

Surface Expression of a Heterologous Phosphatase Complements CD45 Deficiency in a T Cell Clone

By David G. Motto,* Michael A. Musci,† and Gary A. Koretzky*‡§

From the *Department of Physiology and Biophysics, the †Graduate Program in Immunology, and the ‡Department of Internal Medicine, University of Iowa College of Medicine, Iowa City, Iowa 52242

Summary

Expression of CD45, the major transmembrane protein tyrosine phosphatase expressed on lymphoid cells, is required for optimal T cell receptor (TCR) signal transduction. We and others recently have demonstrated that surface expression of the cytoplasmic domain of CD45 in the absence of its extracellular and transmembrane domains is sufficient to restore TCR-mediated signaling events in CD45-deficient cell lines. Here we demonstrate that a single domain nonreceptor tyrosine phosphatase from yeast expressed as a chimeric protein with the extracellular and transmembrane domains of a major histocompatibility complex class I molecule also is able to restore proximal and distal TCR-mediated signal transduction events in the CD45-deficient T cell line J45.01. Ligation of the TCR on the cell line expressing the yeast phosphatase chimera results in the induction of protein tyrosine kinase activity, soluble inositol phosphate generation, and expression of the CD69 activation antigen. Furthermore, a phosphatase-inactive version of this molecule is unable to restore signal transduction, providing the first formal evidence that plasma membrane associated tyrosine phosphatase activity is required for TCR-mediated signaling.

A number of studies from several laboratories have demonstrated that surface expression of the CD45 tyrosine phosphatase is critical for optimal signal transduction initiated by ligation of the T cell antigen receptor (TCR) (1–5). Studies from our laboratory and others have shown that the TCR is uncoupled from its signaling pathways in CD45-deficient cell lines (2–5). Transfection of wild-type CD45 (2, 5, 6), or chimeric molecules containing the cytoplasmic domain of CD45 (7–9), restores signaling ability to several CD45-deficient cell lines.

One model for the role of CD45 in the regulation of TCR-mediated signal transduction suggests that this molecule functions to control the state of phosphorylation of the negative regulatory COOH-terminal tyrosine residue present in the lck and/or fyn protein tyrosine kinases (PTK)¹. A large number of studies have shown that ligation of the TCR induces a series of biochemical signaling events, the earliest of which is the activation of a PTK (10, 11). As the TCR does not possess intrinsic kinase activity, one or more cellular PTK must be recruited. Candidates for the TCR-activated PTK include two members of the src family, lck and fyn, and a syk family kinase, ZAP-70. Increasing evidence suggests that members of both PTK families are important in T cell activation (12–16). Furthermore, recent studies suggest that ac-

tivation of members of the PTK families is sequential, with activation of lck and/or fyn preceding ZAP-70 recruitment (17). As hyperphosphorylation of src family PTK on their COOH-terminal tyrosine results in decreased enzymatic activity (18, 19), the model predicts that in the absence of CD45 the most proximal signaling events associated with TCR ligation would be impaired.

We have found this to be the case in J45.01, a CD45-deficient variant of the Jurkat human T cell leukemic line. Stimulation of the TCR on this cell fails to result in the induction of PTK activity (3); and as predicted, also fails to result in the appearance of second messengers downstream of the PTK, including soluble inositol phosphates (IP), and increased levels of intracellular free calcium (3). Transfection of full-length CD45 cDNA corrects the signaling defect (6). Additionally, we and others recently have demonstrated that surface expression of the cytoplasmic domain of CD45, in the absence of its extracellular and transmembrane domains, also rescues proximal (7–9) and distal (20) TCR-initiated signaling events, although not to wild-type levels, thus suggesting that the cytoplasmic domain of CD45 is necessary and sufficient for TCR-mediated signal transduction.

There is considerable evidence to support the model for the role of CD45 described above. CD45 has been shown to dephosphorylate lck in vitro (21). Additionally, in a number of CD45-deficient cells, src family kinases have been shown to be hyperphosphorylated on their COOH-terminal tyrosine residues (5, 21–23). Finally, coimmunoprecipitation studies

¹ Abbreviations used in this paper: IP, inositol phosphates; PLC γ 1, phospholipase C γ 1; PTK, protein tyrosine kinase.

from our laboratory and others have shown that CD45 associates with lck in a number of human T cell lines (24–27). Similar to essentially all transmembrane tyrosine phosphatases, the cytoplasmic domain of CD45 contains two tandem catalytic domains that are responsible for the enzymatic activity of the molecule (28). There are to date, however, no studies formally demonstrating that it is the enzymatic activity of CD45 that is essential for its function to regulate signaling via the TCR. Similarly, there are as yet no data addressing whether there are features of CD45 independent of its enzymatic activity which are required for the support of TCR function.

In an effort to investigate these questions more thoroughly, we transfected J45.01 with a cDNA encoding a chimeric transmembrane protein consisting of a single domain nonreceptor tyrosine phosphatase from yeast (29) coupled to the extracellular and transmembrane domains of the HLA-A2 allele of the major histocompatibility complex class I molecule. Here we report that ligation of the TCR on the cell line expressing the yeast phosphatase chimera results in the induction of PTK activity, soluble IP generation, and expression of the CD69 activation antigen. However, the efficiency of TCR signaling in the transfectant expressing the yeast chimera is reduced in comparison to signaling demonstrated by our transfectants expressing a similar chimeric molecule containing CD45 sequences for its cytoplasmic domain. Furthermore, we demonstrate that a phosphatase-inactive version of the yeast chimera is unable to restore TCR signal transduction. These data provide the first formal evidence that plasma membrane-associated tyrosine phosphatase activity is required for TCR-mediated signaling.

Materials and Methods

Cells and Cell Culture. The wild-type human T cell leukemic line Jurkat, and the CD45-deficient Jurkat derivative, J45.01, have been described previously (3), and were maintained in RPMI supplemented with 10% FCS, penicillin (1,000 U/ml), streptomycin (1,000 U/ml), and glutamine (20 mM). J45/CH11 (7), J45/YP1.6, and J45/YPCA4 are transfectants of J45.01, and were maintained in the above medium supplemented additionally with G418 (2 mg/ml).

Antibodies and Flow Cytometric Analysis. The following antibodies were used in this study: anti-CD45 mAb, 9.4 (American Type Culture Collection [ATCC, Rockville, MD]); anti-CD3 mAb, Leu4 (Becton Dickinson & Co., Mountain View, CA); anti-CD3 mAb, OKT3 (ATCC); anti-TCR mAb, C305 ([30], gift of A. Weiss, Howard Hughes Medical Institute and University of California–San Francisco, San Francisco, CA); anti-A2 mAb, CR11-351 (gift of C. Lutz, University of Iowa, Iowa City, IA); anti-PLC γ 1 mAb (Upstate Biotechnology, Inc., Lake Placid, NY); anti- ζ antisera, 387 ([31], gift of L. Samelson, Bethesda, MD); antiphosphotyrosine mAb, 4G10 (gift of B. Drucker, Oregon Health Sciences University, Portland, OR); anti-CD69 FITC conjugated mAb, Leu23 (Becton Dickinson & Co.); negative IgG2b control mAb, MOPC-195 (Cappel Laboratories, Malvern, PA); negative IgG control FITC-conjugated mAb (Becton Dickinson & Co.); and goat anti-mouse FITC-conjugated secondary mAb (Cappel Laboratories). For flow cytometric analysis, cells were stained with the indicated monoclonal antibodies followed by a goat anti-mouse FITC-conjugated secondary mAb if necessary, and analyzed with either a flow cytometer

(Epics 753; Coulter Electronics Corp., Hialeah, FL) at the University of Iowa Flow Cytometry Facility, or a FACScan[®] (Becton Dickinson & Co.).

cDNA Constructs. cDNA encoding the A2/YP chimeric molecule was generated using the polymerase chain reaction (PCR) using a pBS vector containing the yeast phosphatase cDNA (gift of R. Deschenes, University of Iowa, Iowa City, IA) as a template, and the following primers: sense (5'GCGAGATCTTATATAGACAGCGTGAC-3'), containing a 5' BglII site, and antisense, (3'-TGT-CGATATATTTCAAGGAGATCTGCG-5'), containing a 3' XbaI site. The resulting product (from tyrosine seven through the stop codon of the yeast phosphatase) was digested with BglII and XbaI, gel purified, and ligated into the pGEM A2/CD45 vector (7) previously gel purified after digestion with the same restriction enzymes to remove CD45 sequences. The resulting plasmid was designated pGEM A2/YP. The fidelity of the PCR was verified by automated DNA sequencing by the DNA core facility at the University of Iowa. cDNA encoding the A2/YP chimera was then subcloned into the pAW Neo3 expression vector. To construct the cDNA encoding the A2/YPCA phosphatase-inactive chimeric molecule, the plasmid pRS-YP1CA containing the yeast phosphatase cDNA bearing the cysteine to alanine point mutation (gift of R. Deschenes) was digested with the restriction enzymes MluI and NsiI, and the appropriate fragment was gel purified and ligated into the plasmid pGEM A2/YP (above), which previously had been gel purified after digestion with the same restriction enzymes. Verification that the resulting plasmid contained the point mutation was provided by automated DNA sequencing of the appropriate region by the DNA core facility at the University of Iowa. cDNA encoding the A2/YPCA chimera was then subcloned into the pAW Neo3 expression vector.

Transfections and FACS[®]. Transfections were performed by electroporation with a Gene Pulser (Bio-Rad Laboratories, Richmond, CA) set at 360 mV and 960 μ F. Drug-resistant cells transfected with the A2/YP chimeric molecule were sorted with an Epics 753 flow cytometer (Coulter Electronics Corp.) three times with the mAb CR11-351 for high expression of the A2 epitope, and once with the anti-CD3 mAb OKT3, resulting in establishment of the J45/YP1.6 cell line. Drug resistant cells transfected with the A2/YPCA chimeric molecule were sorted four times with the mAb CR11-351 to establish the J45/YPCA4 cell line.

Northern Analysis and Metabolic Labeling. Total RNA was isolated from Jurkat, J45.01, J45/CH11, and J45/YP1.6 with Tri-Reagent (Molecular Research Center, Cincinnati, OH). PolyA⁺ RNA (1.5 μ g) extracted with the PolyAtract system (Promega Corp., Madison, WI) was then subjected to electrophoresis in a formaldehyde-agarose gel and transferred to a Hybond N blotting membrane (Amersham Corp., Arlington Heights, IL). The yeast phosphatase probe was prepared by random priming (Pharmacia LKB, Piscataway, NJ) the EcoRI fragment of the A2/YP cDNA. Hybridization was performed at 65°C for 12 h, and the blot was washed with SSC solutions increasing in stringency to 2 \times . Metabolic labeling with [³⁵S]methionine (160 μ Ci/50 \times 10⁶ cells [Amersham Corp.]) was performed for 6 h in methionine-free RPMI supplemented with dialyzed 10% FCS, penicillin (1,000 U/ml), streptomycin (1,000 U/ml), and glutamine (20 mM).

Immunoprecipitations. Cells were left unstimulated or were stimulated for the indicated times with C305 ascites at a final concentration of 1:2,500. Cell lysates prepared in either NP-40 (150 mM NaCl, 1% NP-40, 10 mM Tris, pH 7.4) or RIPA (150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 50 mM Tris, pH 7.5) lysis buffers including protease (50 μ g/ml aprotinin, 10 μ g/ml leupeptin, 50 μ g/ml pepstatin A, 1 mM PMSF) and phosphatase (400 nM

sodium vanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate) inhibitors were then subjected to immunoprecipitation with the indicated antibodies conjugated to protein A (Sigma Chemical Co., St. Louis, MO) for 2 h at 4°C. Immunoprecipitates were washed four times in high-salt (500 mM NaCl) lysis buffer, subjected to SDS-PAGE either for autoradiography, or for transfer to nitrocellulose for immunoblotting with the indicated antibodies followed by an alkaline phosphatase conjugated secondary antibody (Bio-Rad Laboratories). Immunoreactive proteins were detected by developing with 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) as recommended by the supplier.

Measurement of Soluble Inositol Phosphate Generation. Cells were loaded with [³H]myoinositol (Amersham Corp.), cultured overnight, and then stimulated with media alone or with media plus C305 ascites at a final concentration of 1:2,500. The cells were then lysed at the indicated times in a 1:1 mixture of chloroform/methanol, and soluble IP were isolated by anion-exchange chromatography as described (32).

GST Fusion Protein Production and Tyrosine Phosphatase Assays. cDNAs encoding the yeast phosphatase portions of the A2/YP and A2/YPCA chimeras were subcloned into the bacterial expression vector pGEX-2T (Pharmacia LKB). GST/YP and GST/YPCA fusion proteins were induced and isolated as described (33), and aliquots containing equivalent amounts of protein were incubated with 10 mM *o*-phospho-L-tyrosine (Sigma Chemical Co.) and liberated phosphate was measured by colorimetric assay as described (3).

Results

Characterization of the Cell Line Expressing the Yeast Phosphatase Chimera. To define more precisely the requirements for tyrosine phosphatase activity in TCR-mediated signal transduction, we transfected the CD45-deficient cell line, J45.01, with a cDNA encoding a chimeric molecule consisting of a single domain cytosolic tyrosine phosphatase from yeast (29) in frame with the transmembrane and extracellular domains of the A2 major histocompatibility class I molecule (Fig. 1 A). This chimeric molecule, termed A2/YP, thus contains extracellular and transmembrane sequences serving to target the cytosolic yeast phosphatase to the plasma membrane. After transfection, drug-resistant cells were sorted by flow cytometry for high level expression of the A2 epitope, and the J45/YP1.6 cell line was established (Fig. 1 B). Flow cytometric analysis revealed that J45/YP1.6 expressed a similar low level of CD45 compared with J45.01, and a similar level of TCR when compared with J45/CH11, a previously reported transfectant of J45.01 that expresses an A2/CD45 chimeric molecule (Fig. 1 B). Northern analysis with a probe specific for the yeast phosphatase demonstrated hybridization only with RNA obtained from J45/YP1.6 (Fig. 1 C); and anti-A2 immunoprecipitates from [³⁵S]methionine-labeled J45/YP1.6

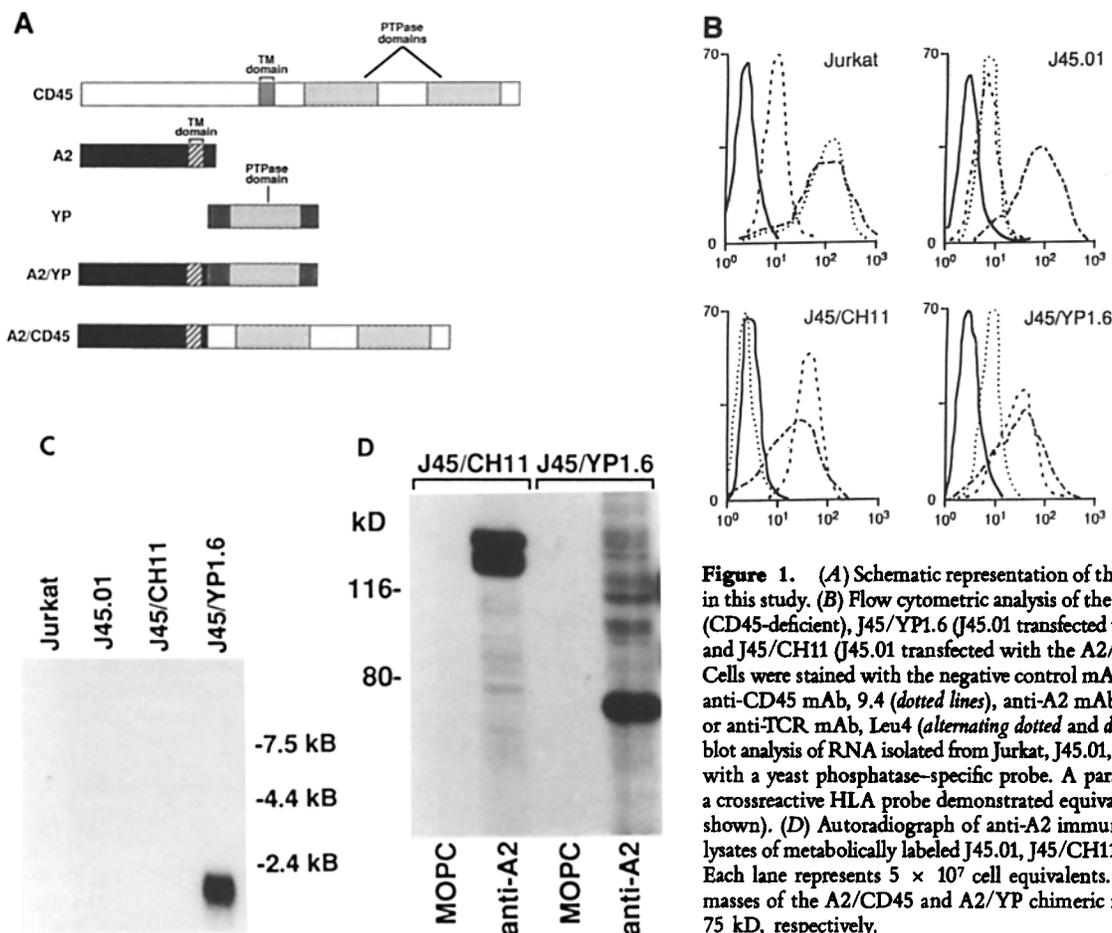


Figure 1. (A) Schematic representation of the chimeric molecules used in this study. (B) Flow cytometric analysis of the Jurkat (wild-type), J45.01 (CD45-deficient), J45/YP1.6 (J45.01 transfected with the A2/YP chimera), and J45/CH11 (J45.01 transfected with the A2/CD45 chimera) cell lines. Cells were stained with the negative control mAb, MOPC 195 (solid lines), anti-CD45 mAb, 9.4 (dotted lines), anti-A2 mAb, CR11.351 (dashed lines), or anti-TCR mAb, Leu4 (alternating dotted and dashed lines). (C) Northern blot analysis of RNA isolated from Jurkat, J45.01, J45/CH11, and J45/YP1.6 with a yeast phosphatase-specific probe. A parallel blot hybridized with a crossreactive HLA probe demonstrated equivalent mRNA loading (not shown). (D) Autoradiograph of anti-A2 immunoprecipitates from RIPA lysates of metabolically labeled J45.01, J45/CH11, and J45/YP1.6 cell lines. Each lane represents 5×10^7 cell equivalents. The predicted molecular masses of the A2/CD45 and A2/YP chimeric molecules are 118 kD and 75 kD, respectively.

cells visualized by SDS-PAGE and autoradiography possessed a specific band at 75 kD, the calculated molecular mass of the A2/YP chimera (Fig. 1 D). Interestingly, we have found that we can only immunoprecipitate the A2/YP chimera from lysates of cells solubilized in harsh detergents, suggesting that the A2/YP molecule may associate with insoluble cellular components.

Expression of the A2/YP Chimeric Molecule Restores Proximal Signal Transduction. The earliest detectable biochemical event after TCR engagement in wild-type Jurkat is the activation of a PTK resulting in the appearance of multiple newly tyrosine phosphorylated proteins (Fig. 2 A). In J45.01, this event is uncoupled from TCR ligation (Fig. 2 A and reference 3). As reported previously, expression of an A2/CD45 chimeric molecule (Fig. 1 A) on the J45/CH11 cell line (Fig. 1 B) rescues this defect (Fig. 2 A and reference 7). Interestingly, the A2/YP chimera expressed on J45/YP1.6 also rescues TCR-mediated PTK activation, with nearly an identical pattern of phosphoprotein induction (Fig. 2 A).

Two physiologically relevant substrates of the TCR-activated PTK are the phosphoinositide-specific enzyme PLC γ 1 (34, 35) and the ζ chain of the TCR (10, 11). Tyrosine phosphorylation of ζ is thought to be important in TCR-mediated signaling because it allows ζ to associate with the ZAP-70 PTK (15, 17), and the SH2 domain adaptor protein Shc (36). Therefore we investigated the tyrosine phosphorylation state of ζ in J45/YP1.6 in response to increasing periods of TCR stimulation, and compared it with responses generated in Jurkat and J45/CH11 (Fig. 2 B). As shown, ζ becomes tyrosine phosphorylated in J45/YP1.6, but to a lesser degree and with slower kinetics than in either Jurkat or J45/CH11. Tyrosine phosphorylation of ζ peaks after 5 min of TCR stimulation in J45/YP1.6, compared with 1 min of TCR stimulation in both Jurkat and J45/CH11. In the same experiment, an antiphosphotyrosine-reactive protein migrating at 70 kD was observed in each lane phospho- ζ was detected (not shown). This protein had the same kinetics of appearance as phospho- ζ and was presumed to be ZAP-70.

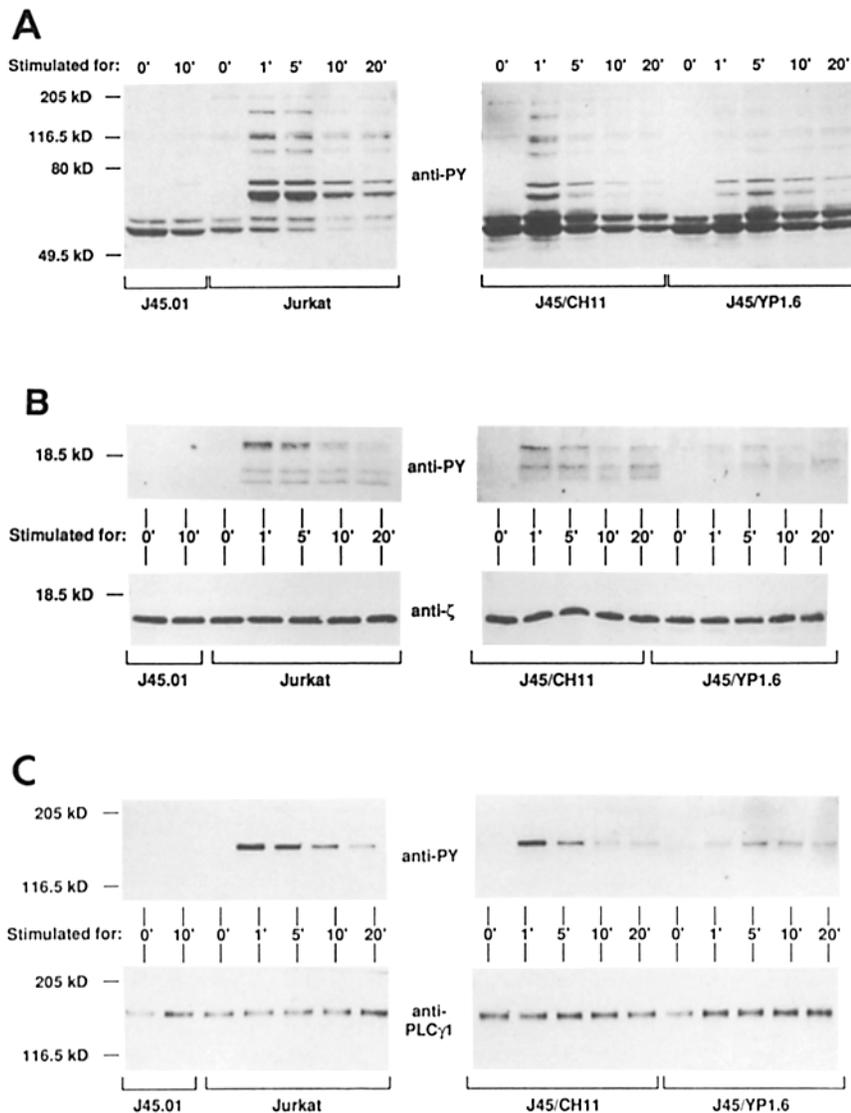


Figure 2. Expression of the A2/YP chimera restores TCR-mediated phosphoprotein induction. (A) Time course of phosphoprotein induction in J45.01, Jurkat, J45/CH11, and J45/YP1.6 after TCR ligation. Cells were left unstimulated or were stimulated for the indicated times with anti-TCR mAb C305 (ascites, final concentration 1:2,500). NP-40 lysates were subjected to 10% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the antiphosphotyrosine mAb 4G10. Each lane represents 10^6 cell equivalents. (B) Time course of ζ phosphorylation in J45.01, Jurkat, J45/CH11, and J45/YP1.6 after TCR ligation. NP-40 cell lysates were subjected to immunoprecipitation with the anti- ζ antiserum 387, subjected to SDS 12% PAGE, transferred to nitrocellulose, and immunoblotted with 387 (bottom, 10^7 cell equivalents/lane) or 4G10 (top, 5×10^7 cell equivalents/lane). (C) Time course of PLC γ 1 phosphorylation in J45.01, Jurkat, J45/CH11, and J45/YP1.6 after TCR ligation. NP-40 cell lysates were subjected to immunoprecipitation with the anti-PLC γ 1 mAb, subjected to SDS 8% PAGE, transferred to nitrocellulose, and immunoblotted with anti-PLC γ 1 mAb (bottom, 10^7 cell equivalents/lane) or 4G10 (top, 5×10^7 cell equivalents/lane).

Tyrosine phosphorylation of phospholipase C γ 1 (PLC γ 1) positively regulates its enzymatic activity (37); therefore this event also is thought to be physiologically relevant in TCR-mediated signaling. In a manner analogous to that described for ζ , we investigated tyrosine phosphorylation of PLC γ 1 in Jurkat, J45.01, J45/CH11, and J45/YP1.6 in response to increasing periods of TCR stimulation (Fig. 2 C). As shown, PLC γ 1 becomes tyrosine phosphorylated in J45/YP1.6 with similar kinetics to those observed for ζ , peaking after 5 min of TCR stimulation, compared with 1 min for both Jurkat and J45/CH11. Additionally, the amount of PLC γ 1 that becomes tyrosine phosphorylated varies considerably between the cell lines, with the bands representing phosphorylated PLC γ 1 from Jurkat being more intense than those from J45/CH11, which in turn are more intense than those from J45/YP1.6. Taken together, these results suggest that expression of the A2/YP chimera is sufficient for the TCR to couple with the PTK signal transduction pathway.

Activation of PLC γ 1 by tyrosine phosphorylation should lead to the generation of phosphatidylinositol-derived second messengers. We examined further the integrity of TCR signaling in J45/YP1.6 by measuring production of soluble IP in response to increasing periods of TCR stimulation (Fig. 3). TCR stimulation in J45/YP1.6 generates a threefold increase in soluble IP after 30 min, compared to a 6-fold induction in J45/CH11, and a 12-fold response in Jurkat. Additionally Jurkat and J45/CH11 demonstrate a response within 5 min, whereas J45/YP1.6 does not respond until 10 min of stimulation. This slower pattern of induction in J45/YP1.6 relative to J45/CH11 and Jurkat may reflect the differences in kinetics of PLC γ 1 tyrosine phosphorylation demonstrated among the cells (Fig. 2 C). Likewise, the differences in total

soluble IP induction may reflect the amount of PLC γ 1 that became tyrosine phosphorylated in each of the cells, which was greater in Jurkat relative to J45/CH11 relative to J45/YP1.6 (Fig. 2 C).

Expression of the A2/YP Chimeric Molecule Restores Distal Signal Transduction. We next assessed TCR-stimulated expression of the CD69 antigen (38, 39) as a more distal marker of activation in the four cell lines (Fig. 4). As shown, TCR ligation results in increased CD69 expression in Jurkat, but not J45.01. Both J45/CH11 and J45/YP1.6 express increased levels of CD69 after TCR ligation, however neither cell expresses this activation antigen to levels seen on the wild-type parent. Interestingly, J45/YP1.6 and J45/CH11 exhibit a similar augmentation of CD69 expression in response to TCR ligation, indicating that the differences in proximal signal transduction observed between the two cell lines do not result in differences in this measure of distal signaling.

A Phosphatase Inactive A2/YP Chimeric Molecule Is Unable to Support Signal Transduction. To address whether the tyrosine phosphatase activity of the A2/YP chimera was responsible for its ability to restore signaling in J45.01, we constructed a phosphatase-deficient version of the molecule. Tyrosine phosphatases possess an active site cysteine residue which is essential for enzymatic activity (40). Thus we generated an A2/YP molecule with the essential cysteine residue mutated to an alanine. To demonstrate that this molecule (A2/YPCA) possessed no enzymatic activity, we subcloned cDNA encoding the yeast phosphatase portion of the chimera into the prokaryotic expression vector pGEX-2T, and subjected bacterial-expressed fusion protein to an *in vitro* phosphatase assay. As shown, the cysteine to alanine mutation completely abrogates the enzymatic activity of the yeast

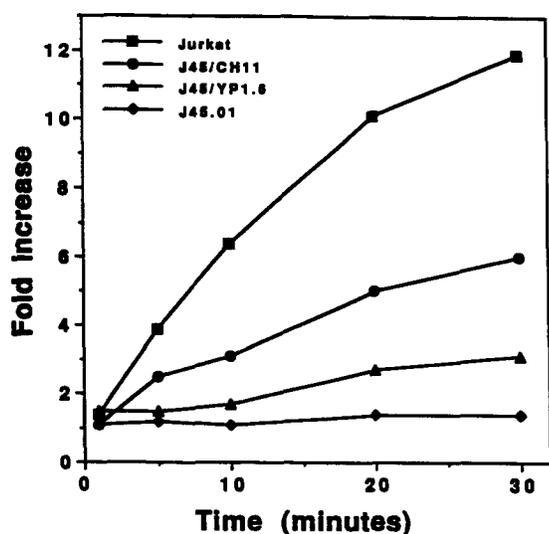


Figure 3. TCR-mediated soluble IP production in Jurkat, J45.01, J45/CH11, and J45/YP1.6. Soluble IP production was measured after stimulation of the four cell lines with medium alone (time = 0), or with anti-TCR mAb (C305 ascites, final concentration 1:2,500) for the indicated periods of time.

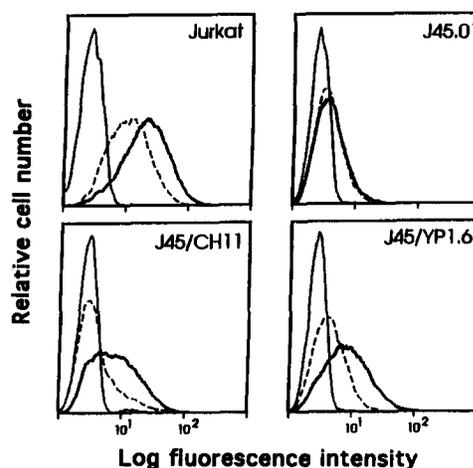


Figure 4. TCR-mediated CD69 expression in Jurkat, J45.01, J45/CH11, and J45/YP1.6. Cells were incubated overnight with media alone, or media plus C305 (ascites, final concentration 1:2,500), stained with control IgG or anti-CD69 mAb Leu23, and analyzed by flow cytometry. *Light solid lines*, control IgG (C305-stimulated cells); *dashed lines*, anti-CD69 (unstimulated cells); *heavy solid lines*, anti-CD69 (C305-stimulated cells).

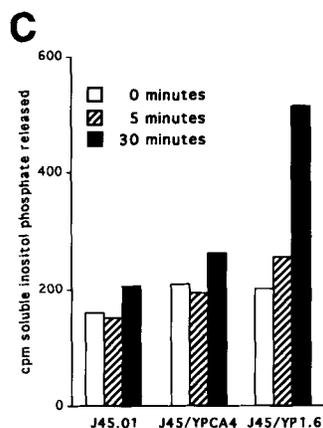
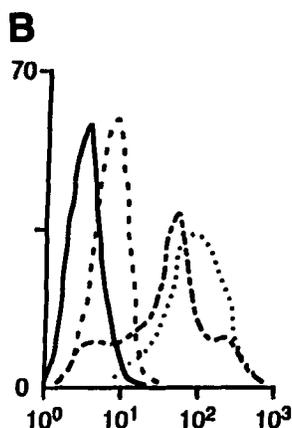
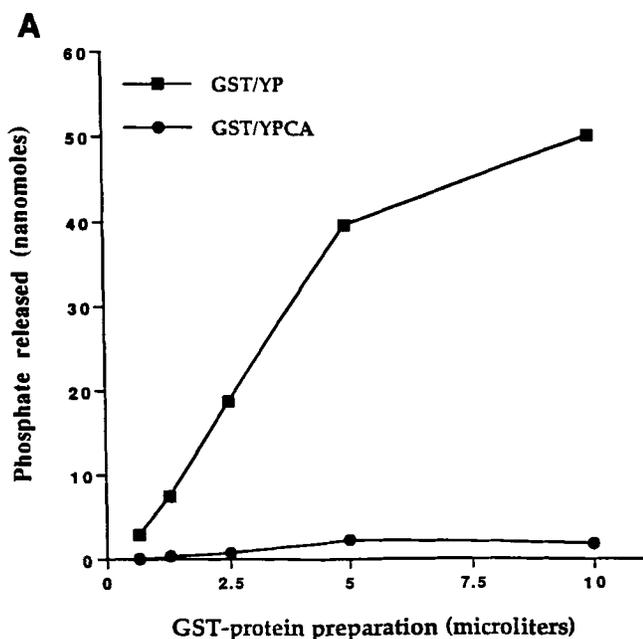


Figure 5. (A) Flow cytometric analysis of J45/YPCA4 (J45.01 transfected with the A2/YPCA chimera). Cells were stained with the negative control mAb, MOPC 195 (solid lines), anti-CD45 mAb, 9.4 (dashed lines), anti-A2 mAb CR11.351 (alternating dotted and dashed lines), or anti-TCR mAb Leu4 (dotted lines). (B) The cysteine to alanine mutation completely abolishes the enzymatic activity of the yeast phosphatase. Bacterial expressed GST/YP (yeast phosphatase) and GST/YPCA (yeast phosphatase containing the cysteine to alanine mutation) were subjected to an *in vitro* tyrosine phosphatase assay. (C) TCR ligation of J45/YPCA4 does not result in production of soluble IP. Soluble IP were measured in J45.01, J45/YPCA4, and J45/YP1.6 after stimulation with medium alone (time = 0), or with anti-TCR mAb (C305 ascites, final concentration 1:2,500) for the indicated periods of time.

phosphatase (Fig. 5 A). We transfected J45.01 with the A2/YPCA construct and established the J45/YPCA4 cell line (Fig. 5 B). Ligation of the TCR on these cells does not result in the activation of a PTK (not shown), nor does it result in the production of soluble IP (Fig. 5 C). These data provide the first formal evidence that plasma mem-

brane-associated tyrosine phosphatase activity is required for the TCR to couple to its signaling machinery.

Discussion

Much has been learned in recent years regarding the role of CD45 in TCR-mediated signal transduction. Evidence generated through the study of CD45-deficient T cell lines has demonstrated the essential role that this molecule plays in the regulation of signals initiated by ligation of the TCR. One proposed signal model for the function of CD45 in TCR-mediated signal transduction is that CD45 is required to dephosphorylate one or more src family PTK on their negative regulatory COOH-terminal tyrosine residues, allowing their function in the propagation of the TCR-initiated signal.

The experiments in this report describe J45/YP1.6, a cell line derived from J45.01, a CD45-deficient variant of the Jurkat human T cell leukemic line. J45/YP1.6 is a stable line transfected with cDNA encoding a chimeric protein consisting of the extracellular and transmembrane domains of the A2 allele of the MHC class I molecule in frame with a single domain nonreceptor tyrosine phosphatase from yeast (A2/YP). Expression of the A2/YP chimera partially rescues the TCR signaling defect exhibited by J45.01. Stimulation of the TCR on J45/YP1.6 results in the induction of PTK activity with resultant tyrosine phosphorylation of numerous cellular proteins including the ζ chain of the TCR and PLC- γ 1. As expected, these events result in the production of soluble inositol phosphates, leading eventually to more distal events associated with T cell activation such as the expression of the CD69 activation antigen. The signaling phenotype of J45/YP1.6 is in marked contrast to that of J45.01 which exhibits no response to TCR engagement. These data thus demonstrate that a molecule which has the appropriate enzymatic activity, but little other homology to CD45, can function to support TCR signaling.

Additionally, experiments in this report provide the first formal demonstration of the requirement for surface tyrosine phosphatase activity for effective TCR signal transduction. Data supporting this conclusion results from experiments using a second transfectant, J45/YPCA4. This cell line, also derived from J45.01, expresses a protein identical to the A2/YP chimera except for a single amino acid change that abolishes enzymatic activity. Although J45/YPCA4 expresses similar amounts of chimeric molecule as does J45/YP1.6, TCR stimulation fails to result in the appearance of second messengers.

Throughout the course of these experiments we noted differences in signaling ability, both between our transfectants when compared with each other, and between our transfectants when compared with wild-type Jurkat. The efficiency of TCR-mediated signal transduction in J45/YP1.6 is considerably reduced in comparison to both Jurkat, and to J45/CH11, a previously described transfectant of J45.01 which expresses an A2/CD45 chimeric molecule. Although J45/YP1.6 and J45/CH11 both demonstrate decreased signaling efficiency in comparison with Jurkat, the defect exhibited by

J45/YP1.6 is considerably greater than that demonstrated by J45/CH11, even though both transfectants express similar levels of A2 chimeric protein and TCR. One potential explanation for why both the A2/YP and A2/CD45 chimeras fail to restore TCR signaling to wild-type levels is that the extracellular and/or transmembrane domains of CD45 mediated protein-protein interactions with other molecules that may potentiate the TCR signaling response. Likewise, one possible explanation for the observed discrepancy of signaling in J45/CH11 relative to J45/YP1.6 is that the yeast phosphatase lacks CD45 cytoplasmic sequences that optimize additional protein-protein interactions, possibly including those that direct interactions with physiological substrates.

An additional potential explanation for the discrepancy in signaling observed between J45/YP1.6 and J45/CH11 is that the A2/YP molecule possesses only one enzymatically active tyrosine phosphatase domain, while CD45 and the A2/CD45 chimeric molecule each contained two tandem tyrosine phosphatase catalytic domains. Although the relative contribu-

tion of each domain to the overall activity of CD45 remains controversial, evidence suggests that both domains may play important roles. However, preliminary evidence from our laboratory indicates that transfection of J45.01 with an A2/CD45 chimera bearing a cysteine to alanine substitution in the second phosphatase domain also rescues TCR signal transduction (data not shown). Currently we are performing quantitative signaling assays to determine if there are consistent differences in signaling ability of our cells containing various CD45 cytoplasmic domain mutations. Additionally, we are constructing a cDNA which encodes extracellular and transmembrane domains from A2 in frame with two tandem yeast phosphatase domains with a spacer of similar length to the CD45 interphosphatase region. Transfection of these cDNAs into CD45-deficient cell lines followed by evaluation of TCR function should help to clarify further the structural requirements for tyrosine phosphatases involved in the TCR signaling pathway.

We thank S. Ross for her excellent technical assistance.

This work was supported by National Institutes of Health grants CA-56843 and CD-56050 and the Carver Trust at the University of Iowa (G. Koretzky) and National Institutes of Health Training Grants HL-07638 (D. Motto) and AI-07260 (M. Musci).

Address correspondence to Dr. Gary A. Koretzky, Department of Internal Medicine, University of Iowa College of Medicine, 200 Hawkins Drive, Iowa City, IA 52242-1081.

Received for publication 4 May 1994 and in revised form 22 June 1994.

References

1. Pingel, J.T., and M.L. Thomas. 1989. Evidence that the leukocyte-common antigen is required for antigen-induced T lymphocyte proliferation. *Cell*. 58:1055.
2. Koretzky, G.A., J. Picus, M.L. Thomas, and A. Weiss. 1990. Tyrosine phosphatase CD45 is essential for coupling T cell antigen receptor to the phosphatidylinositol pathway. *Nature (Lond.)*. 346:66.
3. Koretzky, G.A., J. Picus, T. Schultz, and A. Weiss. 1991. The tyrosine phosphatase CD45 is required for T cell antigen receptor and CD2-mediated activation of a protein tyrosine kinase and interleukin 2 production. *Proc. Natl. Acad. Sci. USA*. 88:2037.
4. Volarević, S., B.B. Niklinska, C.M. Burns, H. Yamada, C.H. June, F.J. Dumont, and J.D. Ashwell. 1992. The CD45 tyrosine phosphatase regulates phosphotyrosine homeostasis and its loss reveals a novel pattern of late T cell receptor-induced Ca^{2+} oscillations. *J. Exp. Med.* 176:835.
5. Shiroo, M., L. Goff, M. Biffen, E. Shivnan, and D. Alexander. 1992. CD45-tyrosine phosphatase-activated p59^{lck} couples the T-cell antigen receptor to pathways of diacylglycerol production, protein kinase-C activation and calcium influx. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:4887.
6. Koretzky, G.A., M.A. Kohmetscher, T. Kadleck, and A. Weiss. 1992. Restoration of T cell receptor mediated signal transduction of CD45 cDNA into a CD45-deficient variant of the Jurkat T cell line. *J. Immunol.* 149:1138.
7. Hovis, R.R., J.A. Donovan, M.A. Musci, D.G. Motto, F.D. Goldman, S.E. Ross, and G.A. Koretzky. 1993. Rescue of signaling by a chimeric protein containing the cytoplasmic domain of CD45. *Science (Wash. DC.)*. 260:544.
8. Volarevic, S., B.B. Niklinska, C.M. Burns, C.H. June, A.M. Weissman, and J.D. Ashwell. 1993. Regulation of TCR signaling by CD45 lacking transmembrane and extracellular domains. *Science (Wash. DC)*. 260:541.
9. Desai, D.M., J. Sap, J. Schlessinger, and A. Weiss. 1993. Ligand-mediated negative regulation of a chimeric transmembrane receptor tyrosine phosphatase. *Cell*. 73:541.
10. June, C.H., M.C. Fletcher, J.A. Ledbetter, and L.E. Samelson. 1990. Increases in tyrosine phosphorylation are detectable before phospholipase C activation after T cell receptor stimulation. *J. Immunol.* 144:1591.
11. Samelson, L.E., M.D. Patel, A.M. Weissman, J.B. Harford, and R.D. Klausner. 1986. Antigen activation of murine T cells induces tyrosine phosphorylation of a polypeptide associated with the T cell antigen receptor. *Cell*. 46:1083.
12. Straus, D.B., and A. Weiss. 1992. Genetic evidence for the in-

- volvement of the *lck* tyrosine kinase in signal transduction through the T cell antigen receptor. *Cell*. 70:585.
13. Appleby, M.W., J.A. Gross, M.P. Cooke, S.D. Levin, Z. Quan, and R.M. Perlmutter. 1992. Defective T cell receptor signaling in mice lacking the thymic isoform of p59^{lck}. *Cell*. 70:751.
 14. Karnitz, L., S.L. Sutor, T. Torigoe, J.C. Reed, M.P. Bell, D.J. McKean, P.J. Leibson, and R.T. Abraham. 1992. Effects of p56^{lck} deficiency on the growth and cytolytic effector function of an interleukin-2-dependent cytotoxic T-cell line. *Mol. Cell. Biol.* 12:4521.
 15. Chan, A.C., M. Iwashima, C.W. Turck, and A. Weiss. 1992. ZAP-70: a 70 kd protein-tyrosine kinase that associates with the TCR ζ chain. *Cell*. 71:649.
 16. Arpaia, E., M. Shahar, H. Dadl, A. Cohen, and C.M. Roifman. 1994. Defective T cell receptor signaling and CD8+ thymic selection in humans lacking Zap-70 kinase. *Cell*. 76:947.
 17. Iwashima, M., B.A. Irving, N.S.C. van Oers, A.C. Chan, and A. Weiss. 1994. Sequential interactions of the TCR with two distinct cytoplasmic tyrosine kinases. *Science (Wash. DC)*. 263:1136.
 18. Cooper, J.A., K.A. Gould, C.A. Cartwright, and T. Hunter. 1986. Tyr 527 is phosphorylated in pp60^{c-src}: implication for regulation. *Science (Wash. DC)*. 231:1431.
 19. Abraham, N., and A. Veillette. 1990. Activation of p56^{lck} through mutation of a regulatory carboxy-terminal tyrosine residue requires intact sites of autophosphorylation and myristylation. *Mol. Cell. Biol.* 10:5197.
 20. Donovan, J.A., F.D. Goldman, and G.A. Kortezy. 1994. Restoration of CD2-mediated signaling by a chimeric membrane protein including the cytoplasmic sequence of CD45. *Hum. Immunol.* 40:123.
 21. Ostergaard, H.L., D.A. Shackelford, T.R. Hurley, P. Johnson, R. Hyman, B.M. Sefton, and I.S. Trowbridge. 1989. Expression of CD45 alters phosphorylation of the *lck*-encoded tyrosine protein kinase in murine lymphoma T-cell lines. *Proc. Natl. Acad. Sci. USA*. 86:8959.
 22. Hurley, T.R., R. Hyman, and B.M. Sefton. 1993. Differential effects of expression of the CD45 tyrosine protein phosphatase on the tyrosine phosphorylation of the *lck*, *fyn*, and *c-src* tyrosine protein kinases. *Mol. Cell. Biol.* 13:1651.
 23. Cahir McFarland, E.D., T.R. Hurley, J.T. Pingel, B.M. Sefton, A. Shaw, and M.L. Thomas. 1993. Correlation between *Src* family member regulation by the protein-tyrosine-phosphatase CD45 and transmembrane signaling through the T-cell receptor. *Proc. Natl. Acad. Sci. USA*. 90:1402.
 24. Kortezy, G.A., M. Kohmetscher, and S. Ross. 1993. CD45-associated kinase activity requires *lck*, but not T cell receptor expression in the Jurkat T cell line. *J. Biol. Chem.* 268:8958.
 25. Ross, S.E., B. Schraven, F.E. Goldman, J. Crabtree, and G.A. Kortezy. 1994. The association between CD45 and *lck* does not require CD4 or CD8 and is independent of T cell receptor stimulation. *Biochem. Biophys. Res. Comm.* 198:88.
 26. Schraven, B., H. Kirchgessner, B. Gaber, Y. Samstag, and S. Meuer. 1991. A functional complex is formed in human T lymphocytes between the protein tyrosine phosphatase CD45, the protein tyrosine kinase p56^{lck} and pp32, a possible common substrate. *Eur. J. Immunol.* 21:2469.
 27. Schraven, B., A. Schirren, H. Kirchgessner, B. Siebert, and S.C. Meuer. 1992. Four CD45/P56^{lck}-associated phosphoproteins (pp29-pp32) undergo alterations in human T cell activation. *Eur. J. Immunol.* 22:1857.
 28. Tonks, N.K., H. Charbonneau, C.D. Diltz, E.H. Fischer, K.A. Walsh. 1988. Demonstration that the leukocyte common antigen CD45 is a protein tyrosine phosphatase. *Biochemistry*. 27:8695.
 29. Guan, K., R.J. Deschenes, H. Qiu, and J.E. Dixon. 1991. Cloning and expression of a yeast protein tyrosine phosphatase. *J. Biol. Chem.* 266:12964.
 30. Weiss, A., and J.D. Stobo. 1984. Requirement for the coexpression of T3 and the T cell antigen receptor on a malignant human T cell line. *J. Exp. Med.* 160:1284.
 31. Orloff, D.G., S.J. Frank, F.A. Robey, A.M. Weissman, and R.D. Klausner. 1989. Biochemical characterization of the η chain of the T-cell receptor: a unique subunit related to ζ . *J. Biol. Chem.* 264:14812.
 32. Goldsmith, M.A., D.M. Desai, T. Schultz, and A. Weiss. 1989. Function of a heterologous muscarinic receptor in T cell antigen receptor signal transduction mutants. *J. Biol. Chem.* 264:17190.
 33. Smith, D.B., and K.S. Johnson. 1988. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene*. 67:31.
 34. Weiss, A., G. Kortezy, R. Schatzman, and T. Kadlecik. 1991. Functional activation of the T-cell antigen receptor induces tyrosine phosphorylation of phospholipase C- γ 1. *Proc. Natl. Acad. Sci. USA*. 88:5484.
 35. Secrist, J.P., L. Karnitz, and R.T. Abraham. 1991. T-cell antigen receptor ligation induces tyrosine phosphorylation of phospholipase C γ 1. *J. Biol. Chem.* 266:12135.
 36. Ravichandran, K.S., K.K. Lee, Z. Songyang, L.C. Cantley, P. Burn, and S.J. Burakoff. 1993. Interaction of Shc with the ζ chain of the T cell receptor upon T cell activation. *Science (Wash. DC)*. 262:902.
 37. Nishibe, S., M.I. Wahl, S.M.T. Hernandez-Sotomayor, N.K. Tonks, S.G. Rhee, and G. Carpenter. 1990. Increase of the catalytic activity of phospholipase C- γ 1 by tyrosine phosphorylation. *Science (Wash. DC)*. 250:1253.
 38. Hara, T., L.K.L. Jung, J.M. Bjorndahl, and S.M. Fu. 1985. Human T cell activation. III. Rapid induction of a phosphorylated 28 kD/32 kD disulfide-linked early activation antigen (EA 1) by 12-*o*-tetradecanoyl phorbol-13-acetate, mitogens, and antigens. *J. Exp. Med.* 164:1988.
 39. Testi, R., J.H. Phillips, and L.L. Lanier. 1989. Leu 23 induction as an early marker of functional CD3/T cell antigen receptor triggering: Requirement for receptor crosslinking, prolonged elevation of intracellular [Ca²⁺] and stimulation of protein kinase C. *J. Immunol.* 142:1854.
 40. Guan, K., and J.E. Dixon. 1991. Evidence for protein-tyrosine-phosphatase catalysis proceeding via a cysteine-phosphate intermediate. *J. Biol. Chem.* 266:17026.