalling events. Restoration of TCR/CD3 signalling events in CD45$^-$ T-cells by transfection of chimaeric CD45 molecules has demonstrated that the extracellular and transmembrane regions of CD45 are not essential but that the phosphatase activity of CD45, present at the cell membrane, is an absolute requirement [25–29]. Furthermore, other studies of chimaeras have shown that the membrane-targeted expression of a yeast tyrosine phosphatase restored TCR/CD3 signalling capabilities, although not to the

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; GST, glutathione S-transferase; PNPP, *p*-nitrophenyl phosphate; PTP, protein tyrosine phosphatase; TCR, T-cell receptor.

[1] To whom correspondence should be addressed.

same extent as CD45 [30]. These results suggested that the major contribution of CD45 to TCR signal transduction was its intrinsic tyrosine phosphatase activity and that its requirement in T-cells could be replaced by another membrane-associated phosphatase.

RPTP$\alpha$ is a widely expressed transmembrane two-domain phosphatase that is expressed at low levels in lymphoid tissues [31–35]. The entire cytoplasmic domain of RPTP$\alpha$ shares approx. 37 % sequence identity with that of CD45: 47 % sequence identity is shared between the first PTP domains and 33 % between the second PTP domains. Overexpression of RPTP$\alpha$ in rat embryo fibroblasts and embryonic carcinoma cells resulted in the activation of pp60$^{c\text{-}src}$ and its dephosphorylation at the negative regulatory tyrosine phosphorylation site (Tyr-527) [36,37]. RPTP$\alpha$ has also been shown to dephosphorylate this site *in vitro*, implying that pp60$^{c\text{-}src}$ is a substrate for RPTP$\alpha$ [36,37]. This phosphatase thus shares many attributes with CD45; they both have similar cytoplasmic domain structures, significant sequence identity within their cytoplasmic regions (particularly within the catalytic PTP domain I), and both phosphatases can dephosphorylate the negative regulatory sites of Src family kinases.

To understand more about the role of CD45 in T-cells and to assess the potential substrate specificities of the PTPs CD45 and RPTP$\alpha$, RPTP$\alpha$ was expressed to high levels in the CD45$^-$ BW5147 T-cell line [38]. Despite the overall similarities between RPTP$\alpha$ and CD45, RPTP$\alpha$ did not detectably alter the tyrosine phosphorylation state or activity of either p56$^{lck}$ or p59$^{fyn}$ and did not restore proximal or distal TCR/CD3-mediated signalling events in these cells. Subsequent determination of the catalytic activity of RPTP$\alpha$ and CD45 isolated from these cells and in recombinant form indicated differences that help to explain why RPTP$\alpha$ cannot compensate for the absence of CD45 in the CD45$^-$ BW5147 T-cells.

## EXPERIMENTAL

### Antibodies and cells

CD3$\epsilon$ (145-2C11), and TCR $\beta$ (H57-597) antibodies were from the American Type Culture Collection (Rockville, MD, U.S.A.); the CD45 rat antibody, I3/2, which recognizes all mouse CD45 isoforms, was a gift from I. S. Trowbridge; the monoclonal antibody G3, specific for the mouse CD3$\zeta$ chain, was a gift from H.-S. Teh [39]; the anti-phosphotyrosine antibody (4G10) was from Upstate Biotechnology (Lake Placid, NY, U.S.A.); biotinylated anti-mouse early activation marker (CD69) was from Pharmingen (San Diego, CA, U.S.A.). Rabbit antisera (R-49 and R-54) were specific for residues 34–150 of p56$^{lck}$ [40]. p59$^{fyn}$ antiserum was a gift from R. Perlmutter [41] and A. Veillette [42]. Rabbit antiserum specific for the extracellular domain of RPTP$\alpha$ (PTP$\alpha$-ext) was used for FACS analysis, and rabbit antiserum specific for the cytoplasmic domain of RPTP$\alpha$ (PTP$\alpha$-2) was used for immunoprecipitation. Rabbit antiserum specific for the cytoplasmic domain of CD45 (R01.1) was used to immunoprecipitate CD45 (J. Felberg, W. Schoorl, W. Jefferies and P. Johnson, unpublished work). Bacterially expressed and purified recombinant six-histidine-tagged CD45 cytoplasmic domain protein, glutathione S-transferase (GST)–RPTP$\alpha$ cytoplasmic domain fusion protein and recombinant GST–p56$^{lck}$ protein were generated as previously described [40,43]. The GST portion was then removed from recombinant RPTP$\alpha$ by thrombin cleavage. Protein A–Sepharose was purchased from Pharmacia Biotech (Baie D'Urfé, Quebec, Canada) and Repligen (Cambridge, MA, U.S.A.). Fluoresceinated (FITC-labelled) goat anti-rat IgG was from Pierce (Rockford, IL, U.S.A.). FITC-labelled goat anti-hamster, mouse and rabbit IgG, FITC-labelled streptavidin, and horseradish peroxidase-conjugated goat anti-mouse IgG were purchased from Southern Biotechnology Associates (Birmingham, AL, U.S.A.). Horseradish peroxidase-conjugated protein A was purchased from Bio-Rad Laboratories (Mississauga, Ontario, Canada).

The following were used as substrates in the phosphatase assays: Fyn pY531 peptide (TATEPQpYQPGENL), Src pY416 peptide (LIEDNEpYTARQGA), Src pY527 peptide (TSTEPQpYQPGENL), provided by I. Clark-Lewis [44], and PNPP (Pierce) and autophosphorylated recombinant GST–p56$^{lck}$ [40].

BW5147 CD45$^+$ and BW5147 CD45$^-$ mouse T lymphoma cells, kindly provided by R. Hyman (also available from the ATCC), were transfected with CD3$\delta$ and CD3$\zeta$ cDNA species, which resulted in the expression of surface TCR/CD3 ([45], and this study). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10 % (v/v) horse serum supplemented with 100 i.u./ml penicillin, 100 $\mu$g/ml streptomycin, 0.25 $\mu$g/ml amphotericin B and 3 mM histidinol to maintain the expression of the transfected plasmids. Cells were checked routinely by FACS analysis to ensure that similar levels of TCR/CD3 levels were expressed in the CD45$^+$ and CD45$^-$ cell lines. It was observed that whereas the TCR/CD3 expression in the CD45$^-$ BW5147 cells was quite stable, expression in the CD45$^+$ cells declined over time. Human RPTP$\alpha$ [31], which shares 99 % sequence identity with the cytoplasmic domain of mouse RPTP$\alpha$, was subcloned into the expression vector pBCMGSneo [46] and transfected into CD45$^-$ BW5147 cells by electroporation. Positive clones were selected in 1 mg/ml active G418 (Gibco BRL, Burlington, Ontario, Canada) and tested for surface expression of the TCR/CD3 and human RPTP$\alpha$ by FACS analysis.

### FACS analysis

Cells ($2 \times 10^5$) were incubated with 100 $\mu$l of 145-2C11 and H57-597 tissue culture supernatant, a 1:10 dilution of I3/2 tissue culture supernatant, a 1:100 dilution of RPTP$\alpha$ antisera (PTP$\alpha$-ext) or a 1:200 dilution of anti-CD69 monoclonal antibody for 20 min on ice. Cells were washed with PBS containing 2.5 % (v/v) horse serum, then incubated for 20 min on ice with 100 $\mu$l of a 1:100 dilution of the appropriate FITC-labelled secondary antibody or FITC-labelled streptavidin. Cells were washed and analysed on a FACScan (Becton Dickinson, Mississauga, Ontario, Canada).

### Stimulation of BW5147 cells

Cells ($10^7$) were resuspended in 100 $\mu$l of DMEM/0.25 % fetal calf serum (FCS) equilibriated at 37 °C. Purified anti-CD3 (145-2C11) (10 $\mu$g) was added at zero time to stimulate the cells. For detection of phosphotyrosine-containing proteins in whole cell lysates, cells were incubated at 37 °C for the appropriate amount of time and then lysed by adding ice-cold 10 × TNE lysis buffer [10 % (v/v) Triton X-100/1.5 M NaCl/0.2 M Tris/HCl (pH 7.5)/20 mM EDTA/5 mM sodium orthovanadate/2 mM sodium molybdate/2 mM PMSF/10 mg/ml leupeptin/10 mg/ml aprotinin/10 mg/ml pepstatin]. For CD3$\zeta$ immunoprecipitations, cells were stimulated for 30 min at 37 °C (stimulated samples) or on ice (unstimulated samples). Cells were washed once in PBS and then lysed in 0.5 ml of 1 × TNE lysis buffer. For experiments monitoring the expression of the early activation marker (CD69), $10^5$ BW5147 cells, resuspended in 200 $\mu$l of DMEM/10 % (v/v) fetal calf serum, were stimulated with 0.1 $\mu$g of 145-2C11. Cells were then taken at 0, 24 and 48 h and analysed for CD69 expression.

### Detection of phosphotyrosine proteins

Cell lysates ($1.5 \times 10^6$ cell equivalents) were run on an SDS/10 % (w/v) polyacrylamide gel, transferred to PVDF membrane (Millipore Canada, Mississauga, Ontario, Canada) in a transblot apparatus (Bio-Rad) in accordance with the manufacturers' instructions and blotted with 4G10 antibody at 1:2000 dilution in 5 % (w/v) BSA/TTBS [0.1 % Tween-20/0.15 M NaCl/20 mM Tris/HCl (pH 7.5), with protease and phosphatase inhibitors] for 45 min after first blocking the membrane for 1 h in the same buffer, but without antibody. The blots were washed in 0.1 % Tween-20/0.15 M NaCl/20 mM Tris/HCl (pH 7.5) and incubated with 1:10000 horseradish peroxidase-conjugated goat anti-mouse IgG in 5 % (w/v) BSA/TTBS for 45 min, then washed thoroughly and developed with the enhanced chemiluminescence (ECL) assay in accordance with the manufacturer's instructions (ECL kit; Amersham Canada, Oakville, Ontario, Canada). In all cases, prestained molecular mass markers (New England Biolabs, Mississauga, Ontario, Canada) were run on the gel and transferred to the PVDF membrane.

### Immunoprecipitation of surface CD3ζ

Stimulated and unstimulated cell lysates were placed on ice, then centrifuged at 12000 ***g*** for 10 min to remove the insoluble pellet. Protein A–Sepharose beads (10 μl) were added to each 0.5 ml lysate sample and left to rotate at 4 °C for 1 h. Beads were then washed three times in 1 × TNE lysis buffer, and $5 \times 10^6$ cell equivalents were loaded on an SDS/15 % (w/v) polyacrylamide gel. Proteins were subsequently transferred to a PVDF membrane and Western blot analysis was performed with an anti-phosphotyrosine antibody (4G10) or an anti-CD3ζ antibody (G3) [39].

### Cell labelling and immunoprecipitation of CD45 and RPTPα

Cells ($5 \times 10^6$) were incubated for 30 min in methionine-free and cysteine-free medium before incubation for 8 h with 200 μCi of [$^{35}$S]methionine/cysteine (1175 Ci/mmol; DuPont–NEN, Boston, MA, U.S.A.) in 5.0 ml of DMEM (methionine-free and cysteine-free) supplemented with 10 % (w/v) dialysed fetal calf serum. Cells were then lysed in 1.0 ml of lysis buffer [0.5 % Triton X-100/10 mM Tris (pH 7.5)/0.15 M NaCl/1 mM EDTA, with protease inhibitors] and the amount of radioactivity incorporated was determined after precipitation of a 50 μl aliquot of the lysate with trichloroacetic acid. CD45 and RPTPα were then precipitated from cell lysates containing equal amounts of radioactivity. Lysates from approx. $2 \times 10^6$ cells were precleared with Sepharose beads and used to immunoprecipitate CD45 and RPTPα with 20 μl of Protein A–Sepharose beads precoupled with either 2 μl of CD45 antiserum (R01.1) or 10 μl of RPTPα antiserum (PTPα-2). These amounts had previously been optimized to ensure maximum immunoprecipitation of the relevant proteins. After 2 h of incubation at 4 °C the immunoprecipitates were washed twice with lysis buffer containing 0.2 % Triton X-100 and then subjected to SDS/PAGE in which [$^{35}$S]methionine/cysteine-labelled proteins were observed after autoradiography of the dried gel after treatment with Amplify (Amersham Canada). Alternatively, unlabelled immunoprecipitates were washed twice more in PTP buffer [20 mM Tris (pH 7.4)/1.0 mM EDTA/0.1 % (v/v) 2-mercaptoethanol, with protease inhibitors] and used in PTP assays. Relative amounts of the [$^{35}$S]methionine/cysteine-labelled proteins were measured by densitometry scanning (PDI Systems, New York, U.S.A.) and quantitative phosphorimaging techniques (Molecular Devices, Sunnyvale, CA, U.S.A.)

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

Cells ($9 \times 10^6$) were lysed in 1 ml of lysis buffer containing 1 % Triton X-100 (see above) and the supernatant was recovered after centrifuging for 10 min at 12000 ***g***. The cell lysate was then precleared with 10 μl of Protein A–Sepharose and incubated at 4 °C for 1.5 h with 10 μl of Protein A–Sepharose that had been precoupled with 1 μl of R-49 or R-54 p56$^{lck}$ antiserum or with 0.4 μl of p59$^{fyn}$ antiserum. These amounts had previously been determined as being sufficient to precipitate p56$^{lck}$ or p59$^{fyn}$ from $10^7$ BW5147 cells. Immunoprecipitates were washed three times with the lysis buffer before being divided into aliquots; $3 \times 10^6$ cell equivalents were run on SDS/7.5 % or 10 % (w/v) polyacrylamide gels, transferred to PVDF membrane and blotted with either the antiphosphotyrosine antibody 4G10, with p56$^{lck}$ antiserum (R49 or R54) or with p59$^{fyn}$ antiserum in 5 % (w/v) BSA/TTBS at dilutions of 1:2000, 1:2500 and 1:1000 respectively. Then $3 \times 10^6$ cell equivalents were washed twice in kinase buffer [40 mM Pipes (pH 7.2)/10 mM $MnCl_2$] and used in a kinase assay *in vitro*.

### Kinase assay *in vitro*

Immunoprecipitated p56$^{lck}$ or p59$^{fyn}$ from $3 \times 10^6$ cells was resuspended in a final volume of 20 μl of kinase buffer containing 10 μCi of [γ-$^{32}$P]ATP (more than 3000 Ci/mmol; Amersham Canada), 1 μM ATP (Pharmacia Biotech), and 5 μg of acid-denatured enolase (Boehringer Mannheim, Laval, Quebec, Canada). Kinase reactions were incubated *in vitro* at 30 °C for 0–10 min. The reactions were stopped with 25 mM EDTA, pH 8.0, and immunoprecipitates were separated by centrifugation from enolase. Immunoprecipitates were washed three times with kinase buffer and run on an SDS/7.5 % or 10 % (w/v) polyacrylamide gel. The gel was dried and then exposed to Kodak BioMax film (InterScience, Markham, Ontario, Canada).

### Phosphatase assay *in vitro*

Phosphatase assays were performed as described previously [44]. Immunoprecipitated bead suspension (10 μl), containing immunoprecipitated protein from $10^5$ cell equivalents, was dispensed into half-area (100 μl) 96-well microtitre plates at 30 °C. The reaction was initiated by the addition of 10 μl of PTP buffer containing 7 mM Fyn pY531 peptide (TATEPQpYQPGENL). The microtitre plate was agitated at 120 rev./min during the course of the assay. Three time points were taken for each sample by stopping the reaction with the addition of 80 μl of filtered Malachite Green reagent (1 part 0.135 % Malachite Green oxalate salt in distilled water, 1 part 4.2 % (w/v) ammonium molybdate in 4.0 M HCl, 2 parts distilled water, and Tween-20 to a final concentration of 0.01 %). Release of $P_i$ was measured by determining the absorbance at 650 nm with a multiwell microtitre plate reader (Molecular Devices) and compared with a standard curve for $P_i$ obtained with $KH_2PO_4$. Malachite Green phosphatase assays were also performed in 20 μl with 20 ng of recombinant cytoplasmic CD45 or RPTPα protein, with different concentrations of various phosphopeptides as substrates. Phosphatase assays with PNPP as substrate in 100 μl of PTP buffer were performed in half-area 96-well microtitre plates at 30 °C. The absorbance at 405 nm was monitored; a molar absorption coefficient of $1.78 \times 10^4$ $M^{-1} \cdot cm^{-1}$ was used to calculate the concentration of the *p*-nitrophenolate ion produced in the reaction. For phosphatase assays on intact recombinant p56$^{lck}$ protein, approximately equimolar amounts of recombinant en-

zymes (50 ng for CD45 and 44 ng for RPTPα) were added to 200 ng of tyrosine-phosphorylated recombinant GST–p56$^{lck}$ protein [40] in a total volume of 10 μl of PTP buffer. Reactions were performed at 30 °C and stopped at various time points from 1 to 32 min by immersion in a solid-$CO_2$/ethanol bath. These samples were then boiled in SDS sample buffer before being subjected to SDS/PAGE and transferred to PVDF membrane. The tyrosine phosphorylation state of GST–p56$^{lck}$ was then analysed by blotting with the antiphosphotyrosine monoclonal antibody 4G10.

## RESULTS

### Transfection and expression of RPTPα in BW5147 CD45⁻ T-lymphoma cells

CD45⁻ BW5147 T-lymphoma cells were originally derived from the parental CD45⁺ BW5147 T lymphoma by chemical mutagenesis [38]. Although the exact nature of the mutation(s) has not been identified, fusion of these cells with a rat cell line suggested that there is a *cis* mutation in the mouse CD45 gene [38]. Transfection of CD45 into these CD45⁻ BW5147 T-cells expressing CD4 and the TCR/CD3 complex has demonstrated that the expression of CD45 can fully restore the TCR signalling of these cells in response to antigen [47,48]. In this study the CD45⁻ BW5147 T-cells were transfected with CD3δ and CD3ζ to express endogenous TCR. These cells were then transfected with RPTPα and positive clones were identified by FACS analysis with RPTPα antisera. Clones (2-10 and 3-11) expressing high levels of RPTPα and comparable levels of TCR/CD3 to untransfected cells and to CD45⁺, TCR/CD3⁺ BW5147 T-cells [45] were selected for further study. The cell-surface expression of CD45, RPTPα, CD3ε and TCRβ on CD45⁺-, CD45⁻- and RPTPα-transfected CD45⁻ BW5147 T-cells is shown in Figure 1.

To compare the level of expression of RPTPα in the transfected CD45⁻ BW5147 cells with that of CD45 in the parental CD45⁺ BW5147 cells, both cell lines were metabolically labelled with [$^{35}$S]methionine/cysteine and the phosphatases were immunoprecipitated (Figure 2). CD45 and RPTPα (which are predicted to have molecular masses of 180 and 125 kDa and contain 48 and 33 methionine and cysteine residues respectively) were found to be expressed at similar levels in the respective cells. The transfected clones 2-10 and 3-11 expressed slightly higher molar amounts (1.2-fold) of RPTPα than CD45 expressed by the parental cell.

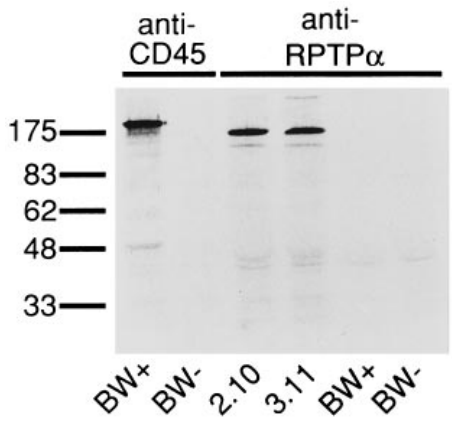



**Figure 2 Immunoprecipitation of CD45 (anti-CD45) and RPTPα (anti-RPTPα) from BW5147 cells**

Cells were labelled metabolically with [$^{35}$S]methionine/cysteine for 8 h at 37 °C. RPTPα and CD45 proteins were immunoprecipitated from BW5147 CD45⁺ T-cells (BW⁺), BW5147 CD45⁻ T-cells (BW⁻) and RPTPα-transfected BW5147 CD45⁻ T-cells (2-10 and 3-11) as indicated. Proteins were immunoprecipitated from cell lysates containing equivalent amounts of radioactivity by using polyclonal rabbit anti-(RPTPα cytoplasmic domain) antiserum (PTPα-2) or polyclonal rabbit anti-(CD45 cytoplasmic domain) antiserum (R01.1) (see the Experimental section for details). Immunoprecipitated proteins were analysed after separation by SDS/PAGE [10% (w/v) gel]. The positions and sizes (in kDa) of the prestained molecular-mass markers are indicated on the left.

### Effect of RPTPα on TCR/CD3-mediated stimulation in BW5147 CD45⁻ T-cells

Because CD45 has been implicated in the initiation of TCR/CD3-mediated signalling events, TCR/CD3-induced tyrosine-phosphorylated proteins were examined in RPTPα-transfected cells (clone 2-10) and compared with those observed in the parental CD45⁻ and CD45⁺ BW5147 T-cells. Cells were stimulated with an anti-CD3 antibody and tyrosine-phosphorylated proteins were detected at various time points by SDS/PAGE and Western blotting with the anti-phosphotyrosine antibody 4G10. The rapid induction of tyrosine phosphorylation of specific proteins was observed in the CD45⁺ cells at the earliest time point measured, 1.5 min (Figure 3A). A major phosphorylated protein was observed at an apparent molecular mass of 38 kDa, and minor bands were observed between approx. 70 and 205 kDa

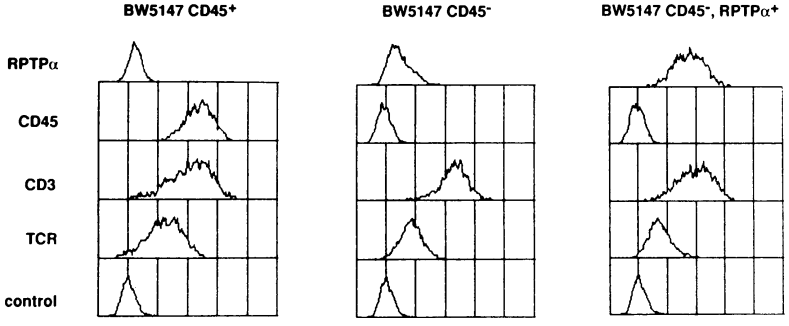



**Figure 1 FACS analysis of BW5147 CD45⁺ T-lymphoma cells, and CD45⁻- and RPTPα-transfected CD45⁻ BW5147 T-lymphoma cells**

The control shown is FITC-labelled secondary antibody alone. Samples were analysed with the FACScan (Becton Dickinson). The *x*-axis denotes the amount of fluorescence intensity (log scale) and the *y*-axis cell number.

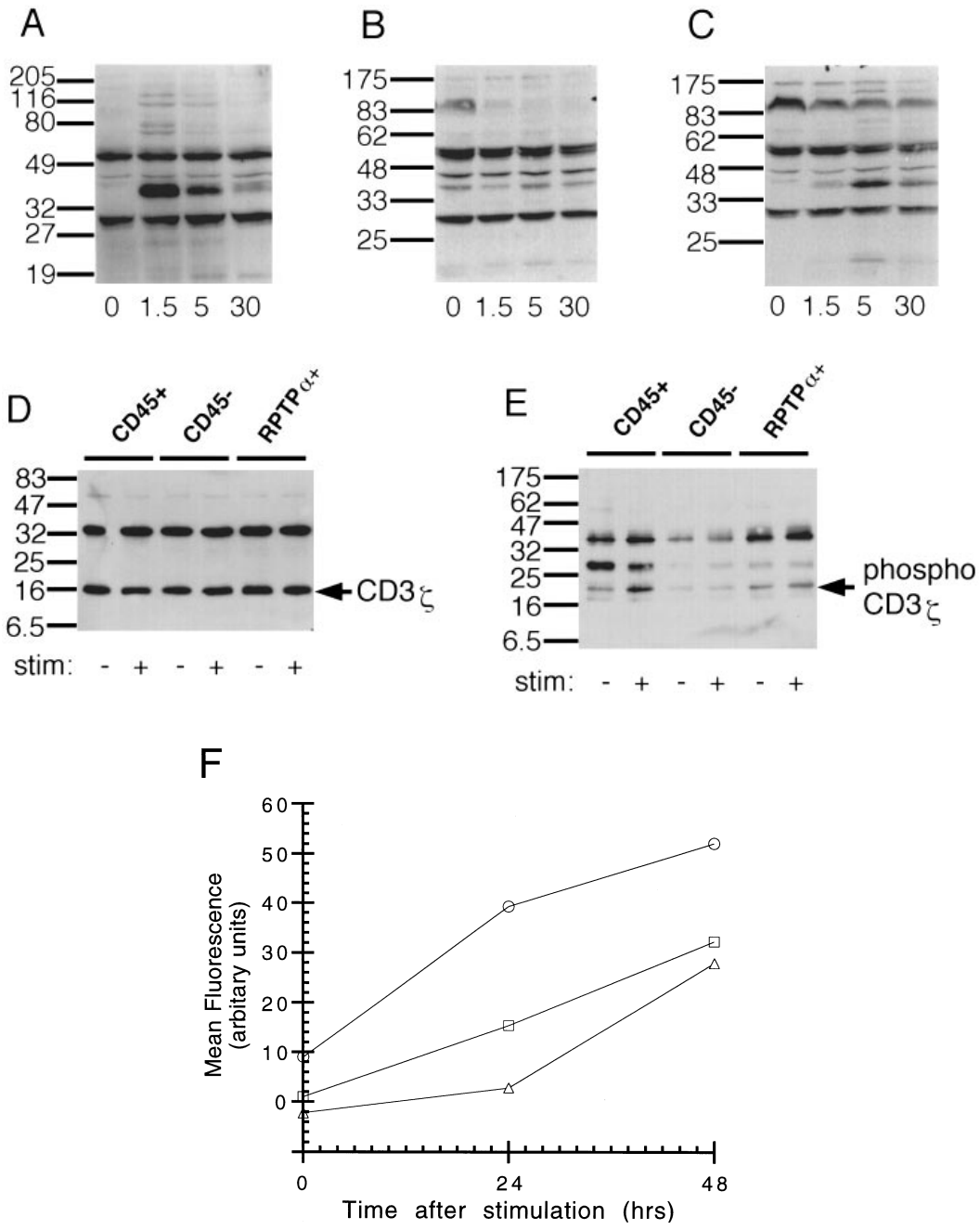
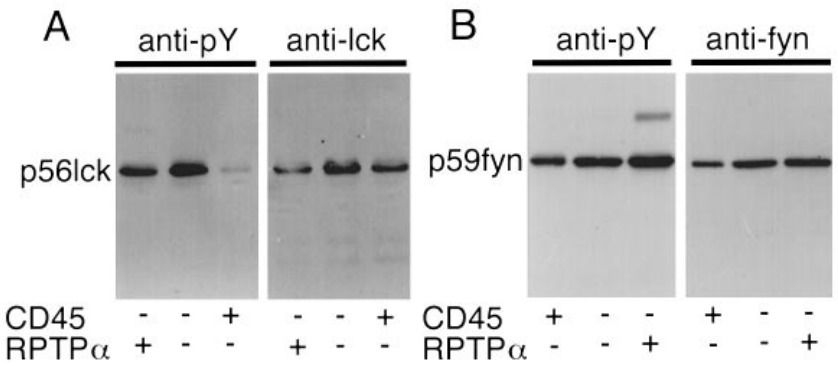



**Figure 3 Effect of CD45 and RPTPα on TCR/CD3-mediated stimulation in BW5147 cells**

BW5147 CD45$^+$ (**A**), BW5147 CD45$^-$ (**B**) and clone 2.10 CD45$^-$, RPTPα$^+$ (**C**) cells were stimulated by the addition of purified anti-CD3ε monoclonal antibody (145-2C11) and incubated at 37 °C for 0, 1.5, 5 and 30 min, as indicated, before lysis; $1.5 \times 10^6$ cell equivalents was then subjected to SDS/PAGE [10% (w/v) gel], transferred to PVDF membrane and probed with the anti-phosphotyrosine monoclonal antibody 4G10. Surface CD3ζ was immunoprecipitated by the addition of 145-2C11 to all three cell lines for stimulated (30 min at 37 °C) or unstimulated (30 min on ice) samples, and $5.0 \times 10^6$ cell equivalents was subjected to SDS/PAGE [15% (w/v) gel], transferred to PVDF membrane and probed with either the anti-CD3ζ monoclonal antibody G3 (**D**) or the anti-phosphotyrosine monoclonal antibody 4G10 (**E**). (**F**) Cells were incubated with the purified 145-2C11 for 0, 24 or 48 h and then analysed for CD69 expression by flow cytometry. The graph shows the mean fluorescence intensity of CD69 expression over time for BW5147 CD45$^+$ (○), BW5147 CD45$^-$(□) and clone 2.10 CD45$^-$, RPTPα$^+$ (△) cells. This graph represents results from one of four separate experiments. The positions and sizes (in kDa) of the prestained molecular mass markers are indicated on the left in (**A**–**E**).

and between 19 and 27 kDa. In contrast, the induction of tyrosine-phosphorylated proteins after CD3 stimulation in BW5147 CD45$^-$ cells was virtually absent (Figure 3B). Transfection of RPTPα into the BW5147 CD45$^-$ cells partly restored their ability to tyrosine phosphorylate proteins in response to the addition of anti-CD3 antibody, although this response was both delayed and less efficient (Figure 3C). Maximal induction of tyrosine phosphorylation (particularly noticeable with the major 38 kDa phosphorylated protein) was observed at the 5 min time point but not at 1.5 min. Similar results were also observed with the 3-11 RPTPα clone (results not shown). The repeated observations of an intense band at an apparent molecular mass of




C

| kinase | Cell Line | Cell Description | Phosphorylation/ Protein Amount |
|---|---|---|---|
| p56lck | CD45- | CD45-,RPTPα- | 1.0 |
| | CD45+ | CD45+,RPTPα- | 0.15 ± 0.1 |
| | 2.10/3.11 | CD45-, RPTPα- | 1.1 ± 0.1 |
| p59fyn | CD45- | CD45-,RPTPα- | 1.0 |
| | CD45+ | CD45+,RPTPα- | 1.2 ± 0.5 |
| | 2.10/3.11 | CD45-, RPTPα- | 1.2 ± 0.5 |

**Figure 4 Phosphotyrosine levels of p56$^{lck}$ and p59$^{fyn}$ isolated from BW5147 cells**

p56$^{lck}$ (**A**) or p59$^{fyn}$ (**B**) was immunoprecipitated from CD45$^{+}$-, CD45$^{-}$- and RPTPα-transfected BW5147 CD45$^{-}$ T-cells. Immunoprecipitates were subjected to SDS/PAGE [10% (w/v) gels] and transferred to PVDF membrane. The membranes containing p56$^{lck}$ protein were probed with an anti-phosphotyrosine monoclonal antibody (4G10) (anti-pY) or with an anti-p56$^{lck}$ antiserum (R49 or R54) (anti-lck), whereas membranes containing p59$^{fyn}$ were probed with 4G10 (anti-pY) or an anti-p59$^{fyn}$ antisera (anti-fyn). (**C**) Table showing the mean values and standard deviations of a ratio value calculated from the band intensities of the phosphotyrosine content divided by the protein amount. Ratio values for BW5147 CD45$^{-}$ cells were normalized to 1.0 and the values are representative of results collected from five separate experiments involving p56$^{lck}$, and three separate experiments involving p59$^{fyn}$. Band intensities ($D \times mm^2$) were obtained by densitometry scanning (PDI Systems).

83 kDa was inconsistent and not reproducible in all BW5147 clones tested. To determine further the effect of RPTPα on proximal TCR/CD3-induced signalling events, the phosphorylation state of CD3ζ was examined. As shown in Figures 3(D) and 3(E), RPTPα expression did not result in increased phosphorylation of CD3ζ after stimulation with TCR/CD3. Thus the expression of RPTPα to levels at least equivalent to that observed for CD45 could not restore the proximal T-cell signalling events observed in the CD45$^{+}$ BW5147 cells.

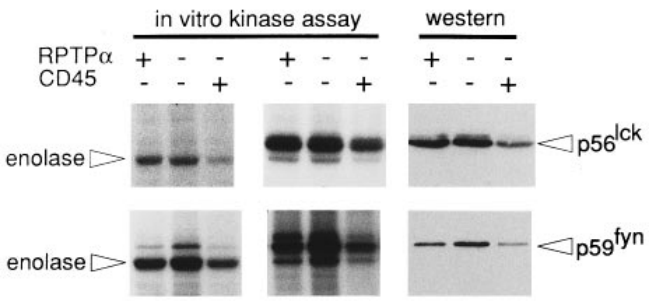
To evaluate whether the partial induction of tyrosine phosphorylation observed in TCR/CD3-stimulated RPTPα$^{+}$ cells was sufficient to stimulate downstream events, the induction of CD69 expression, a known distal marker of T-cell activation [49,50], was determined by flow cytometry. It was observed that there was a distinct difference in CD69 up-regulation between the CD45$^{+}$ BW5147 T-cells when compared with both the CD45$^{-}$- and RPTPα-transfected BW5147 T-cells (Figure 3F). This indicates that the presence of RPTPα had no significant effect on the induction of CD69 expression in response to stimulation with TCR/CD3.

### Effect of RPTPα on the phosphorylation state and activity of p56$^{lck}$ and p59$^{fyn}$

As the earliest detectable signalling events were not restored in RPTPα-transfected BW5147 CD45$^{-}$ cells, it was next determined whether overexpression of RPTPα affected the phosphorylation state or kinase activity of p56$^{lck}$ and p59$^{fyn}$, as both have been implicated in the initiation of TCR/CD3 signalling events and in the phosphorylation of CD3ζ. These kinases were precipitated from CD45$^{+}$-, CD45$^{-}$- and RPTPα-transfected CD45$^{-}$ cells and their tyrosine-phosphorylation states were compared (see Figures 4A and 4B). Densitometric scanning of autoradiographs provided a comparison of tyrosine-phosphorylation levels for equivalent amounts of p56$^{lck}$ and p59$^{fyn}$ (Figure 4C). In keeping with previous results [17], the presence of CD45 in these cells greatly decreased the overall level of tyrosine phosphorylation of p56$^{lck}$ (Figure 4A). However, overexpression of RPTPα in the CD45$^{-}$ BW5147 T-cells did not decrease the overall level of tyrosine phosphorylation of p56$^{lck}$, suggesting that RPTPα has a different substrate specificity from CD45. The effect of CD45 on the overall phosphorylation state of p59$^{fyn}$ was less apparent (Figure 4B). This is also consistent with previous results, which indicate that the phosphorylation state of p59$^{fyn}$ is affected to a smaller extent than that of p56$^{lck}$ by CD45 in some cell lines [23,24]. In these experiments the overexpression of RPTPα in the BW5147 T-cells did not cause a detectable change in the overall tyrosine-phosphorylation state of p59$^{fyn}$.

To address whether overexpression of RPTPα altered the kinase activity of p56$^{lck}$ or p59$^{fyn}$, these proteins were immunoprecipitated from each cell line, and activity was assessed in a kinase assay *in vitro* with both exogenous (enolase) and endo-

A




B

| Kinase | Cell Line | Enolase | Autophosphorylation |
|---|---|---|---|
| p56lck | CD45- | 1.0 | 1.0 |
| | CD45+ | 1.1 ± 0.3 | 1.0 ± 0.4 |
| | 2.10/3.11 | 1.0 ± 0.1 | 1.2 ± 0.2 |
| p59fyn | CD45- | 1.0 | 1.0 |
| | CD45+ | 2.5 ± 0.4 | 4.6 ± 2.6 |
| | 2.10/3.11 | 0.9 ± 0.2 | 1.2 ± 0.1 |

**Figure 5 Kinase activity *in vitro* of p56$^{lck}$ and p59$^{fyn}$ isolated from BW5147 T-cells**

(**A**) p56$^{lck}$ and p59$^{fyn}$ were immunoprecipitated from BW5147 cells containing RPTPα protein, CD45 protein or neither protein, and resuspended in a final volume of 20 μl of kinase buffer containing 5.0 μg of enolase (see the Experimental section). Reactions were initiated by the addition of [$\gamma$-$^{32}$P]ATP, stopped by the addition of 25 mM EDTA (pH 8.0), and immunoprecipitates were separated from enolase by centrifugation. Immunoprecipitates were then washed in kinase buffer and samples subjected to SDS/PAGE. Gels were dried and exposed to autoradiographic film. Immunoprecipitated p56$^{lck}$ and p59$^{fyn}$ were also analysed by Western blot with anti-p56$^{lck}$ antiserum (R49) or anti-p59$^{fyn}$ antiserum to indicate the relative amounts precipitated and used in the kinase assays. (**B**) Table comparing relative kinase activities of equivalent amounts of either p56$^{lck}$ or p59$^{fyn}$ with both an exogenous substrate (enolase) and an endogenous substrate (autophosphorylation). The numbers are ratios of relative amounts of radioactivity incorporated divided by the relative protein amount and were derived from the relative band intensities of the *in vitro* kinase autoradiograph and the Western blot. The ratio values for the BW5147 CD45$^-$ cells were standardized to 1.0 and the results (means ± S.D. were collected from five separate experiments involving p56$^{lck}$ and three separate experiments involving p59$^{fyn}$. Band intensities ($D \times$ mm$^2$) were obtained by densitometry scanning (PDI Systems).

genous (autophosphorylation) substrates (Figure 5A). Various time points were taken and the initial rates of reaction were measured and compared. Relative activities for equivalent protein amounts were compared and no significant differences were observed between the kinases isolated from the BW5147 CD45$^-$ cells and the RPTPα-transfected cells (Figure 5B). Despite the major difference in the tyrosine-phosphorylation state of p56$^{lck}$ between CD45$^+$ and CD45$^-$ cells, no significant difference was observed in the kinase activity *in vitro*, suggesting that either kinase activity *in vitro* is not an accurate reflection of kinase activity inside the cell or that dephosphorylation of p56$^{lck}$ does not affect its intrinsic kinase activity. Conversely, although the overall phosphorylation state of p59$^{fyn}$ was not significantly altered by the presence of CD45, a 2–5-fold increase in kinase activity *in vitro* was observed for exogenous and endogenous substrates. Hence the presence of CD45 in these cells had distinct effects on p56$^{lck}$ and p59$^{fyn}$. In contrast, RPTPα had no detectable effect on either the phosphorylation state or kinase activities of either p56$^{lck}$ or p59$^{fyn}$, implying that CD45 is specifically required to dephosphorylate p56$^{lck}$ and stimulate p59$^{fyn}$ kinase activity and that this, in turn, might be required for the initiation of optimal TCR/CD3-induced signalling events.

**Table 1 Comparison of the phosphatase activity of CD45 and RPTPα immunoprecipitated from BW5147 T-cell lines**

Phosphatase activity was measured with saturating amounts of the Fyn phosphopeptide (3.5 mM Fyn pY531) as substrate. Results for each cell line (means ± S.D.) were derived from three separate experiments. Undetectable levels of CD45 were immunoprecipitated from CD45$^-$ BW5147 cells; hence no phosphatase activity was detected in these samples (n.d.).

| Cell line | Cell description | Precipitating antiserum | PTP activity (nmol/min per $2 \times 10^5$ cells) |
|---|---|---|---|
| CD45$^-$ | CD45$^-$, RPTPα$^-$ | CD45 | n.d. |
| CD45$^+$ | CD45$^+$, RPTPα$^-$ | CD45 | 0.44 ± 0.03 |
| 2.10 | CD45$^-$, RPTPα$^+$ | RPTPα | 0.057 ± 0.003 |
| 3.11 | CD45$^-$, RPTPα$^+$ | RPTPα | 0.073 ± 0.007 |

## Comparison of phosphatase activities of RPTPα and CD45 isolated from BW5147 T-cells

To investigate further the potential differences between these two PTPs, CD45 and RPTPα were immunoprecipitated from the respective BW5147 T-cells and their relative phosphatase activities measured. RPTPα immunoprecipitated from equivalent cell numbers was approx. one-sixth to one-eighth as catalytically active as immunoprecipitated CD45 when a phosphorylated Fyn peptide (Y531) was used as the substrate in a phosphatase assay *in vitro* (Table 1). When the amount of specific phosphatase expressed by each cell was taken into account, CD45 was approximately 7–10-fold more active than RPTPα on a molar basis. A similar result was also obtained with PNPP as the substrate, indicating that the Fyn peptide was not acting as a preferential substrate for CD45 (results not shown). This suggested that the reason for RPTPα's inability to substitute fully for the activities of CD45 might be its lower phosphatase activity. To investigate further whether cellular factors such as post-translational modifications were responsible for these observed differences in phosphatase activity, the activities and specificities of recombinant CD45 and RPTPα proteins were determined.

## Activities and specificities *in vitro* of recombinant RPTPα and CD45 proteins

Phosphatase activities *in vitro* of purified recombinant cytoplasmic CD45 and RPTPα were first determined with GST–p56$^{lck}$ as a substrate (Figure 6A). At physiological pH, CD45 dephosphorylated p56$^{lck}$ within 1 min, whereas only a small percentage was dephosphorylated by RPTPα after 30 min (Figure 6B). This demonstrates that intrinsic differences in activity or specificity exist between these phosphatases, irrespective of whether the phosphatase was isolated from a T-cell or produced as a recombinant protein in *Escherichia coli*.

To try to distinguish between qualitative and quantitative differences in activity, the $V_{max}$ and $K_m$ for each phosphatase were determined with Src family phosphopeptides and PNPP as substrates (Table 2). First, in agreement with the enzymic activities determined from the immunoprecipitated phosphatases, the activity of the recombinant cytoplasmic domain RPTPα was approximately one-seventh to one-tenth as active as the recombinant cytoplasmic domain CD45 when either saturating amounts of phosphopeptides or PNPP were used as substrates,

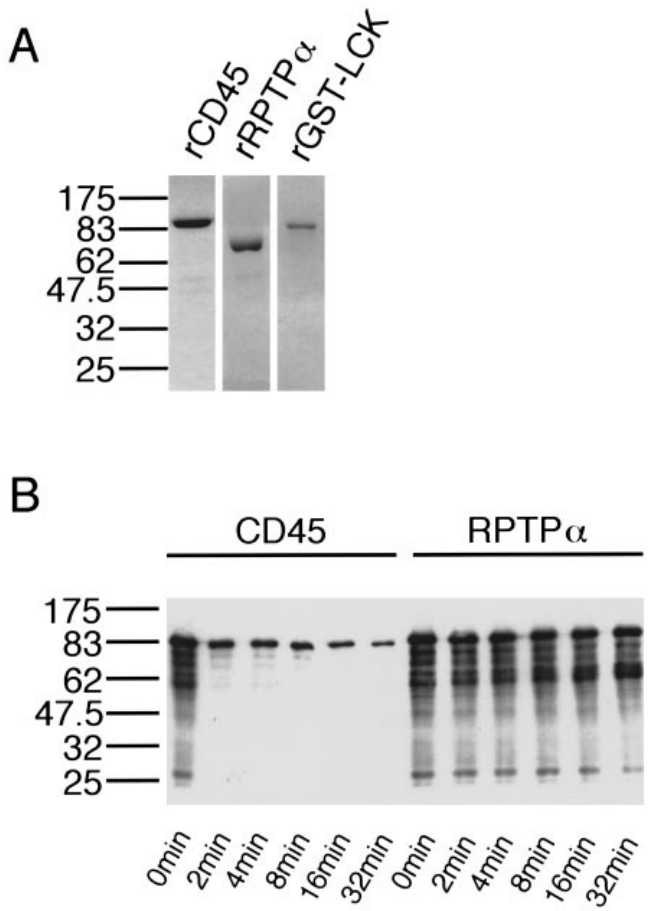



**Figure 6 Phosphatase assay *in vitro* of recombinant cytoplasmic CD45 and recombinant cytoplasmic RPTPα with recombinant GST–p56$^{lck}$ protein as a substrate**

(**A**) Coomassie Blue staining of purified recombinant six-histidine-tagged cytoplasmic CD45 domain protein, cytoplasmic RPTPα domain protein (cleaved from GST with thrombin) and GST–p56$^{lck}$ protein. (**B**) Phosphatase assays *in vitro* were initiated by adding approx. 50 ng of recombinant cytoplasmic CD45 or 44 ng of recombinant cytoplasmic RPTPα to 200 ng of recombinant GST–p56$^{lck}$ in a final volume of 10 μl of PTP buffer (see the Experimental section). Reactions were stopped at various time points (2–32 mins) by immersing samples in a solid-$CO_2$/ethanol bath. Samples were subsequently subjected to SDS/PAGE [10% (w/v) gel] and transferred to PVDF membrane; the level of phosphotyrosine remaining on GST–p56$^{lck}$ was monitored by Western blotting with the anti-phosphotyrosine antibody 4G10. The predicted size for the GST–p56$^{lck}$ fusion protein is approx. 83 kDa.

**Table 2 Comparison of $V_{max}$ and $K_m$ values between recombinant cytoplasmic CD45 and RPTPα enzymes**

$V_{max}$ and $K_m$ values were determined from Lineweaver–Burk and Eadie–Hofstee plots of the data and are expressed in units of μmol/min per mg and mM respectively (means ± S.D.). *n* is the number of experiments from which the results were calculated.

| | | CD45 | | RPTPα | |
|---|---|---|---|---|---|
| Substrate | *n* | $V_{max}$ (μmol/min per mg) | $K_m$ (mM) | $V_{max}$ (μmol/min per mg) | $K_m$ (mM) |
| PNPP | 4 | 0.53 ± 0.06 | 0.22 ± 0.01 | 0.05 | 0.22 ± 0.02 |
| Fyn pY531 | 2 | 58.2 ± 0.8 | 0.23 ± 0.01 | 8.3 ± 0.2 | 1.07 ± 0.03 |
| Src pY527 | 4 | 60.6 ± 0.7 | 0.23 | 7.6 ± 0.4 | 1.16 ± 0.14 |
| Src pY416 | 2 | 66.0 ± 2.4 | 0.22 ± 0.01 | 7.1 ± 0.2 | 0.77 ± 0.02 |

illustrating that RPTPα is intrinsically less active than CD45. The fact that RPTPα was consistently one-sixth to one-eleventh as active as CD45 with all substrates tested suggested that quantitative rather than qualitative differences accounted for its inability to dephosphorylate p56$^{lck}$ effectively. Secondly, although the $K_m$ values for recombinant RPTPα and recombinant CD45 were similar when PNPP was the substrate, $K_m$ values were 3–5-fold higher for recombinant RPTPα when Src family phosphopeptides were used as substrates, indicating that CD45 had a higher affinity for these peptides than did RPTPα. As a measure of overall enzyme efficiency, $k_{cat}/K_m$ values ($M^{-1} \cdot s^{-1}$) were calculated to be approx. 10000 for RPTPα and approx. 370000 for CD45 with the Src family phosphopeptides as substrates, confirming that CD45 was a more effective enzyme against these peptide substrates. Thirdly, when the PTP activity of either phosphatase was examined individually, both enzymes were more active against Src family phosphopeptides than the PNPP substrate. No major substrate preferences were observed for either phosphatase between the Src family phosphopeptides tested. Thus recombinant RPTPα dephosphorylated recombinant p56$^{lck}$, Src and Fyn phosphopeptides and PNPP all less effectively than CD45.

Taken together, these experiments show that overexpression of RPTPα to levels similar to that observed for CD45 in BW5147 cells was not sufficient to restore fully the proximal or distal signalling events seen on stimulation with CD3. In particular, RPTPα was unable to cause any detectable dephosphorylation or activity change in either p56$^{lck}$ or p59$^{fyn}$. Immunoprecipitated RPTPα isolated from CD45$^-$ BW5147 T-cells was found to be one-seventh to one-tenth as catalytically active as equimolar amounts of immunoprecipitated CD45 isolated from the parental CD45$^+$ BW5147 T-cells, suggesting that differences in activity might account for the differences observed in T-cells. This difference in activity was also reproduced *in vitro* with purified, recombinant phosphatases, indicating that post-translational modifications or cellular factors were not responsible for this difference in catalytic activity. Thus RPTPα might not effectively substitute for CD45 in T-cells because it is one-seventh to one-tenth as catalytically active.

## DISCUSSION

Expression of RPTPα in CD45$^-$ BW5147 T-cells to levels equivalent to those observed for CD45 in CD45$^+$ BW5147 T-cells resulted in a partial induction of tyrosine-phosphorylated proteins on stimulation with TCR/CD3, occurring with less intensity and with slower kinetics. Consistent with this decreased level of induction, no increase was observed in the tyrosine phosphorylation state of CD3ζ after stimulation with TCR/CD3. Phosphorylation of CD3ζ is thought to be mediated by p56$^{lck}$, a tyrosine kinase involved in the initiation of TCR/CD3 signalling events [51]. To function effectively in this process, p56$^{lck}$ needs to be dephosphorylated at its negative regulatory tyrosine residue; this occurs in CD45$^+$ cells but not in CD45$^-$ T-cells [17,23,24]. p56$^{lck}$ remained in its phosphorylated form in RPTPα-transfected T-cells, indicating that it was not an efficient substrate for RPTPα. The inability of phosphorylated p56$^{lck}$ to participate effectively in TCR-mediated signal transduction is consistent with the observed lack of CD3ζ phosphorylation and the overall decreased induction of tyrosine phosphorylation. Analysis of CD69 up-regulation, a distal event associated with T-cell activation, indicated that expression of RPTPα in the CD45$^-$ BW5147 cells was also not able to restore downstream TCR/CD3-mediated signalling events. Taken together, these results suggest that the tyrosine phosphatase CD45 is specifically

required for the efficient dephosphorylation of $p56^{lck}$ and its participation in TCR/CD3-initiated signalling events. CD45, but not RPTPα, is required for the optimal phosphorylation of CD3ζ, the generation of a rapid and strong induction of tyrosine phosphorylation on stimulation by TCR/CD3 and for the induction of downstream signalling events, as illustrated by the expression of the T-cell activation antigen CD69. In this study we have shown that RPTPα cannot substitute effectively for CD45 in any of these TCR/CD3-associated signalling events; this might be due to its inability to dephosphorylate $p56^{lck}$ effectively.

It was also clear from this result that CD45 had a distinct effect on $p59^{fyn}$. Although no significant difference was observed in the overall phosphorylation state of $p59^{fyn}$ between $CD45^+$ and $CD45^-$ BW5147 T-cells, a consistent difference in kinase activity was observed *in vitro*. Once again, RPTPα was unable to cause any detectable change in either the phosphorylation state or the kinase activity of $p59^{fyn}$, indicating that RPTPα cannot mimic the effect of CD45 on $p59^{fyn}$ in these cells. However, owing to the sensitivity of the assays, it is difficult to exclude the possibility that RPTPα might have had a slight effect on $p56^{lck}$ or $p59^{fyn}$ that might, in turn, have led to the low level of induction of tyrosine-phosphorylated proteins seen in Figure 3(C).

By expressing RPTPα at similar levels to CD45 in BW5147 T-cells, we were able to make a direct comparison of the effects of the two phosphatases. Comparison of the catalytic activities of these two phosphatases immunoprecipitated from BW5147 T-cells indicated that, when tested against two artificial substrates, CD45 was approx. 7–10-fold more active than RPTPα. This difference in catalytic efficiency between CD45 and RPTPα was also observed *in vitro* at physiological pH with the use of recombinant enzymes purified from *E. coli*, indicating that intrinsic factors alone could account for the difference in activity observed in the BW5147 T-cells. However, it is possible that additional factors such as post-translational modifications, cellular locale or cellular interactions might further contribute towards CD45-specific functions in the T-cell.

The ability of CD45, but not RPTPα, to significantly dephosphorylate relatively low concentrations (approx. 0.2 μM) of recombinant GST–$p56^{lck}$ was consistent with the lower activity and lower affinity of RPTPα for $p56^{lck}$ than CD45. Even when 10-fold more RPTPα was used to generate equivalent $V_{max}$ values with phosphopeptide substrates, RPTPα was still unable to dephosphorylate GST–$p56^{lck}$ as efficiently as CD45 (results not shown). These results indicated that, both in T-cells and *in vitro*, $p56^{lck}$ was preferentially dephosphorylated by CD45 and not by RPTPα, suggesting differences in substrate specificity. However, a further comparison of phosphatase activities with phosphopeptide and PNPP substrates indicated that RPTPα was consistently less active than CD45, implying that quantitative differences in phosphatase activity might account for the differential dephosphorylation of $p56^{lck}$. Taken together, these results suggest that the high catalytic efficiency of CD45, coupled with its abundance at the cell surface, contribute towards the specific function of CD45 in T-cells. Recombinant CD45 has also been shown to be catalytically more active than three other recombinant two-domain phosphatases [52], supporting the notion that CD45 is a very efficient phosphatase.

Factors that make one phosphatase more catalytically active than another are currently unknown. Interestingly, RPTPα and CD45 have different pH optima (pH 6.2 for RPTPα and pH 7.2 for CD45) ([44,53,54], and K. W. Harder and F. R. Jirik, unpublished work), suggesting that the environments surrounding their catalytic centres might be different. The activities of the two recombinant phosphatases at pH 7.4 were consistent with the observed activities of the immunoprecipitated phosphatases. Comparison of phosphatase activities at their respective pH optima indicated that CD45 was still 4–5-fold more catalytically active than RPTPα on the substrates tested (results not shown). A pH optimum of 6.2, which is not particularly close to physiological pH, has led other researchers to propose that RPTPα might exist in two conformations, a high-affinity form and a low-affinity form [53]. If this is so, it is possible that RPTPα is present in its catalytically low-affinity form in BW5147 T-cells.

In this study, equivalent levels of RPTPα could not substitute effectively for CD45 in TCR-mediated signalling events. RPTPα, unlike CD45, did not cause the dephosphorylation of $p56^{lck}$ or the activation of $p59^{fyn}$. The fact that RPTPα immunoprecipitated from BW5147 T-cells was found to be one-seventh to one-tenth as active as CD45 provided one explanation for the observed effects. Studies *in vitro* with purified recombinant enzymes also showed a 7–10-fold difference in activity, illustrating that the observed difference in activity between the phosphatases isolated from T-cells was not due to cellular inactivation or post-translational mechanisms. These results demonstrate that CD45 and RPTPα do have distinct activities in T-cells and that this fact can be attributed, at least in part, to quantitative differences in phosphatase activity. This highlights the possibility that different levels of phosphatase expressed in cells could affect the extent of substrate dephosphorylation and cautions the interpretation of studies derived from cell lines expressing non-physiological levels of enzyme.

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