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Differential Association of CD45 Isoforms with CD4 and CD8 Regulates the Actions of Specific Pools of p56^{lck} Tyrosine Kinase in T Cell Antigen Receptor Signal Transduction*

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An investigation into the role of CD45 isoforms in T cell antigen receptor signal transduction was carried out by transfecting CD45-negative CD4$^+$CD8$^+$ HPB-ALL T cells with the CD45R0, CD45RBC, and CD45RABC isoforms. Fluorescence resonance energy transfer analysis showed that the CD45R0 isoform, but not the CD45RBC or CD45RABC isoforms, was found as homodimers and also preferentially associated with CD4 and CD8 at the cell-surface. A comparison was therefore made of T cell antigen receptor signaling between sub-clones expressing either CD45R0 or CD45RBC. Under basal conditions CD4-associated p56^{lck} tyrosine kinase activity and cellular protein tyrosine phosphorylation levels were higher in the CD45R0$^+$ than in the CD45RBC$^+$ sub-clones. Upon CD3-CD4 ligation, TCR-ζ phosphorylation, ZAP-70 recruitment to the p21/p23 TCR-ζ phosphoisomers, ZAP-70 phosphorylation, as well as p56^{lck}, c-Cbl and Slp-76 phosphorylation, were all markedly increased in CD45R0$^+$ compared with CD45RBC$^+$ cells. T cell antigen receptor (TCR) stimulation alone also promoted c-Cbl phosphorylation in CD45R0$^+$ but not in CD45RBC$^+$ cells. Our results are consistent with a model in which association of CD45R0 with CD4 generates a more active pool of CD4-associated p56^{lck} kinase molecules. Upon CD3-CD4 co-ligation, the active p56^{lck} increases the intensity of T cell antigen receptor signal transduction coupling by promoting TCR-ζ chain phosphorylation and ZAP-70 recruitment.

The CD45 tyrosine phosphatase regulates the threshold of T cell antigen receptor (TCR)[1] signaling by modulating the actions of the p56^{lck} and p59^{fyn} tyrosine kinases (1–4). The p56^{lck} kinase associates with the CD4 and CD8 co-receptors and initiates TCR signaling cascades by phosphorylation of immunoreceptor tyrosine-based activation motifs located in the TCR-ζ and CD3-ϵ chains (5, 6). In CD45$^{-/-}$ mice T cell development is severely affected (7, 8), and elevated TCR signaling thresholds are marked by dysfunctional p56^{lck}, together with defective TCR-ζ chain phosphorylation and ZAP-70 recruitment (9). CD45 exerts a positive effect on p56^{lck} actions by de-phosphorylating the inhibitory C-terminal pTyr-505 (10–13), but can also negatively regulate the kinase by dephosphorylating the pTyr-394 autophosphorylation site (14, 15). The actions of CD45 on p56^{lck} in CD4$^+$ T cells are directed selectively at the CD4-associated pool of the kinase (16). The dominant effect of CD45 in the T-lineage appears to be at pTyr-505, since T cell development in CD45$^{-/-}$ mice can be largely restored by backcrossing to mice expressing the mutant lck^{Y505F}-active transgene (17, 18).

Alternative splicing generates up to eight different CD45 isoforms (19, 20) of which five are expressed at significant levels in T cells (21). All T cells express more than one CD45 isoform, and differential isoform expression is tightly controlled during thymic development and the activation of mature T cells (reviewed in Ref.1). However, the molecular consequences of differential CD45 isoform expression for TCR signaling, if any, are not yet well understood. Reconstitution of a murine CD45$^-$ cell line with CD45 isoforms suggested that the CD45R0 isoform (lacking the A, B, and C exon-encoded segments of the ectodomain) promoted greater interleukin-2 secretion upon engagement of the TCR with the cognate major histocompatibility complex-peptide as compared with other isoforms (22). Co-capping experiments in these cells revealed preferential CD4-CD45R0 association (23). However, more detailed capping and co-immunoprecipitation studies indicated a basal association of CD45R0 with the TCR independent of CD4 expression and suggested that co-capping of CD4 with CD45R0 was mediated by this prior CD45R0-TCR association (24). Nevertheless CD4-CD45 association has also been described in primary CD4$^+$ T cells (25, 26). In Jurkat T cells transfected with different isoforms, the CD45R0 isoform was found to promote greater interleukin-2 secretion in comparison with CD45RABC$^+$ cells, but conversely TCR-stimulated protein ty-

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† Senam Doe sadly died from illness since this work was carried out, and this paper is dedicated to her memory.

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[1] The abbreviations used are: TCR, T cell antigen receptor; FRET, fluorescence resonance energy transfer; mAb(s), monoclonal antibodies; FACS, fluorescence-activated cell sorter; PBS, phosphate-buffered saline; PTPase, tyrosine phosphatase; GAPDH, gylceraldehyde 3-phosphate dehydrogenase.

rosine phosphorylation was generally higher in CD45RABC$^+$ than in CD45R0$^+$ cells (27).

Homodimeric forms of CD45 revealed by chemical cross-linking (28), or by the introduction of cysteine residues into the extracellular domain of CD45 adjacent to the transmembrane domain to stabilize homodimers by the formation of disulfide bonds (29), have also been described. It has been suggested that homodimerization of CD45 may lead to inhibition of its tyrosine phosphatase (PTPase) activity (29, 30). Mutation of the putative inhibitory wedge domain in an epidermal growth factor-CD45 chimeric receptor to prevent epidermal growth factor-induced homodimerization relieved the inhibitory effects of epidermal growth factor on TCR-mediated signaling (30). Introduction of the same point mutation in a mouse line resulted in autoimmunity, consistent with the prevention of inhibition of CD45 PTPase activity (29). It has been hypothesized that the CD45R0 isoform might homodimerize substantially, perhaps due to engagement by a putative exogenous ligand, so preferentially being subject to negative regulation by the inhibitory wedge (29). This model suggests that CD45R0 homodimers should exert a negative effect on TCR signaling.

In the present work we have used the well characterized CD45$^-$ HPB-ALL T cell line (31). In the absence of CD45, TCR signaling in this CD4$^+$CD8$^+$ line is largely ablated but can be restored by transfection of CD45 cDNA (31, 32). We have transfected this line with three different CD45 isoforms and used fluorescence energy resonance transfer (FRET), a technique that obviates the need for co-capping or detergent lysis, to show that CD45R0, but not the CD45RBC or CD45RABC isoforms, exists as homodimers and also associates preferentially with CD4 and CD8. Investigation of TCR signaling shows that the higher activity of CD4-associated p56^{lck} in CD45R0$^+$ cells correlates with elevated basal protein phosphorylation and with enhanced TCR signal transduction intensity.

EXPERIMENTAL PROCEDURES

Materials—The following mAbs were used for routine FACS analysis and cell-sorting: UCHT1 (CD3), pan-CD45-FITC (clone F10–89-4), UCHL1-PE (CD45R0), CD45RA-PE (clone F8–11-13), and CD45RC (clone YTH80.103) (Serotec, Oxford, UK); CD4 (clone 13B8.2) from Beckman Coulter (Bucks, UK); CD8 (clone UCT4) from Sigma; and CD45RB-FITC (clone PD7/26) from DAKO (Cambs, UK). The following mAbs were used for FRET analysis: CD3 (MEM57, IgG2a), CD4 (MEM115, IgG2a), and CD8 (MEM31, IgG2a) from Prof. Vaclav Horejsi (Prague, Czech Republic); pan-CD45 (CD45.2, IgG1) from Prof. S. Meuer (Heidelberg, Germany). The following mAbs were used for stimulation experiments: CD3-biotin (clone MCA463B) and CD4-biotin (clone MCA 1267B) (Serotec). Antibodies were cross-linked with streptavidin (Sigma). Immunoprecipitating antibodies used were Fb2 phosphotyrosine mAb (from Prof. D. Cantrell, Imperial Cancer Research Fund, London, UK), CD4 (QS4120) mAb and goat anti-ζ N39 (CT-11) antiserum (from Prof. C. Terhorst, Boston, MA). Western blotting antibodies were used against phosphotyrosine (4G10), Vav and c-Cbl (Upstate Biotechnology), ERK1 (Santa Cruz, Santa Cruz, CA), ERK2 and Syk (Transduction Laboratories), p59^{fyn} (from Dr. R. Abraham, Mayo Clinic, Rochester, MN), phospho-p44/42 MAP kinase (New England Biolabs, Beverly, MA), LAT and p56^{lck} (from Dr L. Samelson, NIH, Bethesda, MD), SLP-76 (from Dr G. Koretzky, University of Pennsylvania Medical School), ZAP-70 (from Glaxo-Smith-Kline), and TCR-ζ chain (Zymed Laboratories Inc.). Horseradish peroxidase-conjugated secondary antibodies were from DAKO (Ely, UK). The vector expressing the Grb-2 GST-fusion protein was from Prof. D. Cantrell. Constructs containing cDNA encoding human CD45R0, CD45RBC, and CD45RABC isoforms in a retroviral vector pZipNeoSV(X) (33) were from Prof. M. Streuli (Harvard University, Boston, MA).

Cells—The CD45$^-$ HPB-ALL sub-clone has been previously described (31). Cells were cultured in RPMI 1640 with added penicillin, streptomycin, and 5% fetal calf serum. Stable transfectants of CD45 isoforms were sorted by flow cytometry to generate sub-clones with comparable CD3, CD4, CD8, and CD45 expression. Cells were routinely analyzed using CD45 isoform-specific mAbs to confirm the specificity of isoform expression. The CD45R0$_{SD}$ and CD45R0$_{LG}$ sub-clones were transfected with human *Lck* cDNA (from Dr. C. Butcher, The Yamanouchi Institute, Oxford, UK) sub-cloned into pcDNA3.1/Zeo (Invitrogen, Leek, Netherlands). For stimulation experiments, HPB-ALL cells were resuspended in RPMI without fetal calf serum and allowed to rest for 20 min at 37 °C before adding biotin-CD3 and biotin-CD4 mAbs (at 1 μg/ml) or biotin-CD3 alone, for 2 min prior to addition of streptavidin (to 20 μg/ml) for the times shown.

Northern Blot Analysis—Northern blot analysis was carried out using 10 μg of total RNA from each HPB-ALL sub-clone, and blots were probed using a ^{32}P-labeled 1.5-kb *Eco*RI-*Not*I DNA fragment from pCR2.1/hLck. Membranes were stripped and reprobed with a ^{32}P-labeled gylceraldehyde 3-phosphate dehydrogenase (GAPDH) probe as a loading control.

Fluorescence Resonance Energy Transfer—F(ab′) fragments were prepared by digestion of purified IgG with activated papain at 37 °C for 11 min. The enzyme activity was terminated by addition of iodoacetamide. The reaction mixture was passed through a Sephadex G-100 fine column and the collected F(ab′) fractions were further separated from intact IgG on a protein A-Sepharose column. Purified mAbs and F(ab′) fragments were conjugated with 6-(fluorescein-5-carboxamido)-hexanoic acid succinimidyl ester and 6-(tetramethylrhodamine-5-carboxamido)-hexanoic acid succinimidyl ester (Molecular Probes, Eugene, OR) or with sulfoindo-cyanin succinimidyl bifunctional ester derivative of Cy-3 and Cy-5 (Amersham Biosciences, Inc.) as described (34). The dye:protein ratios were separately determined for each labeled aliquot by spectrophotometric measurements and in the case of F(ab′) fragments were ~1. The fluorescently tagged antibodies and F(ab′) fragments retained their binding capacity according to competition assays with identical but unlabeled antibodies. Shortly before use, conjugated F(ab′) fragments were centrifuged at 100,000 × *g* for 30 min to remove any complexes that may have formed during storage. Separation of F(ab′) fragments from supernatants on a sizing column revealed single peaks of the expected molecular weight.

For labeling of cell surface antigens, cells were washed twice in PBS and resuspended in PBS with 0.1% bovine serum albumin. About 10^6 cells in 50 μl of PBS were incubated with fluorescently tagged antibodies or F(ab′) fragments at saturating concentration for 30 min on ice in the dark. The applied antibody concentration (50–100 μg/ml) saturated all available binding sites. The excess mAb or F(ab′) fragment was removed by washing the cells twice in PBS. Cells were either used immediately for measurements or were fixed in 1% formaldehyde. Data obtained with fixed cells did not differ significantly from those of unfixed, viable cells.

FRET measurements were carried out using a Becton Dickinson FACScan flow cytometer to determine FRET efficiency between fluorescein- and rhodamine-conjugated mAb. In brief, the donor fluorescence was excited with the 488 nm line of an argon ion laser, and fluorescence emission of the donor was detected at 530 ± 30 nm. Donor fluorescence of the double-labeled samples was compared with that of samples where the acceptor antibody was replaced by non-labeled antibody to compensate for any competition between the donor and acceptor antibodies. FRET efficiency was calculated from the fractional decrease of the donor fluorescence in the presence of the acceptor.

Flow cytometric FRET measurements to determine homoassociation of Cy-3- and Cy-5-labeled antigens were carried out using a Becton Dickinson FACScalibur flow cytometer. Four fluorescence intensities were measured. Three were excited at 488 nm and detected at 530 ± 30 nm, 585 ± 42 nm, and above 670 nm, whereas the fourth was excited at 635 nm and detected at 661 ± 16 nm. Correction factors for the spectral overlap between the different fluorescence channels were obtained from data measured on unlabeled and single-labeled cells. Forward and side angle light scattering were used to gate out debris and dead cells. We calculated the energy transfer efficiency on a cell-by-cell basis from the four fluorescence intensities on 10,000 cells using list mode data. Correction of autofluorescence was carried out on a cell-by-cell basis to eliminate the effect of low signal to noise ratios.

Calculated values for the energy transfer efficiency were expressed as the ratio of the number of excited donor molecules tunneling their excitation energy to the acceptor, to the number of all excited molecules. The transfer efficiency is related to the inverse sixth power of the separation between the donor and the acceptor when the orientation factor of the donor's excitation and the acceptor's emission dipoles can be considered as a statistical average of random orientation (35). The calculated energy transfer efficiency has an extremely high sensitivity to changes in distance within the range 1–10 nm. Larger energy transfer efficiencies may reflect closer physical association of the donor- and

acceptor-labeled antibodies or a larger density of acceptor-labeled antibodies in the vicinity of donor-labeled antibodies (36, 37).

Immunoprecipitation and Immunoblotting—Stimulated cells were lysed for 10 min at 4 °C in 1% Nonidet P-40, 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EGTA, 1 mM sodium orthovanadate, and protease inhibitors (protease inhibitor mixture tablets, Roche Molecular Biochemicals, Sussex, UK). Nuclei and cellular debris were removed by centrifugation for 10 min at 10,000 rpm at 4 °C. For analysis of whole cell lysates by immunoblotting, samples (equivalent to 10^6 cells) were added to Laemmli's buffer and boiled for 5 min. For TCR-ζ immunoprecipitation the lysates were pre-cleared by rotating at 4 °C for 30–60 min with 40 μl of 50% packed protein G-Sepharose beads (Pharmacia Biotech). Pre-cleared lysates were incubated at 4 °C for 60 min or overnight with immunoprecipitating antibody. Immune complexes were recovered by incubation with 40 μl of 50% packed protein G-Sepharose beads for 1 h at 4 °C. Pellets were washed three times with lysis buffer, resuspended in Laemmli's buffer, and boiled for 5 min prior to separation using 7.5–15% gradient gels. For CD4 immunoprecipitates, cells were coated with CD4 mAb (QS4120) at 5 μg/ml for 30 min on ice prior to immunoprecipitation using protein G-Sepharose beads as above. For *in vitro* kinase assay, immune complexes were washed once in kinase buffer (50 mM Pipes, pH 7.4, 10 mM $MnCl_2$, 10 mM $MgCl_2$, 1 mM sodium orthovanadate, and 1 mM AEBSF). The kinase reaction was initiated by the addition of 30 μl of kinase buffer containing 1 mM dithiothreitol, 5 μM unlabeled ATP, and 1 μCi of [γ-^{32}P]ATP (PerkinElmer Life Sciences). Samples were incubated for 10 min at room temperature, and the reaction stopped by the addition of 30 μl of Laemmli's buffer and boiling for 5 min. Quantification of immunoblots and autoradiographs was carried out using a LAS-1000 CCD camera and AIDA imaging software (Fuji Photo Film Company).

RESULTS

A CD45$^-$ sub-clone of HPB-ALL T leukemia cells (31) was used to generate stable transfectants expressing specific CD45 isoforms. Two CD45R0$^+$ sub-clones were obtained from the same CD45$^-$ parental cell-line, generated in two different laboratories (CD45R0$_{SD}$ and CD45R0$_{LG}$). Two further sub-clones were made expressing either the CD45RABC or CD45RBC isoforms. Initial experiments (data not shown) revealed that upon TCR stimulation or CD3-CD4 ligation, protein tyrosine phosphorylation and other signals were greatly reduced in both of the CD45R0$^+$ sub-clones in comparison with cells expressing either CD45RABC or CD45RBC. This result was surprising in light of previously published findings suggesting that CD45R0 may promote TCR signaling (22). However, as Fig. 1 illustrates, whereas the expression of the important signaling molecules Syk, ZAP-70, and p59^{fyn} were found to be comparable between the HPB-ALL sub-clones (Fig. 1*a*), both CD45R0$^+$ sub-clones expressed very low levels of p56^{lck}, up to 20-fold lower than the expression level in the CD45RBC$^+$ sub-clone (Fig. 1*b, upper panel*). Furthermore, p56^{lck} was expressed 2-fold higher in the CD45RBC$^+$ than in the CD45RABC$^+$ sub-clone. Northern analysis revealed that Lck mRNA levels closely matched the protein expression differences (Fig. 1*b, lower panel*). The parental CD45$^-$ sub-clone also had low p56^{lck} expression, although not as low as the two CD45R0$^+$ sub-clones. Since the CD45R0$^+$ sub-clones were independently generated, we cannot exclude the possibility that differential CD45 isoform expression regulates Lck mRNA levels, but the low p56^{lck} expression in the CD45R0$^+$ sub-clones might also be coincidental.

Since p56^{lck} is critical as the kinase that promotes TCR-ζ phosphorylation, thereby initiating downstream signaling cascades, the two CD45R0$^+$ sub-clones were stably transfected with p56^{lck} to generate a large number of clones expressing varying p56^{lck} levels. Sub-clones were picked to match the CD45RBC$^+$ and CD45RABC$^+$ sub-clones for p56^{lck} expression, as well as for CD3, CD45, CD4, and CD8 expression (Fig. 2). We then measured the differential association of CD45 isoforms with CD4 and CD8 at the cell surface by FRET analysis. This technique has the advantage that it detects associations by

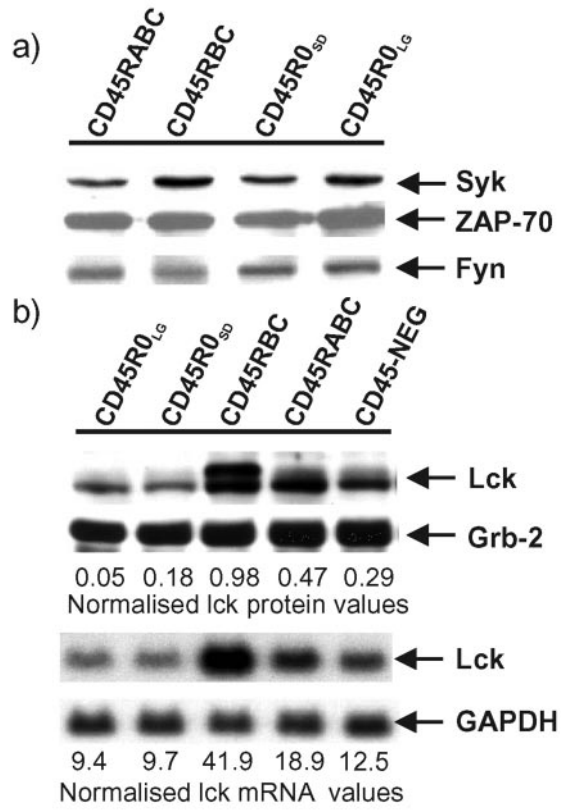

FIG. 1. **Expression of signaling molecules in HPB-ALL sub-clones.** *a,* immunoblotting of whole cell lysates for Syk, ZAP-70, and p59^{fyn}. *b,* immunoblotting of whole cell lysates for p56^{lck} and Grb-2 protein (*upper panel*) and Northern blotting for p56^{lck} and GAPDH mRNA (*lower panel*). The values shown are the relative expression levels of p56^{lck} protein normalized for Grb-2 (*upper panel*) and of p56^{lck} mRNA normalized for GAPDH (*lower panel*).

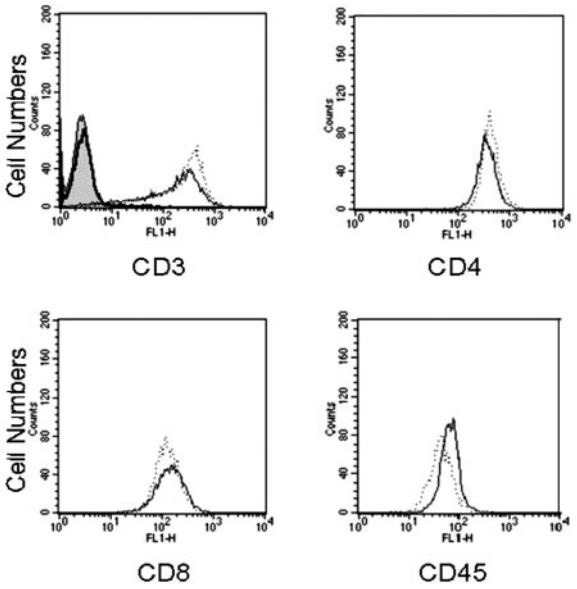

FIG. 2. **Antigen cell-surface expression in CD45R0$^+$ and CD45RBC$^+$ HPB-ALL sub-clones.** CD45R0$^+$ (*continuous line*) or CD45RBC$^+$ (*dotted line*) HPB-ALL cells were stained for CD3, CD4, CD8, and CD45 as shown and analyzed by FACS. The *filled area* in the CD3 histogram shows the staining of isotype-matched control mAbs.

FACS measurements using intact cells and avoids any requirement to expose the CD45 molecules to detergent. Measurements were carried out using F(ab)′ fragments bound to fluorophores rather than intact IgG molecules to avoid any cross-

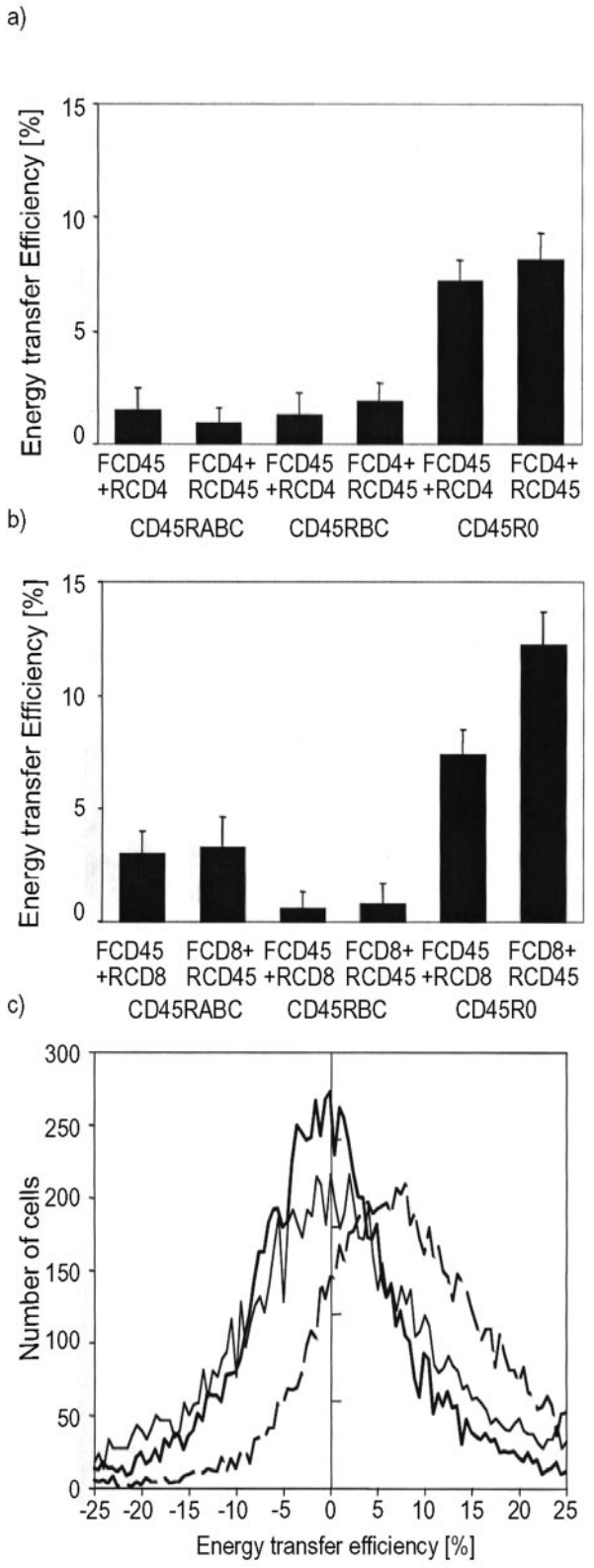

FIG. 3. **FRET analysis of CD45 isoform associations.** HPB-ALL sub-clones expressing CD45RABC, CD45RBC, or CD45R0 were labeled with F(ab′) fragments covalently bound to fluorescein (*F*) or rhodamine (*R*) used as either donor or acceptor. Associations were estimated by FACS analysis as described under "Experimental Procedures". *a* and *b* show the mean energy transfer efficiency values ± S.D. calculated from six independent experiments for CD4 and CD8 associations, respectively. *c,* homodimerization analysis of CD45R0 (*dashed line*), CD45RABC (*thick solid line*), and CD45RBC (*thin solid line*) by FACS analysis of cells labeled with 50:50 mixtures of Cy-3/Cy-5-conjugated F(ab′) fragments. The data show are representative of four independent experiments.

linking of the molecules under investigation. Either fluorescein or rhodamine were used in each experiment as fluorescence donor or acceptor molecules, thereby generating two energy transfer efficiency values for each antibody pair. Energy transfer efficiencies below 5% were ignored as "background noise". Fig. 3, *a* and *b* show that there was substantial association between CD45R0 and CD4 and CD8, respectively, but not between CD45RBC or CD45RABC with CD4 or CD8. The energy transfer values for CD45R0-CD4/CD8 association were found to be in the range 7.2–12.2%, demonstrating that a pool of CD45R0 molecules are within 10 nm at the cell-surface of a pool of CD4 or CD8 molecules. However, it should be noted that these values do not provide information about the percentage of molecules that associate. To confirm that these results were not unique to a particular HPB-ALL sub-clone, the FRET analysis for CD4-CD45R0 association was repeated on a total of five, and the CD8-CD45R0 association repeated on three, independent CD45R0$^+$ sub-clones, with comparable results (data not shown).

In light of recent reports that CD45 homodimerization may regulate CD45 function, it was also of interest to determine whether the various CD45 isomers homodimerized. To investigate this question, FRET analysis using the FACS was carried out on the different sub-clones labeled with a 50:50 mixture of Cy3- and Cy5-CD45 F(ab)′ fragments. Fig. 3*c* shows that of the three CD45 isoforms under investigation, only CD45R0 was found as homodimers, generating energy transfer efficiency values of 11.0 ± 4.4 based on four independent experiments, in contrast to 1.2 ± 0.4 and 1.8 ± 1.4 for the CD45RBC$^+$ and CD45RABC$^+$ sub-clones, respectively.

The homodimerization of CD45R0, together with the preferential association of CD45R0 with CD4 and CD8 expressed on the same cells, provided an interesting system for testing whether CD45R0 might exert a dominantly positive effect on signaling mediated by the TCR-CD4/CD8 complex, or whether the possible inhibition of CD45 tyrosine phosphatase activity by CD45R0 homodimerization might exert a negative effect on TCR signaling. To distinguish these possibilities we carried out a detailed investigation on the strength of TCR signal transduction in the CD45RBC$^+$ sub-clone compared with CD45R0$_{SD7}$, a sub-clone matched for p56^{lck}, CD3, CD45, CD4, and CD8 expression (Fig. 2). We surmised that the close association between CD45R0 and CD4 molecules (Fig. 3*a*) might generate a pool of CD4-associated p56^{lck} that was more active in the CD45R0$^+$ than in the CD45RBC$^+$ sub-clone, due to dephosphorylation of the inhibitory p56^{lck} pTyr-505 by CD45. To investigate this possibility, *in vitro* kinase assays were carried out in CD4 immunocomplexes derived from cell-surface CD4 molecules, and p56^{lck} activity was assessed by autophosphorylation. Fig. 4*a* illustrates two representative experiments demonstrating that the CD4-associated p56^{lck} kinase activity was, on average, 2-fold higher in the CD45R0$^+$ than in the CD45RBC$^+$ sub-clone. Consistent with this finding, basal protein tyrosine phosphorylation levels were consistently higher in the CD45R0$^+$ than in the CD45RBC$^+$ cells (Fig. 4*b*). Among the hyper-phosphorylated proteins were the proto-oncogene c-Cbl (38), p56^{lck}, and the p21 TCR-ζ phosphoisomer (*arrowed* in Fig. 4*b*; identification of the molecules is illustrated in Fig. 5). The increased basal level of p21 TCR-ζ phosphorylation is consistent with the established role of p56^{lck} in phosphorylating TCR-ζ (5, 6). The cell-surface expression of CD45R0$^+$ was routinely slightly higher than CD45RBC$^+$ as revealed by FACS staining using a pan-CD45 mAb (Fig. 2). When the CD45R0$^+$ cells were sorted to select a population with exactly the same CD45 expression as the CD45RBC$^+$ sub-clone, after a few days in culture the CD45R0$^+$ expression became again slightly

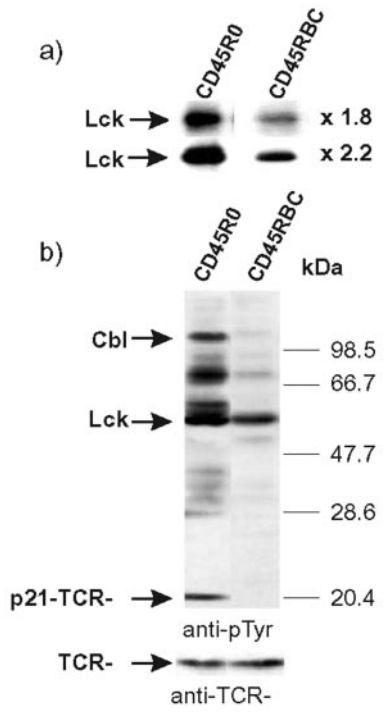

FIG. 4. **CD4-associated p56^{lck} kinase activity and protein phosphorylation under basal conditions.** *a,* p56^{lck} kinase autophosphorylation assays were carried out in CD4 immunocomplexes. The results illustrate two representative experiments, and the values shown are the ratios of p56^{lck} kinase values between CD45R0$^+$ and CD45RBC$^+$ HPB-ALL sub-clones. *b,* whole cell lysates were immunoblotted for phosphotyrosine (*upper panel*) and for the 16-kDa TCR-ζ chain as a loading control (*lower panel*).

higher than the CD45RBC$^+$ expression. To confirm that the observed phosphorylation differences observed were not due to subtle differences in CD45 expression, we therefore FACS-sorted both the CD45R0$^+$ and CD45RBC$^+$ sub-clones on the same day to generate two populations with identical CD45 expression and then immediately repeated the phosphorylation assays. Comparable differences were observed between the two sub-clones as illustrated in Fig. 4*b* (data not shown). We therefore conclude that the small difference in expression of the two isoforms was not the cause of the increased basal protein phosphorylation in the CD45R0$^+$ compared with CD45RBC$^+$ sub-clone.

Signaling assays were carried out to demonstrate that the greater activity of the CD4-associated p56^{lck} pool from CD45R0$^+$ cells as measured *in vitro* was functionally more effective in increasing the intensity of TCR signal transduction coupling *in vivo*. We have previously shown that CD3-CD4 co-ligation greatly amplifies protein tyrosine phosphorylation events in HPB-ALL cells in comparison with CD3-CD3 ligation (31), presumably by enforced juxtaposition of CD4-p56^{lck} with its TCR polypeptide and ZAP-70 substrates. Immunoprecipitation of the TCR-ζ chain followed by immunoblotting for phosphotyrosine showed that upon CD3-CD4 co-ligation, generation of the TCR-ζ p21 and p23 phosphoisomers was amplified in the CD45R0$^+$ compared with the CD45RBC$^+$ sub-clone (Fig. 5*a*). Recruitment of ZAP-70 was also increased, as was tyrosine phosphorylation of the kinase (Fig. 5*a*, *upper panels*). Consistent with these results, CD3-CD4 co-ligation also increased the tyrosine phosphorylation levels of multiple proteins to a greater extent in CD45R0$^+$ compared with CD45RBC$^+$ cells, as shown by immunoblotting whole cell lysates for phosphotyrosine (Fig. 5*b*). This assay was also repeated for cells freshly sorted for identical CD45 expression, as described above, with comparable results (data not shown). Furthermore, amplified CD3-CD4-induced protein tyrosine phosphorylation was also observed in two other independent CD45R0$^+$ sub-clones matched for cell-surface expression of CD3, CD4, CD8, and CD45 (data not shown). A major phosphorylated protein that migrated in the same position as p56^{lck}, as shown by immunoblotting (Fig. 5*b*, *middle panel*), displayed both higher basal and inducible tyrosine phosphorylation in CD45R0$^+$ than in CD45RBC$^+$ cells. Comparison of p56^{lck} protein levels in the two sub-clones, normalized by reference to Vav used as a loading control (Fig. 5*b*, *lower panel*), confirmed that p56^{lck} expression levels were comparable. In contrast to CD3-CD4 co-ligation, ligation of CD3 alone induced very little increase in protein tyrosine phosphorylation in either the CD45R0$^+$ or CD45RBC$^+$ sub-clones (data not shown). However, as Fig. 5*c* illustrates, an exception was the ability of CD3-stimulation to increase the phosphorylation of a 120-kDa protein identified as c-Cbl (Fig. 5*d*). Whereas the increased Cbl phosphorylation was readily detectable in CD45R0$^+$ cells, neither basal nor inducible phosphorylation of Cbl occurred in CD45RBC$^+$ cells (Fig. 5*c*).

Identification of the major proteins tyrosine phosphorylated upon CD3-CD4 co-ligation was carried out by immunoblotting phosphotyrosine Fb2 mAb immunocomplexes and Grb-2 fusion protein precipitates using specific antibodies. Fig. 5, *d* and *e* identify the major 120-kDa protein illustrated in Fig. 5, *b* and *c* as c-Cbl and confirms that Cbl phosphorylation was considerably higher in the CD45R0$^+$ compared with the CD45RBC$^+$ sub-clone. Likewise ZAP-70 and p56^{lck} proteins were both identified and confirmed using this approach as displaying increased phosphorylation in the CD45R0$^+$ cells (Fig. 5*d*). Furthermore, when the important signaling molecule Slp-76 was precipitated using the Grb-2 fusion protein, inducible phosphorylation of the protein was greater in CD45R0$^+$ but not in CD45RBC$^+$ cells (Fig. 5*e*). An exception to this general pattern was LAT, which had no detectable tyrosine phosphorylation under basal conditions in either sub-clone, but showed comparable phosphorylation levels following CD3-CD4 co-ligation in both sub-clones (Fig. 5*d*). Further investigation, however, showed that this anomaly was readily explained by the much reduced expression of LAT protein in the CD45R0$^+$ compared with the CD45RBC$^+$ sub-clone (Fig. 5*d*, *lowest panel*), whereas Cbl (Fig. 5*d*), p56^{lck} (Fig. 5*b*), and ZAP-70 (Fig. 1 and data not shown) expression levels were all comparable.

Overall these results show that the intensity of TCR signal transduction coupling was much greater when CD45R0$^+$ rather than CD45RBC$^+$ was expressed at the cell-surface.

DISCUSSION

The most important finding presented in this paper is that CD45R0, but not the CD45RBC or CD45RABC isoforms, forms heterodimers with CD4 and CD8 at the cell-surface of HPB-ALL T cells and also exists as CD45R0 homodimers (Fig. 3), associations that correlate with elevated basal kinase activity of CD4-associated p56^{lck} (Fig. 4) and increased intensity of TCR signaling phosphorylation events (Fig. 5). These findings represent the first report that CD45R0 preferentially forms homodimers and the first time that CD4/CD8-CD45R0 associations have been described using non-invasive FRET technology, obviating the need for antibody-induced co-capping or for exposing cells to detergent. A concern in co-capping studies is that the molecular composition of the cap, normally examined after prolonged periods (10–30 min) of antigen cross-linking (23, 24), might not reflect basal-state associations between the same molecules. Furthermore, a very large range of cell-surface molecules have been reported to associate with CD45 following co-immunoprecipitation from detergent cell lysates (reviewed in Ref. 1); CD45 is an abundant and heavily

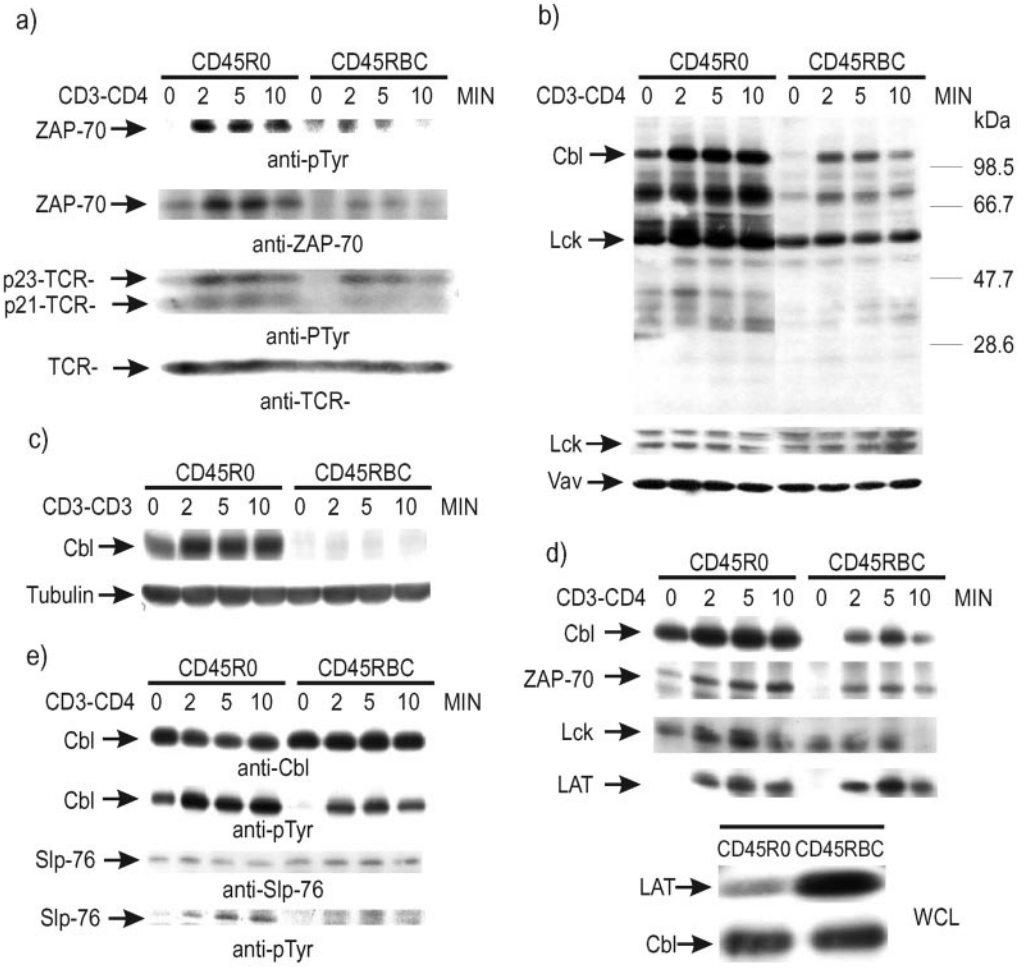

FIG. 5. **CD3-CD4 and CD3-CD3 stimulated phosphorylation events in CD45R0$^+$ compared with CD45RBC$^+$ HPB-ALL sub-clones.** *a,* cells were stimulated by CD3-CD4 co-ligation for the times shown before immunoprecipitating TCR-ζ and blotting for phosphotyrosine; the blot was then stripped and re-probed for ZAP-70 and TCR-ζ. *b,* cells were stimulated by CD3-CD4 co-ligation before immunoblotting whole cell lysates for phosphotyrosine (*upper panel*), p56^{lck} (*middle panel*), or for Vav (*lower panel*) as a loading control. *c,* cells were stimulated using a cross-linked CD3 mAb for the times shown, and whole cell lysates immunoblotted for phosphotyrosine (*upper panel*) and for tubulin (*lower panel*) as a loading control. The phosphorylated band was identified as c-Cbl as shown in *d* and *e*. *d,* cells were stimulated by CD3-CD4 co-ligation, and tyrosine phosphorylated proteins were then immunoprecipitated using the Fb2 phosphotyrosine mAb followed by immunoblotting for c-Cbl, ZAP-70, p56^{lck}, and LAT as indicated (*upper panel*). The lower panel shows immunoblotting of whole cell lysates for LAT using c-Cbl as a loading control. *e,* cells were stimulated by CD3-CD4 co-ligation followed by precipitation of proteins bound to a Grb-2 fusion protein. Precipitates were blotted for phosphotyrosine, and the blot was then stripped and re-probed for c-Cbl and Slp-76.

glycosylated molecule, and the possibility that nonspecific associations may occur during the immunoprecipitation process becomes correspondingly high. Nevertheless, our findings based on FRET are consistent with the previously described co-capping of CD4 with CD45R0 (23) and the co-immunoprecipitation of CD4 and CD45 from detergent lysates of primary resting CD4$^+$ T cells (26), although not with the reported preferential association between CD45R0 and the TCR (24). Interestingly, an earlier study also using FRET analysis reported that association of CD45 with CD4 and CD8 only occurred following 72–96 h of TCR stimulation (25). Although specific CD45 isoforms were not characterized in this investigation, it is known that CD45R0 expression markedly increases on activated T cells (39), and it therefore seems very likely that the observed CD4/CD8-CD45 associations detected involved the CD45R0 isoform. A further possibly relevant observation is that whereas CD4 was found clustered with TCR-ζ at the central initiating locus of the immune synapse (the "c-SMAC"), within a few minutes the CD4 moved to a more peripheral location (40). It will be interesting to determine whether the relatively small CD45R0 isoform may be localized in the c-SMAC with CD4 prior to re-localization into a less central position. It is possible that the reported exclusion of CD45 from the immune synapse (41) might selectively apply to CD45 isoforms encoding A/B/C exon products rather than to CD45R0.

The mechanism of CD45R0 association with CD4 and CD8 remains unknown. The association could be mediated by an intermediary molecule such as the transmembrane leukocyte phosphatase-associated molecule (LPAP) (42), but it is not apparent how leukocyte phosphatase-associated molecule might mediate the selectivity of the interactions and LPAP$^{-/-}$ mice do not display an obvious phenotype (43). More likely are direct interactions between binding motifs in the respective polypeptide back-bones and/or oligosaccharide interactions. The CD45 A, B, and C exon products contribute much of the heavy glycosylation that characterizes CD45, and the absence of this region causes a large reduction in the size of the CD45 ectodomain (44). It is possible that CD45-CD4/CD8 binding represents the "default" association state and that this is prevented by the A-C exon products. Similarly CD45R0 homoassociation might represent the default position of CD45 molecules that is also prevented, for example, by the heavy sialylation associated with the A-C exon region. We are cur-

rently investigating the mechanisms involved in the associations revealed by FRET analysis.

The finding that CD45R0 homodimers, as well as CD4-CD45R0 and CD8-CD45R0 heterodimers, were present at the cell-surface of HPB-ALL cells (Fig. 3) raises the question as to whether CD4 and/or CD8 are in competition with CD45R0 to decrease the extent of CD45R0 homodimerization. The expression of both CD4 and CD8 is high in these cells (Fig. 2), but whether homodimerization might increase as CD4/CD8 expression levels are decreased is not yet known. It also remains formally possible that the CD45R0 that associates with CD4 and/or CD8 is homodimeric, a possibility currently under investigation. Our findings do not exclude the possibility that CD45R0 homodimerization inhibits the CD45 PTPase activity (29), but the dominant effect of CD45R0 in the present experimental context was to associate with CD4/CD8 (Fig. 3) and to amplify TCR signal transduction coupling by generating a more active pool of CD4-associated $p56^{lck}$ kinase molecules (Fig. 4*a*). Nevertheless it remains possible that the increased TCR signaling intensity observed may be explained by secondary changes in other "third party" signaling molecules regulated by differential CD45 isoform expression. The downstream consequences of increased intensity of TCR signal transduction coupling are expected to reflect the balance between concomitant amplification of both positive and negative signals. For example, c-Cbl, a major tyrosine kinase substrate in HPB-ALL cells (Fig. 5), is well established as a negative regulator of immune receptor signal transduction (38), and its coupling to downstream effectors appears to be tyrosine phosphorylation-dependent (45, 46). A comparison of downstream signaling pathways in HPB-ALL cells expressing different isoforms will be described in a separate paper.

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REFERENCES

1. Alexander, D. R. (1997) in *Lymphocyte Signalling: Mechanisms, Subversion, and Manipulation* (Harnett, M. M., and Rigley, K. P., eds) pp 107–140, John Wiley & Sons, New York
2. Thomas, M. L., and Brown, E. J. (1999) *Immunol. Today* **20,** 406–411
3. Ashwell, J. D., and Doro, U. (1999) *Immunol. Today* **20,** 412–416
4. Alexander, D. R. (2000) *Sem. Immunol.* **12,** 349–359
5. Chan, A. C., Desai, D. M., and Weiss, A. (1994) *Annu. Rev. Immunol.* **12,** 555–592
6. Van Oers, N. S. C., Killeen, N., and Weiss, A. (1996) *J. Exp. Med.* **183,** 1053–1062
7. Kishihara, K., Penninger, J., Wallace, V. A., Kundig, T. M., Kawai, K., Wakeham, A., Timms, E., Pfeffer, K., Ohashi, P. S., Thomas, M. L., Furlonger, C., Paige, C. J., and Mak, T. W. (1993) *Cell* **74,** 143–156
8. Byth, K. F., Conroy, L. A., Howlett, S., Smith, A. J. H., May, J., Alexander, D. R., and Holmes, N. (1996) *J. Exp. Med.* **183,** 1707–1718
9. Stone, J. D., Conroy, L. A., Byth, K. F., Hederer, R. A., Howlett, S., Takemoto, Y., Holmes, N., and Alexander, D. R. (1997) *J. Immunol.* **158,** 5773–5782
10. Ostergaard, H. L., Shackelford, D. A., Hurley, T. R., Johnson, P., Hyman, R., Sefton, B. M., and Trowbridge, I. S. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 8959–8963
11. McFarland, E. D. C., Hurley, T. R., Pingel, J. T., Sefton, B. M., Shaw, A., and Thomas, M. L. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 1402–1406
12. Hurley, T. R., Hyman, R., and Sefton, B. M. (1993) *Mol. Cell. Biol.* **13,** 1651–1656
13. Sieh, M., Bolen, J. B., and Weiss, A. (1993) *EMBO J.* **12,** 315–321
14. Doro, U., and Ashwell, J. D. (1999) *J. Immunol.* **162,** 1879–1883
15. Baker, M., Gamble, J., Tooze, R., Higgins, D., Yang, F. T., O'Brien, P. C. M., Coleman, N., Pingel, S., Turner, M., and Alexander, D. R. (2000) *EMBO J.* **19,** 4644–4654
16. Biffen, M., McMichael-Phillips, D., Larson, T., Venkitaraman, A., and Alexander, D. R. (1994) *EMBO J.* **13,** 1920–1929
17. Pingel, S., Baker, M., Turner, M., Holmes, N., and Alexander, D. R. (1999) *Eur. J. Immunol.* **29,** 2376–2384
18. Seavitt, J. R., White, L. S., Murphy, K. M., Loh, D. Y., Perlmutter, R. M., and Thomas, M. L. (1999) *Mol. Cell. Biol.* **19,** 4200–4208
19. Ralph, S. J., Thomas, M. L., Morton, C. C., and Trowbridge, I. S. (1987) *EMBO J.* **6,** 1251–1257
20. Streuli, M., Hall, L. R., Saga, Y., Schlossman, S. F., and Saito, H. (1987) *J. Exp. Med.* **166,** 1548–1566
21. Rogers, P. R., Pilapil, S., Hayakawa, K., Romain, P. L., and Parker, D. C. (1992) *J. Immunol.* **148,** 4054–4065
22. Novak, T. J., Farber, D., Leitenberg, D., Hong, S. C., Johnson, P., and Bottomly, K. (1994) *Immunity* **1,** 109–119
23. Leitenberg, D., Novak, T. J., Farber, D., Smith, B. R., and Bottomly, K. (1996) *J. Exp. Med.* **183,** 249–259
24. Leitenberg, D., Boutin, Y., Lu, D. D., and Bottomly, K. (1999) *Immunity* **10,** 701–711
25. Mittler, R. S., Rankin, B. M., and Kiener, P. A. (1991) *J. Immunol.* **147,** 3434–3440
26. Bonnard, M., Maroun, C. R., and Julius, M. (1997) *Cell. Immunol.* **175,** 1–11
27. McKenney, D. W., Onodera, H., Gorman, L., Mimura, T., and Rothstein, D. M. (1995) *J. Biol. Chem.* **270,** 24949–24954
28. Takeda, A., Wu, J. J., and Maizel, A. L. (1992) *J. Biol. Chem.* **267,** 16651–16659
29. Majeti, R., Xu, Z., Parslow, T. G., Olson, J. L., Daikh, D. I., Killeen, N., and Weiss, A. (2000) *Cell* **103,** 1059–1070
30. Majeti, R., Bilwes, A. M., Noel, J. P., Hunter, T., and Weiss, A. (1998) *Science* **279,** 88–91
31. Shiroo, M., Goff, L., Biffen, M., Shivnan, E., and Alexander, D. (1992) *EMBO J.* **11,** 4887–4897
32. Koretzky, G. A., Picus, J., Thomas, M. L., and Weiss, A. (1990) *Nature* **346,** 66–68
33. Streuli, M., Morimoto, C., Schrieber, M., Schlossman, S. F., and Saito, H. (1988) *J. Immunol.* **141,** 3910–3914
34. Hederer, R. A., Guntermann, C., Miller, N., Nagy, P., Szollosi, J., Damjanovich, S., Hale, G., and Alexander, D. R. (2000) *Int. Immunol.* **12,** 505–516
35. Damjanovich, S., Gaspar, R., and Pieri, C. (1997) *Quart. Rev. Biophys.* **30,** 67–106
36. Szollosi, J., Tron, L., Damjanovich, S., Helliwell, S. H., Arndtjovin, D., and Jovin, T. M. (1984) *Cytometry* **5,** 210–216
37. Tron, L., Szollosi, J., Damjanovich, S., Helliwell, S. H., Arndtjovin, D. J., and Jovin, T. M. (1984) *Biophys. J.* **45,** 939–946
38. Lupher, M. L., Rao, N., Eck, M. J., and Band, H. (1999) *Immunol. Today* **20,** 375–382
39. Akbar, A. N., Terry, L., Timms, A., Beverley, P. C. L., and Janossy, G. (1988) *J. Immunol.* **140,** 2171–2178
40. Krummel, M. F., Sjaastad, M. D., Wulfing, C., and Davis, M. M. (2000) *Science* **289,** 1349–1352
41. Bromley, S. K., Burack, W. R., Johnson, K. G., Somersalo, K., Sims, T. N., Sumen, C., Davis, M. M., Shaw, A. S., Allen, P. M., and Dustin, M. L. (2001) *Annu. Rev. Immunol.* **19,** 375–396
42. Schraven, B., Schoenhaut, D., Bruyns, E., Koretzky, G., Eckerskorn, C., Wallich, R., Kirchgessner, H., Sakorafas, P., Labkovsky, B., Ratnofsky, S., and Meuer, S. (1994) *J. Biol. Chem.* **269,** 29102–29111
43. Ding, I., Bruyns, E., Li, P., Magada, D., Paskind, M., Rodman, L., Seshadri, T., Alexander, D., Giese, T., and Schraven, B. (1999) *Eur. J. Immunol.* **29,** 3956–3961
44. McCall, M. N., Shotton, D. M., and Barclay, A. N. (1992) *Immunol.* **76,** 310–317
45. Yokouchi, M., Kondo, T., Sanjay, A., Houghton, A., Yoshimura, A., Komiya, S., Zhang, H., and Baron, R. (2001) *J. Biol. Chem.* **11,** 11
46. Kirsch, K. H., Georgescu, M. M., Shishido, T., Langdon, W. Y., Birge, R. B., and Hanafusa, H. (2001) *J. Biol. Chem.* **276,** 4957–4963