The CD45 Tyrosine Phosphatase Regulates Phosphotyrosine Homeostasis and Its Loss Reveals a Novel Pattern of Late T Cell Receptor-induced Ca²⁺ Oscillations

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Summary

CD45 is a transmembrane tyrosine phosphatase implicated in T cell antigen receptor (TCR)-mediated activation. In T cell variants expressing progressively lower levels of CD45 (from normal to undetectable), CD45 expression was inversely related to spontaneous tyrosine phosphorylation of multiple proteins, including the TCR β chain, and was directly correlated with TCR-driven phosphoinositide hydrolysis. The Ca²⁺ response in these cells was altered in an unexpected fashion. Unlike wild-type cells, stimulated CD45⁻ cell populations did not manifest an early increase in intracellular Ca²⁺, but did exhibit a delayed and gradual increase in mean intracellular Ca²⁺.Computer-aided fluorescence imaging of individual cells revealed that CD45⁻ cells experienced late Ca²⁺ oscillations that were not blocked by removal of extracellular Ca²⁺. CD45 revertants had the signaling properties of wild-type cells. Thus, CD45 has a profound influence on both TCR-mediated signaling and phosphotyrosine homeostasis, and its loss reveals a novel role for this tyrosine phosphatase in Ca²⁺ regulation.

Stimulation of T cells via the TCR invokes both early and late activation events. Classical early events include phosphoinositide (PI)¹ hydrolysis, increases in intracellular calcium ([Ca²⁺]), activation of protein kinase C, and induction of protein tyrosine kinase (PTK) activity. The complex relationships among these events have recently been demonstrated in a number of ways. PTK activity is detectable before either PI hydrolysis or increases in [Ca²⁺] (1), raising the possibility that tyrosine phosphorylation of intracellular substrates may be the primary signaling event initiated via the TCR. Furthermore, two drugs that antagonize PTK activity also inhibit activation-induced increases in inositol phosphates and [Ca²⁺] (2, 3). More support for a direct role of PTKs in T cell activation comes from studies in which v-src was introduced into T cell hybridomas (4). Cells expressing the active pp60v-src PTK spontaneously produced IL-2, even after depletion of protein kinase C. Moreover, their [Ca²⁺] was constitutively higher than in wild-type cells, and increased to much higher levels upon TCR crosslinking (5). Together, these data suggest that tyrosine phosphorylation has a fundamental role in regulating T cell [Ca²⁺] and cellular activation.

The extent to which intracellular substrates are phosphorylated on tyrosine residues is also determined by tyrosine phosphatases. By removing phosphate groups from tyrosines, phosphatases can specifically counter the actions of PTKs. Furthermore, tyrosine phosphatases can have the opposite effect, actually enhancing PTK activity by removing phosphates from negative regulatory tyrosines (6-10). The best characterized lymphocyte tyrosine phosphatase is CD45 (also known as leukocyte common antigen, T200, B220, and Ly-5). This molecule is present on most hematopoietic cells and exists as multiple isoforms. The isoforms have primary amino acid sequence differences (due to alternate splicing of at least three 5' exons) and different N- and O-linked glycosylation patterns (11). In contrast to the extracellular portion, the intracellular piece of CD45 is invariant. It consists of two homologous domains that are similar in sequence to a placental

¹ Abbreviations used in this paper: GAPDH, glyceraldehyde phosphate dehydrogenase; PI, phosphoinositide; PTK, protein tyrosine kinase.
tyrosine phosphatase, and CD45 has intrinsic tyrosine phosphatase activity (12). Recent work has shown that CD45-deficient T cells are aberrant in their ability to proliferate or, in the case of cytotoxic T cells, to kill target cells when stimulated with antigen (13, 14). Furthermore, decreases have been noted in TCR-mediated PI hydrolysis, Ca\(^{2+}\) flux, and PTK activation (15, 16).

In the present report we examine a series of murine T cell CD45 loss variants. The analyses indicate a critical role for CD45 in normal tyrosine phosphorylation homeostasis. Furthermore, these variants reveal the existence of both CD45-deficient and CD45-independent mechanisms of mobilizing intracellular Ca\(^{2+}\). The latter appears to be a novel pathway that predominantly involves late oscillations of Ca\(^{2+}\) derived from intracellular stores.

Materials and Methods

Cells The YAC-1 wild-type T lymphoma cell (designated WT) was derived from an A/Sn mouse that had been inoculated with Moloney leukemia virus (17), and was obtained from the American Type Culture Collection (Rockville, MD). To generate CD45-deficient variants, the YAC-1 bulk population was treated with ethyl methanesulfonate (500 µg/ml) overnight. The cells were washed and cultured in medium alone for 5 d. After this time the cells were stained with the anti-CD45 antibody M1/9.3 (Boehringer Mannheim, Indianapolis, IN) plus FITC-goat anti-mouse F(ab')\(_2\), and the dullest 2% of the population were sorted by flow cytometry (FACS IV®; Becton Dickinson & Co., Mountain View, CA). A total of six such sterile sorts were performed, each occurring 3–7 d after the previous sorting, and the dullest 2% of the cells were selected each time. After the sixth sorting the cells were subcloned at 0.3 cells/well; the N1 and N2 subclones were isolated from different wells. To obtain cells expressing intermediate amounts of CD45, the CD45 low population obtained after the sixth sorting was sorted once again, but this time the brightest 2% of the cells were selected, and then subcloned at 0.3 cells/well in 96-well microtiter plates (3596; Costar, Cambridge, MA); the D17 and D4 cells were isolated from different wells. After ∼2 mo of continuous culture it was noted by flow cytometry that a small fraction (1–2%) of the CD45 low N2 cells once again expressed wild-type levels of CD45. The CD45 low cells in this population were incubated with anti-CD45 and selected with magnetic beads (Dynabeads M-450; Dynal, Inc., Great Neck, NY) coated with sheep anti-rat IgG, and subcloned at 0.3 cells/well. Approximately one-third of the subclones expressed high levels of CD45. Three such CD45 revertant subclones, N2.R23, N2.R33, and N2.R47, were selected for further study. LK 35.2 cells were Fc receptor-bearing B cell hybridomas (18). All cells were grown in medium consisting of RPMI 1640 plus 10% FCS, 5 × 10\(^{-5}\) 2-ME, 2 mM glutamine, and antibiotics (complete medium).

Antibodies and Reagents. The following antibodies were used for staining of cell surface molecules: biotinylated M1/89.18.7.HK (M1/89, anti-CD45) (19); 145-2C11 (2C11; anti-CD3-ε) (20); 500A2 (anti-CD3-ε) (21); G7 (anti-Thy-1) (22); FD441.8 (anti-LFA-1) (23). FITC-anti-CD4 (Caltag Laboratories, San Francisco, CA), FITC-anti-CD8 (Becton Dickinson & Co.), FITC-goat anti-mouse F(ab')\(_2\) (FITC-GAM; Dako Corp., Glostrup, Denmark), and FITC-goat anti-hamster F(ab')\(_2\) (Jackson ImmunoResearch, West Grove, PA) were purchased. Biotinylated rat anti-mouse IgG was the kind gift of Dr. Lori Jones (National Cancer Institute, Bethesda, MD). 30H12 (IgG2b) is a rat anti–mouse Thy-1.2 (24). Thapsigargin was the kind gift of Dr. H. Okano (Johns Hopkins University, Baltimore, MD).

Flow Cytometry. Flow cytometric analysis was performed with a FACSscan® (Becton Dickinson & Co.). Approximately 10\(^6\) cells were incubated with the indicated antibodies on ice. After 30 min the cells were washed and stained with either FITC-goat anti–mouse F(ab')\(_2\), FITC-goat anti–hamster F(ab')\(_2\), or PE-avidin (Jackson Immunoresearch). Fluorescence data were collected using logarithmic amplification.

Northern Blot Analysis. Total RNA was extracted by guanidinium thiocyanate and prepared as described (25). After ethanol precipitation, the water-solubilized RNA was quantified by spectrophotometry, and 15 µg of total RNA from each sample was separated by electrophoresis (0.9% agarose gel with 6.5% formaldehyde) for 5 h at 60 V (26). The RNAs were transferred to Nytran membranes (Schleicher & Schuell, Inc., Keene, NH) in 2× SSC (27). After immobilization of nucleic acids by baking for 2 h at 80°C, the filter was prehybridized and then hybridized with specific probes labeled by random hexamer priming (27). A CD45-specific 2-kb HindIII fragment was prepared from the p70ZLC cDNA clone (generously provided by Dr. Matthew Thomas, St. Louis, MO). The same filter was subsequently hybridized with a glyceraldehyde phosphate dehydrogenase (GAPDH) probe as an internal control. After room temperature washes in 2× SSC, 0.1% SDS, and washing at 55°C in 0.1× SSC, 0.1% SDS, the filter was air dried and autoradiography was performed.

Quantitation of Total Inositol Phosphates. Cells were loaded with [3H]myo-inositol (14 Ci/mmol; New England Nuclear, Boston, MA) for 3 h at 37°C as described (28). The cells were thoroughly washed and divided into duplicate groups of 10\(^7\) cells each in tubes containing 10 ml of medium containing 10 mM LiCl. 2C11 (1:30 dilution of culture supernatant, final concentration) was added and incubated for 30 min. After this time the cells were lysed, lipid extracted, and the total water-soluble labeled products of phosphoinositide hydrolysis measured by anion exchange chromatography (28). The mean cpm achieved for each experimental point was divided by the total cpm incorporated by the cells (yielding percent labeled phospholipid), and then the percent labeled phospholipid generated in the absence of 2C11 (background) was subtracted to obtain percent change labeled phospholipid.

Measurement of Tyrosine Phosphatase Activity. Tyrosine phosphatase activity was assayed by measuring the release of \(^3\)P from a tyrosine-containing substrate, [N\(^\beta\)]-angiotensin II (Sigma Chemical Co., St. Louis, MO) as described (29). Briefly, 10\(^7\) cells were lysed in 100 µl of buffer containing 0.5% Triton X-100 in Tris/NaCl buffer, pH 7.4, leupeptin, aprotinin, and PMSF; for 30 min on ice. Either 10 µl of lystate was analyzed directly, or 50 µl of lystate was immunoprecipitated with M1/89 plus protein G-Sepharose beads (Gibco-BRL, Gaithersburg, MD). 10 µl of labeled substrate was added to the lystate or the beads and the reaction was allowed to proceed at 30°C for 60 s and then terminated by the addition of 5% activated charcoal suspended in 20 µM Hepes, pH 7.4. Supernatants were counted in a β scintillation counter.

Phosphotyrosine Immunoblotting. 5 × 10\(^7\) cells were resuspended either in 1 ml of complete medium (5–6 × 10\(^7\) cells/tube) and kept on ice for 30 min (unstimulated) or in 1 ml of 2C11 culture supernatant and incubated for 30 min at 37°C with frequent shaking (stimulated). The cells were then washed in ice-cold PBS with phosphatase inhibitors (0.4 mM sodium orthovanadate, 0.4 mM EDTA, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate; pH 7.6), resuspended in lysis buffer (0.5% Triton X-100, 50 mM Tris, and 150 mM NaCl), and phosphatase inhibitors for 30 min on
ice. After centrifugation, the postnuclear fractions were immunoprecipitated with protein A-agarose beads precoated with either 2C11 or 4G10 (antiphosphotyrosine mAb; Upstate Biotechnology, Inc., Lake Placid, NY) for 2 h on ice with frequent shaking. The beads were boiled in reducing sample buffer (50 mM TRIS, pH 6.8; 1% SDS, 10% glycerol, 3% 2-ME, plus bromophenol blue and phenol red) for 5 min, pelleted, and the supernatants separated on SDS-PAGE. The proteins were transferred to nitrocellulose, immunoblotted (using BSA as a blocking agent) with the 4G10 antibody followed by 125I-protein A-agarose (ICN, Costa Mesa, CA), and autoradiography was performed.

**Measurement of [Ca²⁺]**. Determination of [Ca²⁺] by flow cytometry was modified from the procedure of Rabinovitch and June (30). Briefly, the indicated cells were loaded with 2.5 μM indo-1 (Molecular Probes, Junction City, OR) in HBSS for 25 min at 31°C. After establishing baseline values, purified 2C11 (20 μg/ml final dilution) was added as indicated. Cells were then analyzed for violet/blue fluorescence emission ratio (395 nm/500 nm). A fluorescence image data processing system was used to detect changes in [Ca²⁺] in individual cells by a procedure modified from Poenie et al. (31). Briefly, cells were loaded in 3 μM fura-2 AM (Molecular Probes) at 37°C for 30 min in the presence of 0.0175% F-127. After settling on a glass coverslip, the cells were analyzed in a chamber maintained at 36.5° ± 0.25°C containing 2 ml of medium consisting of HBSS (containing 1 mM CaCl₂), 0.1% FCS, 10⁻² M 2-ME, and 20 mM Hepes. A baseline 350/380 ratio was established, after which purified 2C11 (20 μg/ml final) was added while images were acquired and stored digitally at 15-s intervals. The hardware consists of an image processor (FDS5000; Gould, Fremont, CA), an Axiovert microscope (Carl Zeiss, Inc., Thornwood, NY), and a filter changer (Ludl Electronic Products, Scarsdale, NY) with excitation filters centered at 350 ± 10 and 380 ± 10 nm. The image processor and filter changer are interfaced to a Microvax host computer (Digital Equipment Corporation, Maynard, MA). Images (16 at each wavelength) are acquired at 30/s through a CCD camera (Cohu, San Diego, CA) and image intensifier (VideoScope, Washington, DC) and processed according to code kindly provided by R. Y. Tien. [Ca²⁺] values were calculated from fura-2 ratio by the equation: [Ca²⁺] = K(R - Rₘᵦ)(Rₘᵦ - R); where Rₘᵦ and Rₘᵦ are the 350 nm/380 nm ratios obtained in Ca²⁺ absence or saturation, respectively. K is the product Kₒ(Fₒ/Fₘᵦ), where Kₒ is the effective dissociation constant of fura-2 with respect to Ca²⁺ (224 nM), Fₒ is the 380-nm excitation efficiency in the absence of Ca²⁺, and Fₘᵦ is the 380-nm excitation efficiency at saturating Ca²⁺ concentrations (31-33). The parameters are determined in the bath chamber from solutions containing fura-2-free acid.

**Results**

**Generation of YAC-1 CD45 Loss Variants.** YAC-1 T cells were analyzed by flow cytometry for cell surface expression of molecules involved in T cell activation (Fig. 1 A). The wild-type YAC-1 cells (WT) express both CD45 and the TCR. These cells were chemically mutagenized and CD45-deficient subclones selected by repetitive rounds of cell sorting, as detailed in Materials and Methods. To allow a quantitative evaluation of the relationship between CD45 expression and biological behavior, four YAC-1 subclones that expressed progressively lower levels of CD45 were selected for study. As shown in Fig. 1 A, the expression of CD45 (relative to WT cells as judged by mean fluorescence intensity) was: D17, 25%, D4, 3.5%, N1 and N2, undetectable. The TCR was expressed equally by all of the cells. Similar analyses revealed that all of the cells expressed equivalent levels of Thy-1 and LFA-1; none of the cells expressed detectable CD4, CD8, or Ly-6 (data not shown). The CD45 and CD3 cell surface phenotype of two representative CD45⁺ revertants, N2.R23 and N2.R47, are shown in Fig. 1 B. Similar results were obtained with the N2.R33 subclone (data not shown).

To determine why cell surface CD45 was not expressed, RNA from WT cells and its variants was subjected to Northern blot analysis with a CD45-specific cDNA probe. WT cells expressed easily detectable CD45 mRNA (Fig. 2). The hierarchy of CD45 mRNA expression paralleled the cell surface expression of CD45: WT > D17 > D4 > N1, N2. The two cell surface CD45⁺ variants, N1 and N2, expressed barely detectable CD45 mRNA. Thus, the loss of CD45 from the surface of the YAC-1 variants is due to a defect in transcription or, possibly, in posttranscriptional stabilization of CD45 mRNA.

CD45-specific and total cellular tyrosine phosphatase activity was determined (Fig. 3). WT cells had abundant anti-CD45-precipitable tyrosine phosphatase activity. The amount of activity precipitated from the CD45 variants correlated well with the relative abundance of this molecule as detected by flow cytometry and Northern blot analysis. In particular, no CD45-associated tyrosine phosphatase activity was detected in the N1 and N2 cell lines. Comparison of enzymatic activity in whole cell lysates suggests that ~40% of the total tyrosine phosphatase activity in the YAC-1 cells can be attributed to CD45. Thus, WT cells and the CD45 variants provide a series of cells that have a graded range of cell surface CD45 protein, CD45 mRNA, and CD45-associated tyrosine phosphatase activity.

**Altered Intracellular Steady-State Tyrosine Phosphorylation as a Function of CD45.** TCR-driven and kinase-mediated tyrosine phosphorylation of the TCR ζ chain is an early event in T cell activation (34). Given the close physical proximity of CD45 and the TCR (29), we asked if the loss of the CD45 tyrosine phosphatase influenced the level of ζ phosphorylation in the resting and the activated state. YAC-1 cells, unstimulated or stimulated with 2C11 (anti-CD3), were lysed, and anti-CD3 immunoprecipitates were analyzed for phosphotyrosine-containing species by immunoblotting. YAC-1 cells exhibited two phosphorylated ζ species (Fig. 4). As described (34), tyrosine phosphorylated ζ chains (which have six intracellular tyrosine residues potentially available for phosphorylation [35]) migrate aberrantly on SDS-PAGE, having an apparent relative molecular mass of 21,000 rather than 16,000. The two bands in YAC-1 cells (pp21 and pp23) presumably represent the usual phospho-ζ and a hyperphosphorylated form (the unphosphorylated ζ chain in YAC-1 cells has a mass of 16 kD, data not shown). As shown in Fig. 4 A, unstimulated WT cells expressed very little phospho-ζ of the 21-kD form and none of the 23-kD form. Stimulation with 2C11 caused a marked increase in both phospho-ζ bands. The D17 cell line expressed low but easily detectable pp21...
and pp23 phospho-\(\xi\). Both forms increased substantially with activation. D4 had more resting phospho-\(\xi\) than either WT or D17, and this again increased with activation. In contrast, the N1 and N2 cells had maximally phospho-\(\xi\) in the unstimulated state. If anything, stimulation with 2C11 actually decreased the amount of phospho-\(\xi\) in these cells to a small degree. In another experiment (Fig. 4 b), unstimulated WT cells expressed a small amount of phospho-\(\xi\) that increased in intensity after exposure to 2C11. The CD45- N2 cells had high levels of phospho-\(\xi\) in the absence of stimulation. Two different CD45 revertant cells, N2.R23 and N2.R47, had phospho-\(\xi\) levels that were indistinguishable from the WT cells. These results demonstrate that CD45 expression is inversely related to spontaneous TCR \(\xi\) phosphorylation.

There are tyrosine phosphorylated substrates other than \(\xi\) in activated T cells (36, 16). To determine if the presence or absence of CD45 influenced these substrates as well, lysates of YAC-1 cells were immunoprecipitated with antiphosphotyrosine antibodies, resolved by SDS-PAGE, and immunoblotted with an antiphosphotyrosine antibody (Fig. 5). Fig. 5, A and B, represent the same autoradiogram exposed for different periods of time. The data clearly show that there are relatively few tyrosine-phosphorylated species in unstimulated YAC-1 cells, but that stimulation with 2C11 induces bands of 21 and 23 kD (phospho-\(\xi\)), and 29, 40, and 92 kD. There is an increase in the intensity of a doublet at 59–61 kD, but no change in a band at 79 kD. In contrast to WT cells, the N1 variant had many intense tyrosine-phosphorylated species even in the unactivated state. Among these are the bands induced in YAC-1 cells by 2C11 (phospho-\(\xi\)), the 29, 40, and 92 kD) as well as many other bands not seen in WT cells. Prominent among the new species in the unactivated N1 cells were bands of 26 and 29 kD, as well as three closely spaced bands of 69, 71, and 75 kD. The single 92-kD species that appears in activated WT cells is seen as a doublet in N1 cells. Stimulation of the N1 cells with 2C11 had little effect on overall tyrosine phosphorylation. However, there was a clear increase in the intensity of the 27-kD band and actually a decrease in the intensity of the 29-kD species. No bands were detected when phosphotyrosine was included while the CD45- cells were being immunoblotted with the 4G10 antiphosphotyrosine antibody, while the addition of phosphoserine or phosphothreonine had no effect on the intensity of the bands, confirming this reagent's high degree of specificity (data not shown). Thus, loss of CD45 results in a global alteration of tyrosine phosphorylation status.

TCR-mediated PI Hydrolysis and Ca\(^{2+}\) Flux Vary as a Function of CD45 Expression. Other TCR-coupled signaling pathways were also examined. YAC-1 cells were loaded with [\(3H\)]myoinositol and stimulated with 2C11 or AIFl- (Fig. 6). Both the WT and the D17 cells responded well to TCR crosslinking. D4 cells responded poorly. Neither N1 nor N2 cells gave any detectable response. In contrast, all cells responded equivalently to AIFl-, a reagent that stimulates GTP-binding protein-dependent T cell PI hydrolysis in a TCR-independent fashion (37). The fact that D17 cells were as good as WT cells in their ability to hydrolyze PI (and to mobilize

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**Figure 1.** Cell surface phenotype of WT and the CD45-deficient variants. (A) WT cells and its variants were stained with FITC-GAM, biotinylated anti-CD45 (M1/89) plus PE-avidin, or anti-CD3 (2C11) plus FITC-rabbit anti-hamster F(ab')2, and cell surface fluorescence was quantitated by flow cytometry. (B) WT cells, N2, and 2 revertant subclones derived from N2 cells were stained with biotinylated anti-CD45 (M1/89) plus PE-avidin or anti-CD3 (500A2) followed by FITC-rabbit anti-hamster F(ab')2. The controls for each staining (biotinylated rat anti-mouse IgG plus PE-avidin and FITC-rabbit anti-hamster F[ab']2, respectively) are shown as dotted lines.

**Figure 2.** Northern blot analysis of CD45 expression. Total RNA from WT cells and its CD45-deficient derivatives (15 \(\mu\)g/lane) was separated by electrophoresis, transferred to a Nytran membrane, and probed with a \(^{32}P\)-labeled 2-kb fragment of mouse CD45 cDNA. As a control, the same filter was subsequently hybridized with a GAPDH probe.
Figure 3. Tyrosine phosphatase activity in WT and its derivatives. The YAC-1-derived cell lines were lysed in detergent. (A) After duplicate immunoprecipitation with anti-CD45, the beads were assayed for tyrosine phosphatase activity by measuring the release of \(^{32}P\) from \(^{32}P\text{[Val5]}\text{angiotensin II (192 pmol released by CD45 precipitated from WT cells). (B) Triplicate aliquots of cell lysate were directly assayed for tyrosine phosphatase activity (580 pmol released by WT cell lysate). The error bars indicate the SD for each replicate assay.}

\([\text{Ca}^{2+}];\text{see below}\) indicates that for YAC-1 cells a four- to fivefold decrease in CD45 expression is still sufficient to support normal TCR-mediated responses. Only when the CD45 level falls dramatically (to that of D4 cells, <5% of normal) is a signaling defect revealed.

\(\text{Ca}^{2+}\) flux is another measure of TCR-mediated cell activation. In preliminary experiments it was found that the Fc receptor-negative (based upon staining with the anti-Fc receptor 2.4G2 antibody \([38]\), data not shown) YAC-1 cells responded well to 2C11 in the absence of accessory cells (i.e., no external crosslinking of 2C11 was required). Therefore, YAC-1 cells were loaded with the \(\text{Ca}^{2+}\)-sensitive dye indo-1 and analyzed by flow cytometry (Fig. 7 A). Whereas WT and D17 cells responded to 2C11 with a rapid and vigorous increase in \([\text{Ca}^{2+}]\), D4 cells responded poorly. However, the shape of the D4 response curve was similar to those of the clones that expressed higher levels of CD45 (i.e., there was a rapid and short-lived rise). N1 and N2 cells displayed a very different \(\text{Ca}^{2+}\) response pattern. First, no 2C11-induced early (within 1 min) response was detected. However, during the \(>6\) min of monitoring, there was a slow and steady increase in mean \([\text{Ca}^{2+}]\) (baseline/maximal \([\text{Ca}^{2+}]\) levels: N1, 134 nM/236 nM; N2, 134 nM/265 nM). Monitoring the fraction of responding cells was also revealing (Fig. 7 B). The large majority of WT (84%) and D17 (94%) cells responded at the peak; only about half of the D4 cells responded. Neither N1 nor N2 cells responded by 1 min after activation. However, by 3 min after stimulation, it was evident that a significant number of cells were responding with elevated \([\text{Ca}^{2+}]\), (the fraction of cells with elevated \([\text{Ca}^{2+}]\) \((>2\ SD\ above\ baseline)\) at the peak of the response was \(n = 7\): N1, 15.4%; N2, 18.5%).

**Figure 4.** CD45 expression and phosphorylation of TCR \(\zeta\). (a) WT cells or the CD45-deficient variants were cultured in medium alone or in the presence of 2C11 for 30 min at 37\(^{\circ}\)C. At that time the TCR chains were immunoprecipitated, separated by SDS-PAGE, and immunoblotted with antiphosphotyrosine antibodies. The YAC-1-phosphorylated TCR \(\zeta\) chain appears as a doublet. (b) Phospho-\(\zeta\) was analyzed as in a. Cells were cultured in medium alone (−) or with 2C11 for 30 min at 37\(^{\circ}\)C (+). Because the intensity of the bands varied greatly, two different times of exposure are shown: the lane in group A represents the immunoprecipitate from N2 cells exposed for 10 h; the lanes in group B represent immunoprecipitates from WT and N2 cells, as well as two CD45 revertant cells, exposed for 60 h.

**Figure 5.** Total cellular tyrosine phosphorylation as a function of CD45 expression. WT and N1 cells were cultured in medium alone or in the presence of 2C11 for 30 min at 37\(^{\circ}\)C. Detergent lysates were prepared and immunoprecipitated with an antiphosphotyrosine antibody. The immunoprecipitates were resolved by SDS-PAGE and immunoblotted with the same antiphosphotyrosine antibody. (A) 18-h autoradiogram; (B) same membrane exposed to x-ray film for 5 d.
Changes in \([Ca^{2+}]_i\) were also measured in CD45 revertants. As shown in Fig. 7, C and D, three independent revertant cell lines, N2.R23, N2.R33, and N2.R47, responded to activation with a \(Ca^{2+}\) response that was indistinguishable from the WT cells. In contrast, the N2 cells (from which the revertants were derived) displayed a delayed and rather small increase in \([Ca^{2+}]_i\). Five other CD45 revertants were analyzed, and all were found to express the wild-type pattern of \([Ca^{2+}]_i\) rise; two CD45− subclones derived from N2 at the same time as the CD45+ revertants had delayed \(Ca^{2+}\) responses of the type manifested by N2 (data not shown). Thus, there are two distinct \(Ca^{2+}\) response phenotypes that depend upon the presence or absence of CD45.

\([Ca^{2+}]_i\) in Single Cells. Flow cytometric analysis is limited in that it can only provide a static picture of responding cells. Therefore, we employed computer-aided fluorescence imaging, which allows one to monitor the \([Ca^{2+}]_i\) of individual cells over time, in order to analyze the atypical \(Ca^{2+}\) response of the CD45-deficient cells. WT and the two CD45− subclones were labeled with fura-2 and monitored for changes in \([Ca^{2+}]_i\) after stimulation with 2C11 (Fig. 8). Anti-CD3 induced a rapid (within 1 min) \([Ca^{2+}]_i\) increase in virtually all WT cells. This was followed by \([Ca^{2+}]_i\), os-
cillations, although they were small in amplitude and only manifested by a minor fraction of the cells. Surprisingly, both N1 and N2 cells gave a qualitatively different response. First, 2C11 did not induce a rapid increase in \([Ca^{2+}]_i\). In fact, cells generally did not begin to respond until several minutes after stimulation. Second, unlike the concerted response of the WT cells, the response of the CD45− cells was unsynchronized. Third, whereas the WT cells generally manifested a large early \(Ca^{2+}\) rise with little in the way of oscillations, the N1 and N2 cells exhibited only oscillations, which were much larger in amplitude and more frequent. The fraction of CD45− cells that responded over the course of the observation period varied from experiment to experiment, but was always >50%. In this experiment the fraction of N1 cells that responded was 75% and the fraction of N2 cells that responded was 88%. The responses of seven representative individual cells (WT vs. N2) are shown in Fig. 9. N1 cells had profiles that were indistinguishable from N2 cells (data not shown). EGTA was also used to chelate extracellular \(Ca^{2+}\). This maneuver greatly blunted the early response of the WT cells, and completely abrogated any late response. In contrast, the \(Ca^{2+}\) oscillations by the N2 cells (and N1 cells; data not shown) were hardly affected by the chelation of extracellular \(Ca^{2+}\). The response of the YAC-1 cells to 2C11 was specific; no changes in \([Ca^{2+}]_i\) were detected when Thy-1 was bound by the 30H12 antibody (Fig. 8 B). Furthermore, both CD45+ and CD45− cells labeled equivalently with fura-2 and responded to a TCR-independent stimulus, thapsigargin, which directly elevates \([Ca^{2+}]_i\), by inhibiting the sarcoplasmic or ER ATP-dependent \(Ca^{2+}\) pumps responsible for sequestering \([Ca^{2+}]_i\). Together, these results demonstrate that the absence of CD45 allows T cells to manifest a specific large and periodic TCR-driven release of \(Ca^{2+}\) from intracellular stores.

**Discussion**

The data in this study provide a view of the profound way in which the tyrosine phosphatase CD45 influences T cell signal transduction pathways. Three major and commonly studied signaling pathways were aberrant in CD45− cells. The defects, which consisted of dysregulated cellular homeostatic mechanisms and responses to TCR-mediated activation, were not merely of a quantitative nature. Rather, CD45− cells had qualitative changes in both tyrosine kinase/tyrosine...
lates phosphorylation initiated by some PTKs that are activated by TCR ligation, and we were surprised at the extent of the hyperphosphorylation. The simplest possibility is that constitutive phosphorylation of multiple proteins on tyrosine residues was that TCR-mediated increases in \([\text{Ca}^2+]_i\) were simply either absent or markedly blunted (15, 16). It is possible that this discrepancy is due to the degree to which the YAC-1 cells and the Jurkat variant T cells are "CD45 deficient." Whereas N1 and N2 cells had no detectable cell surface CD45 and no enzymatic activity could be precipitated by anti-CD45, the CD45-deficient Jurkat cells still expressed ~8% of their usual cell surface CD45 (16). It may be that the small amount of CD45 remaining in the CD45-low Jurkat cells was sufficient to maintain tyrosine phosphorylation homeostasis in unactivated cells, although insufficient to allow normal activation-induced increases in PTK activity. This could not account for another case, in which antiphosphotyrosine immunoblotting of whole cell lysates from several CD45- cell lines found them to have a baseline tyrosine phosphorylation pattern similar to that of their CD45+ counterparts, except for hyperphosphorylation of p56\(\kappa\kappa\) (41). We also had difficulty demonstrating differences in tyrosine phosphorylation between CD45+ and CD45- YAC-1 cells when whole cell lysates were used (data not shown); only when antiphosphotyrosine immunoprecipitation was first performed was a clear-cut difference seen upon subsequent immunoblotting. This is not dissimilar from the results of Ostergaard et al. (41), in which p56\(\kappa\kappa\) hyperphosphorylation was inconsistently seen in whole cell lysates from three independent CD45- cell lines, although it was quite marked when p56\(\kappa\kappa\) was specifically immunoprecipitated. We are currently using whole cell lysates or antiphosphotyrosine immunoprecipitates to evaluate the tyrosine phosphorylation status of other CD45-deficient cell lines.

An unexpected finding was the novel late \([\text{Ca}^2+]_i\) oscillations induced by TCR crosslinking. Data obtained with CD45-deficient human HPB-ALL and Jurkat cells suggested that TCR-mediated increases in \([\text{Ca}^2+]_i\) were simply either absent or markedly blunted (15, 16). It should be emphasized that the view one obtains of the aberrant \([\text{Ca}^2+]_i\) response is largely a function of the technique employed (fluorimetry, flow cytometry, or computer-aided fluorescence imaging). Flow cytometric analysis of activated YAC-1 CD45- cells demonstrated a relatively small (~40 nM; \(n = 7\)) increase in baseline Ca\(^{2+}\) and TCR-mediated Ca\(^{2+}\) mobilization.

The most obvious consequence of CD45 loss was the constitutive phosphorylation of multiple proteins on tyrosine residues. Because of the substantial amount of tyrosine phosphatase activity remaining in the YAC-1 CD45-deficient cells, we were surprised at the extent of the hyperphosphorylation. This observation underscores the high degree of tyrosine phosphatase specificity in vivo, and proves that for at least a cohort of PTK substrates (including the TCR \(\gamma\) chain), other tyrosine phosphatases will not substitute to serve CD45's regulatory function. There are a number of possible explanations for the hyperphosphorylation. The simplest possibility is that constitutive CD45 phosphatase activity normally counters the activity of constitutively active PTKs. Although the tyrosine-phosphorylated species detected in N1 and N2 cells include those induced in WT cells by TCR ligation, many new bands also appear, implying that either CD45 regulates phosphorylation initiated by some PTKs that are associated with T cell activation and some that are not, or that T cell activation somehow limits the variety of substrates available for the PTKs, perhaps by regulating their subcellular localization. Another possible explanation for the enhanced spontaneous phosphorylation is that the absence of CD45 activates one or more PTKs. Although there is evidence that CD45 activates the p56\(\kappa\kappa\) PTK by removing a regulatory (inhibitory) phosphate from Tyr-505 (40-42), phosphorylation of some regulatory tyrosines can also enhance enzymatic activity. Tyr-394 may play such a role in p56\(\kappa\kappa\), since site-directed mutagenesis of this residue prevents the enhancement of enzyme activity associated with mutation of Tyr-505 (43). In the case of c-src, mutation of Tyr-416 (a tyrosine kinase target) to a phenylalanine suppresses the transforming ability of this kinase, and diminishes the PTK activity induced by Tyr-527 removal (8). Thus, lack of CD45 could potentially increase PTK activity by allowing unopposed phosphorylation of regulatory (enhancing) tyrosine residues, or by some more complex intermediary set of interactions. Interestingly, the doublet seen at 60 kD in activated WT, which is very prominent in unactivated N1 cells, is in the molecular mass range that is commonly associated with autophosphorylated src family members (44). Efforts are now underway to identify which PTKs are active in the CD45- YAC-1 subclones.

The extreme degree of spontaneous hyperphosphorylation contrasts with previous results reported with CD45-deficient Jurkat cells, in which baseline tyrosine phosphorylation was normal, although tyrosine kinase activity was not induced by TCR crosslinking (16). It is possible that this discrepancy is due to the degree to which the YAC-1 cells and the Jurkat variant T cells are "CD45 deficient." Whereas N1 and N2 cells had no detectable cell surface CD45 and no enzymatic activity could be precipitated by anti-CD45, the CD45-deficient Jurkat cells still expressed ~8% of their usual cell surface CD45 (16). It may be that the small amount of CD45 remaining in the CD45-low Jurkat cells was sufficient to maintain tyrosine phosphorylation homeostasis in unactivated cells, although insufficient to allow normal activation-induced increases in PTK activity. This could not account for another case, in which antiphosphotyrosine immunoblotting of whole cell lysates from several CD45- cell lines found them to have a baseline tyrosine phosphorylation pattern similar to that of their CD45+ counterparts, except for hyperphosphorylation of p56\(\kappa\kappa\) (41). We also had difficulty demonstrating differences in tyrosine phosphorylation between CD45+ and CD45- YAC-1 cells when whole cell lysates were used (data not shown); only when antiphosphotyrosine immunoprecipitation was first performed was a clear-cut difference seen upon subsequent immunoblotting. This is not dissimilar from the results of Ostergaard et al. (41), in which p56\(\kappa\kappa\) hyperphosphorylation was inconsistently seen in whole cell lysates from three independent CD45- cell lines, although it was quite marked when p56\(\kappa\kappa\) was specifically immunoprecipitated. We are currently using whole cell lysates or antiphosphotyrosine immunoprecipitates to evaluate the tyrosine phosphorylation status of other CD45-deficient cell lines.

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**Figure 9.** Ca\(^{2+}\) responses of single cells in the presence or absence of extracellular Ca\(^{2+}\). WT and N1 cells were loaded with fura-2, and the [Ca\(^{2+}\)] of individual cells was monitored as in Fig. 8. Analyses were done in medium alone or in the presence of 3 mM EGTA. The arrows indicate the time of 2CaI addition. Each graph displays the response of an individual cell. Seven representative profiles under each condition were chosen for display.
Reference

The finding that [Ca\textsuperscript{2+}]i did change in response to a TCR-mediated stimulus in the YAC-1 CD45-deficient cells was convincingly obtained only with the sensitive technique of computer-aided fluorescence imaging. Furthermore, by allowing one to monitor individual cells over time, asynchronous oscillations that do little to raise the mean [Ca\textsuperscript{2+}]i of the population can be detected. The finding that this was the case with the CD45\textsuperscript{-} YAC-1 derivatives is consistent with the small but reproducible finding with flow cytometry that, on average, from 15 to 20\% of the cells were responding at any given moment 1 min or more after activation.

The loss of the early rise in [Ca\textsuperscript{2+}]i and the novel pattern of TCR-mediated fluctuations in [Ca\textsuperscript{2+}]i, is intriguing. Experiments using EGTA to chelate extracellular Ca\textsuperscript{2+} indicate that both intracellular and extracellular Ca\textsuperscript{2+} contribute to the early response in YAC-1 cells, while intracellular stores provide most of the Ca\textsuperscript{2+} for the late response. Although not prominent in WT cells, the CD45\textsuperscript{-} cells manifested late Ca\textsuperscript{2+} oscillations that were almost as great in magnitude as the initial response in WT cells. These results suggest that either CD45 normally tends to prevent late oscillations or that loss of CD45 (with the attendant dysregulation of PTK activity) allows atypical late Ca\textsuperscript{2+} oscillations to occur. We have recently shown that tyrosine kinase activity, by itself, can alter intracellular Ca\textsuperscript{2+} regulation both in the absence or the presence of TCR-mediated signaling (5). One attractive possibility is that an altered level of tyrosine phosphorylation in CD45\textsuperscript{-} cells, perhaps due to a change in tyrosine kinase activity, is the cause of the aberrant Ca\textsuperscript{2+} response phenotype. Alternatively, CD45 may serve to couple the TCR to Ca\textsuperscript{2+} responses by directly dephosphorylating critical but uncharacterized substrates. Whatever the exact mechanisms, together with previous studies, these data demonstrate that CD45 profoundly influences all of the well-characterized signaling pathways in T cells. The answer to how a tyrosine phosphatase regulates PI hydrolysis, [Ca\textsuperscript{2+}]i mobilization, and PTKs will ultimately explain a great deal about how the TCR couples to biological function.

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