The CD45 protein tyrosine phosphatase (PTPase) has been shown to regulate the activity of Lck and Fyn protein tyrosine kinases in T cells. However, it is not clear that these constitute the only CD45 substrates. Moreover, the manner by which PTPase activity and substrate recruitment are regulated, is poorly understood. Previous in vitro studies suggest that the first cytoplasmic PTPase domain (D1) of CD45 is the active PTPase, which may be regulated by an enzymatically inactive second PTPase domain (D2). However, the function of CD45 D2 in vivo is unknown. In this study, reconstitution of CD45+ T cells with specific CD45 PTPase mutants allowed demonstration of a critical role for D2 in TCR-mediated interleukin (IL)-2 production. Specifically, replacement of CD45 D2 with that of the LAR PTPase to form a CD45/LAR-D2 chimera, abrogates CD45-dependent IL-2 production. This effect cannot be accounted for by loss of PTPase activity per se. The expression of D1 substrate-trapping mutants reveals an in vivo interaction between CD45 and TCR-ζ that is dependent on CD45 D2. Thus, cells expressing CD45 lacking D2 exhibit abnormal TCR-mediated signaling characterized by hyperphosphorylation of ζ and deficient ZAP-70 phosphorylation. These data suggest an essential role for CD45 D2 in TCR-regulated IL-2 production through substrate recruitment of the ζ chain.

CD45 is a family of transmembrane PTPases1 critically involved in lymphocyte activation. Multiple alternatively spliced isoforms differ in the length and glycosylation of their extracellular domains but share identical cytoplasmic PTPase domains (1, 2). CD45− mutant T cell lines fail to normally phosphorylate cellular proteins or produce IL-2 after TCR ligation (3, 4). Initial studies comparing CD45− mutants to wild-type or revertant cells indicated that CD45 up-regulates Lck and Fyn activity by dephosphorylating their negative regulatory (COOH-terminal) tyrosine sites (5–8). However, subsequent analysis demonstrated that CD45 can also down-regulate Fyn and Lck activity by dephosphorylating their positive regulatory autophosphorylation sites (9, 10). It is also not clear that these PTKs constitute the only CD45 substrates.

Cytoplasmic PTPases appear to be targeted to their appropriate substrates by protein interaction and cellular localization motifs (11). Such interactions with target proteins may also enhance the activity of these PTPases (12, 13). However, for transmembrane PTPases, very little is known about how substrate specificity and activity are regulated. Recent evidence indicates that the extracellular domain is able to regulate signaling through the cytoplasmic PTPase domains (14–17). In this regard, most transmembrane PTPases, including CD45, share tandem PTPase domains (18, 19). Curiously, the second PTPase domain (D2) of most of these molecules appears to have little or no PTPase activity against various in vitro substrates. Furthermore, PTPase consensus sequences in D2 are less well conserved than in domain 1 (D1), and in some cases, key residues required for enzymatic activity (for example, the catalytic center Cys residue), are absent (20, 21). Such findings suggest an alternative role for D2 in transmembrane PTPases.

In the case of CD45, mutation of the D1 catalytic center Cys to Ser (position 828) completely abrogates in vitro PTPase activity (22, 23), whereas, mutation of the analogous Cys to Ser in D2 (position 1144), has no effect. While, deletion of D2 in its entirety destabilizes D1 rendering it inactive (22, 23), small deletions within D2 strikingly alter the relative activity of CD45 against various substrates in vitro. These results suggest that D2 is not an active PTPase but may play a regulatory role for D1 (22). Nevertheless, it has been reported that deletion of large portions of D1 can activate D2 in vitro (24), at least raising the possibility that D2 could have cryptic PTPase activity in vivo.

Although Cys to Ser (CS) mutation of the catalytic center inactivates PTPase activity, substrate binding is preserved and substrate trapping within the enzyme active site is actually promoted (19, 25). Glutathione S-transferase-CD45 fusion proteins containing CS mutation of D1 were able to co-precipitate phospho-ζ from lysates obtained from activated Jurkat cells (26). When D1 was active, phospho-ζ was no longer co-precipitated, suggesting that ζ might be a direct substrate for CD45 D1. Swapping either CD45 D1 or D2 with the corresponding domain from the LAR PTPase prevented this in vitro association. These findings suggested that CD45 D2 may influence D1 by playing a role in substrate recruitment. To establish a regulatory role for D2 among transmembrane PTPases and identify in vivo substrates critical to our understanding of CD45 function, we now address the physiologic role of CD45 D2 and substrate recruitment of ζ in TCR-mediated signal transduction.

We have transfected a CD45− version of the Jurkat human T cell lymphoblastoid line with CS mutation of D2 carrying the D2 domain of LAR and examined the expression of CD45 in these cells by an in vivo interaction assay (25). The results demonstrated that CD45 can also down-regulate Fyn and Lck activity by dephosphorylating their positive regulatory autophosphorylation sites (9, 10). It is also not clear that these PTKs constitute the only CD45 substrates.
leukemic T cell line with either wild-type CD45 or PTPase mutants. Direct comparison of these cell lines demonstrates that IL-2 production is dependent on an intact CD45 D1 PTPase. Importantly, we now show that CD45 D2 is also critical for IL-2 production. However, this is not related to loss of D2 PTPase activity per se, nor by a requirement for D2 in the interaction between CD45 and Lck. While enzymatically inactive substrate-trapping mutants of CD45 D1 coprecipitate phospho-ζ, this interaction is dependent on the presence of CD45 D2. Despite active CD45 D1, replacement of CD45 D2 with that of LAR resulted in hyperphosphorylation of TCR-ζ and deficient activation-induced phosphorylation of ZAP-70. These data support a mechanism whereby CD45 D2 regulates IL-2 production through the recruitment of TCR-ζ as a CD45 substrate.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs**—The CD45(0) construct encodes wild-type (wt) CD45 PTPase domains in the context of the smallest CD45 extracellular domain under control of the SRE promoter (15). Plasmids encoding the isolated CD45 cytoplasmic domains containing CS point mutations of: D1 (position 829), D2 (position 1144), or wt CD45 D1 (amino acids 584–895) fused to wt LAR D2 (amino acids 1500–1881) (26, 27) were kindly provided by Dr. H. Saito (Dana-Farber Cancer Institute, Boston, MA). Using convenient restriction sites, segments of these constructs were used to replace the analogous wt region of CD45(0) generating full-length (transmembrane) CD45 PTPase mutants. A double mutant, containing CS mutations in both D1 and D2, was generated using Psfl sites. Using polymerase chain reaction, a CS mutation was inserted into position 828 of D2:LAR to generate D1:CS/D2:LAR. DNA sequencing (Keck Biotechnology Center, Yale University) confirmed the expected mutation.

**Cell Lines and Transfectants**—The CD4+ CD45 J-AS Jurkat clone lacks endogenous CD45 expression by virtue of a stably integrated antisense gene targeted to the 5′-untranslated region of CD45. The J-AS was transfected with CD45(0) constructs encoding either wt or mutant PTPase domains along with the PGK-bgal vector by electroporation, as described (15). Hygromycin-resistant clones were screened for CD45 expression by immunofluorescence and Western blotting. Two previously described clones expressing the wt CD45(0) isoform, J-0(2) and J-0(3), were also used in these studies (16). All cells were grown in RPMI 1640 media supplemented with 10% iron-fortified calf serum, L-glutamine, and gentamycin. Transfectants were removed from G418 and hygromycin for 7–10 days before use in assays.

**Antibodies and Immunofluorescence Phenotyping**—Cell phenotype was routinely monitored for CD3, CD4, and CD45 expression using commercially available mAbs and analyzed on a FACSTAR IV (Becton Dickinson, Mountain View, CA) (10,000 cells/sample), as described (15). Anti-CD45 (9A4), and anti-CD3 (OKT3) hybridomas were from the ATCC. mAbs were purified from culture supernatants using Protein-A-Sepharose according to standard methods. The following were kindly provided as gifts: anti-phosphotyrosine (anti-Tyr(P)) mAb 4G10 (from Dr. B. Drucker, University of Oregon, Portland, OR), anti-ζ mAb B610.2 (from Dr. A. Weiss, University of California at San Francisco, San Francisco, CA), polyclonal rabbit anti-CD3 (J-3), polyclonal mouse anti-CD45 (ICD-10) obtained from Dr. K. Bottomly, Yale University, New Haven, CT, and polyclonal rabbit anti-Lck (from Dr. K. Ruddle, Dana-Farber Cancer Institute, Boston, MA).

**IL-2 Secretion**—10^5 cells/well in triplicate flat bottom 96-well tissue culture plates were stimulated with anti-CD3 (OKT3) (from 0.1 to 0.01 μg/ml) plus goat anti-mouse cross-linking (at a 1:1 ratio with OKT3). Phorbol 12-myristate 13-acetate (1 ng/ml) was added to all wells, as described (15). IL-2 secretion in 20-h cell culture supernatants was determined by enzyme-linked immunosorbent assay (Genzyme Corp.). In each experiment, the data were normalized to the response of transfectants expressing (wt) CD45(0) to 0.1 μg/ml anti-CD3 (15). IL-2 secretion after stimulation with 1 ng/ml phorbol 12-myristate 13-acetate and 1 μM ionomycin, was used as a positive control.

**Cellular Activation and Lysis**—As described previously (15, 16), cells (5 × 10^5/ml) were stimulated at 37 °C with either anti-CD3 (17 μg/ml) or pervanadate (3 mM H2O2, 100 mM Na3VO4) plus 10 mM phenylarsine oxide, which mimics the effects of TCR ligation (28, 29). After the indicated times, ice-cold stop solution (phosphate-buffered saline with phosphatase inhibitors) was added, cells were pelleted and lysed in ice-cold 1% Brij-97 lysis buffer (CD45 immunoprecipitation) or 1% Nonidet P-40 (ζ immunoprecipitation) containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM aminothiolsulfonyl fluoride (AEBSF), 10 mg/ml aprotinin, 10 mg/ml leupeptin, 10 mM iodoacetamide, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate, as described (15, 16).

**Immunoprecipitation and Immunoblotting**—Immunoprecipitations were performed as described (15, 16). Briefly, after precollecting, postnuclear supernatants containing equivalent amounts of protein (DC protein assay; Bio-Rad) were incubated with antibody, followed by immunoprecipitation with Protein A-Sepharose (UBI). Immunoprecipitates or postnuclear supernatants (whole cell lysates) were subjected to SDS-PAGE and transferred to nitrocellulose. Membranes were blocked (5% nonfat milk in phosphate-buffered saline) and probed with primary antibody followed by horseradish peroxidase-conjugated secondary antibody, and developed with enhanced chemiluminescence.

**In Vitro Kinase Assay**—Cells expressing either CD45(0) or D2:LAR were lysed in 1% digitonin, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, AEBSF, aprotinin, and leupeptin (as above), followed by immunoprecipitation with anti-CD45, anti-Lck, or control rabbit anti-mouse Ab. After washing 4 times, immunoprecipitates were resuspended in 40 μl of kinase buffer (25 mM HEPEs (pH 7.6), 10 mM MnCl₂ plus 10 μM of [γ-32P]ATP), for 15 min, as described (30). Sepharose beads were then washed twice in lysis buffer, boiled in Laemmli sample buffer, run on 12% SDS-PAGE, and subjected to autoradiography.

**PTPase Assay**—After lysis in 0.5% Triton X-100 containing 20 mM Tris (pH 7.5), 150 mM NaCl, 2 mM EDTA, and AEBSF, aprotinin, and leupeptin (as above), immunoprecipitates from each cell line were extensively washed and resuspended in 25 mM imidazole (pH 7.2), 50 mM NaCl, 1 mM EDTA with 5 mM dithiothreitol. Equal amounts of each CD45 protein, as confirmed by Western analysis, were analyzed for PTPase activity using the tyrosine phosphatase assay (Promega Corp., Madison WI) which determines the concentration of free phosphate released from a tyrosyl-phosphorylated peptide substrate by the absorbance of a molybdate-malachite green-phosphate complex. Both phosphopeptide substrates, ENDpYINASL and DADE(pY)LIPQQG, supplied with the assay gave equivalent results.

**RESULTS**

Because of the potential regulatory role of the CD45 extracellular domain (15, 16) and association of LPA with the CD45 transmembrane domain (31, 32), the physiologic function of the CD45 PTPase domains was addressed using intact transmembrane CD45 molecules. To this end, CD45 J-AS Jurkat cells were stably transfected to express either wild-type (wt) CD45, or PTPase mutations in the context of the smallest (CD45RO or CD45(0)) extracellular domain. The J-AS parental cell line was initially generated by means of antisense gene targeting of endogenous CD45, as we described (15). These cells combine specific targeting of CD45 with expression levels that are undetectable by Western analysis. J-AS cells were stably transfected with (wt) CD45(0), inactivating CS point mutation of the catalytic center of CD45 domain 1 (D1:CS), domain 2 (D2:CS), or domains 1 and 2 (D1:CS/D2:CS) (see Fig. 1). In addition, chimeric proteins containing D2 of the LAR PTPase, either in the context of active CD45 D1 (D2:LAR) or inactive CD45 D1 (D1:CS/D2:LAR) were expressed. The transfectants expressed similar levels of CD45, CD3, and CD4 (See Fig. 2). Expression of only a single CD45 isoform of 180 kDa was confirmed by Western blotting (as seen in Fig. 6C). Two independent transfected clones expressing each CD45 construct were selected for these studies.

Before examining the effects of these CD45 mutations on cellular function, the PTPase activity of each was examined in vitro (see Fig. 3). Consistent with previous analysis of isolated cytoplasmic domains, CS mutation of D1 (D1:CS), or D1 and D2 (D1:CS/D2:CS), abrogates the PTPase activity of intact CD45 molecules immunoprecipitated from such transfected cells. Likewise, CS mutation of CD45 D2 alone (D2:CS) was without detectable effect (22, 23). Importantly, comparison of the D2: LAR chimera to wild-type CD45 (CD45(0)) shows that the chimeric protein retains full catalytic activity (Fig. 3). This is not surprising, since D2 of both LAR and CD45 lacks activity in vitro and are believed to share similar structure based on conservation of grouped residues at key positions and ~40%
To examine the in vivo role of CD45 D2, TCR-mediated IL-2 production was assessed (See Fig. 4). In agreement with our previous results, CD45<sup>2</sup> J-AS cells produce only small amounts of IL-2 in response to anti-CD3 stimulation and IL-2 production is reconstituted by expression of the CD45(0) isoform (15). Expression of CD45 containing CS mutations of both D1 and D2 eliminates the restoration of IL-2 production, confirming the principal role of PTPase activity in CD45 function. Consistent with results suggesting that CD45 D1 contains most if not all of the detectable PTPase activity (Fig. 3) (22, 23), mutation of CD45 D1 alone (D1:CS) also prevented IL-2 secretion, whereas, mutation of the D2 catalytic center (D2:CS) resulted in only a small decrease in IL-2 production that was not statistically different from wt CD45(0). Similar results were obtained using concentrations of anti-CD3 ranging from 0.1 to .01 mg/ml (Fig. 4). Each cell line displayed a similar inherent capacity to produce IL-2 when the proximal signaling apparatus was bypassed using phorbol 12-myristate 13-acetate and ionomycin (data not shown). These data indicate that if D2 does exhibit cryptic PTPase activity in vivo, it does not play a significant role in TCR-induced IL-2 production. Concordantly, expression of chimeric proteins containing active CD45 D1 (in the presence of wt or inactive D2) in CD45<sup>2</sup> cells was necessary and sufficient to reconstitute TCR-mediated tyrosine phosphorylation of cellular proteins and dephosphorylate Lck at its carboxyl-terminal regulatory site (34, 36). Taken together, these results cast doubt on physiologically significant PTPase activity of CD45 D2 in vivo.
Construct were examined (*, not significant; †, not significant). J-AS cells were stimulated with anti-CD3 concentrations. These results suggest that CD45 D2 plays an important regulatory role in CD45 function and this is not explained by loss of D2 PTPase activity per se.

One explanation for these findings is that CD45 D2 plays an important role in substrate recruitment. In this regard, Lck acts as a CD45 substrate and co-precipitates with CD45 in non-disruptive detergents (30, 37, 38). Although Lck interacts with the CD45 cytoplasmic domain (39, 40), it is unknown whether CD45 D2 is involved. To determine whether D2 is required for this interaction, we compared overall and CD45-associated Lck activity in cells expressing either CD45(0) or D2:LAR using immune complex kinase assays. As seen in Fig. 5A, equivalent Lck activity, as measured by Lck autophosphorylation (56–60 kDa band), is precipitated from both cell lines. Thus, loss of CD45 D2 does not appear to affect overall Lck activity. CD45 immunoprecipitates from both transfectants reveal autophosphorylated Lck, as well as associated LPAP (32–34 kDa) which undergoes in vitro phosphorylation in this assay, as described (30, 37, 38). While only a small fraction of overall cellular Lck activity is associated with CD45, CD45 immunoprecipitates from cell lines expressing wild-type CD45 and the D2:LAR chimera contain similar Lck activity. Lysates used for immunoprecipitation from both cell lines contained equivalent amounts of CD45 by immunoblotting (Fig. 5B). Thus, Lck association with the cytoplasmic domain of CD45 is not dependent on CD45 D2 and this does not explain the decreased IL-2 production by cells expressing D2:LAR.

Another approach for studying the interaction of CD45 with its substrate is to utilize substrate-trapping PTPase mutants. While point mutation of the PTPase catalytic center Cys to Ser maintains substrate binding, it prevents cleavage of the phosphate ester and inhibits substrate release. Previously, Futukawa et al. (26) showed that glutathione S-transferase-CD45 fusion proteins containing a CS mutation in D1 could precipitate phospho-ζ from lysates obtained from activated Jurkat cells. To determine whether the TCR-ζ chain associates with CD45 in vivo, we examined CD45 immunoprecipitates from transfectants expressing both wild-type and substrate-trapping mutations of CD45(0). (We have previously shown that stimulation of CD45− (J-AS) cells results in tyrosine phosphorylation of the same general spectrum of proteins seen in parental (wt) Jurkat cells, only with delayed kinetics (15). Cells were either unstimulated or stimulated with phenylarsine oxide plus pervanadate (28, 29) to maximize the signal and eliminate any possibility of direct precipitation of signaling components by anti-CD3. Anti-CD45 immunoprecipitates from activated cells expressing D1:CS were immunoblotted with anti-ζ, revealing prominent bands at 21 and 23 kDa, consistent with the migration of phosphorylated ζ (Fig. 6A). In agreement, ζ was only co-precipitated with CD45 after cellular stimulation. Reprobing the same membrane with anti-phosphotyrosine (anti-Tyr(P)) confirms the association of phospho-ζ with CD45.
D1:CS (Fig. 6A). Similar phospho-ζ bands were also co-precipitated with CD45 from cells expressing CD45 D1:CS/D2:CS. In marked contrast, anti-ζ and anti-Tyr(P) immunoblots of CD45 immunoprecipitates from cells expressing wt CD45(0) revealed only trace amounts of material at 23 kDa (Fig. 6, A and B). Likewise, CD45 D2:CS (which contains wt D1) co-precipitates only traces of phospho-ζ. These data strongly suggest that phospho-ζ binds to the catalytic center of CD45 D1 where under normal circumstances, it is dephosphorylated by the wt PTPase and is released. Inactivation of D2 was without notable effect, consistent with a lack of enzyme activity of this domain in vivo.

To determine whether D2 plays a significant role in substrate interactions in vivo, we next examined the ability of CD45 D1:CS substrate-trapping mutants to co-precipitate ζ in the context of the LAR D2 (D1:CS/D2:LAR). As seen in Fig. 6, A and B, this chimera failed to co-precipitate ζ. Thus, CD45 D2 is required for the interaction between CD45 D1 and ζ.

Since the anti-CD45 mAbs examined were unable to recognize CD45 on immunoblots run in reducing conditions, the same membranes were not reprobed with anti-CD45. Rather, equivalent amounts of lysate from each cell line were run on 8% SDS-PAGE under non-reducing conditions and immunoblotted with anti-CD45. As shown (Fig. 6C), lysates used for immunoprecipitation all contained equivalent amounts of CD45. In other experiments, duplicate anti-CD45 immunoprecipitates run under non-reducing conditions corroborated the results obtained from examination of lysates (not shown).

Taken together, these results support the notion that ζ is an in vivo substrate for CD45. Thus, to the extent that ζ phosphorylation by residual PTK activity would be unopposed, inactivation of CD45 PTPase activity might be expected to lead to an accumulation of phosphotyrosine on the ζ chain. To examine this point, TCR-ζ was immunoprecipitated from each of the transfectants and subjected to anti-phosphotyrosine immunoblotting (Fig. 7A). Comparison of immunoprecipitates from unstimulated cells reveals that ζ is constitutively hyperphosphorylated in CD45- J-AS cells, compared with those expressing wt CD45(0) (lane 1 versus lane 3). Consistent with findings that CD45 PTPase activity resides in D1, ζ is also hyperphosphorylated in cells expressing CD45 D1:CS (lane 9 versus lane 7).

As expected, anti-CD3-mediated stimulation of cells expressing wt CD45(0) (Fig. 7, lanes 4 and 8) results in increased phosphorylation of TCR-ζ despite active CD45 PTPase, presumably resulting from an activation-related increase in activity and recruitment of Lck and Fyn. Anti-CD3 stimulation of
J-AS cells also induce increased \( \zeta \) phosphorylation (lane 2). Thus, in response to activation, these CD45 \(^{-}\) Jurkat cells are able to augment the activity and/or recruitment of PTKs sufficiently to allow for further accumulation of phosphotyrosine on TCR-\( \zeta \), at least in the context of defective CD45-mediated \( \zeta \) dephosphorylation. Similar findings are observed in cells expressing CD45 D1:CS (lane 10).

If CD45 D2 is critical for the interaction of the \( \zeta \) chain with CD45, one might predict that cells expressing D2:LAR would also exhibit \( \zeta \) hyperphosphorylation, despite the presence of active CD45 D1 PTPase. Indeed, as shown in Fig. 7A (lanes 5 and 6), this is the case. Reprobing the membranes with anti-\( \zeta \) mAb 6B10 (which preferentially detects non-phosphorylated \( \zeta \) (16 kDa) on immunoblots) confirms immunoprecipitation of similar amounts of \( \zeta \) from each cell line (Fig. 7B). Thus, lack of either CD45 PTPase activity (D1:CS) or ability of CD45 to interact with substrate (D2:LAR) is associated with constitutive hyperphosphorylation of \( \zeta \) as well as decreased IL-2 secretion.

According to the current paradigm of T cell activation, phosphorylation of \( \zeta \) ITAMs results in SH2-mediated interaction with ZAP-70 (41). Subsequent phosphorylation of \( \zeta \)-associated ZAP-70 leads to its activation and propagation of downstream signals. Curiously, in CD45-deficient cells, hyperphosphorylation of \( \zeta \) is associated with decreased IL-2 secretion. Therefore, we next examined the recruitment and phosphorylation of ZAP-70 associated with \( \zeta \) immunoprecipitates from each cell line. As seen in Fig. 7D, after cellular activation, similar amounts of ZAP-70 are recruited by phospho-\( \zeta \) in each cell line. As expected, CD3-mediated activation of cells expressing wt CD45(0), results in significant tyrosine phosphorylation of associated ZAP-70 (Fig. 7C, lanes 3 versus 4 and 7 versus 8). In contrast, despite hyperphosphorylation of \( \zeta \) and equivalent recruitment of ZAP-70, cells lacking CD45 (J-AS) and those expressing inactive CD45 D1 (D1:CS), exhibit a marked decrease in ZAP-70 phosphorylation compared with cells expressing wt CD45. Unexpectedly, even in the presence of active CD45 D1, cells expressing D2:LAR also consistently displayed deficient activation-induced phosphorylation of ZAP-70 (Fig. 7C, lane 6 versus lane 4). Analysis of data from four independent experiments with a scanning densitometer demonstrates that activation-induced phosphorylation of ZAP-70 in cells lacking CD45, expressing inactive CD45 D1 (D1:CS), and those expressing chimeric CD45 containing domain two of LAR (D2:LAR) averages only 11–12.5% of the ZAP-70 phosphorylation observed in cells expressing wt CD45(0) (see Fig. 7E). Thus, optimal signal generation and ZAP-70 phosphorylation depends upon both active D1 PTPase and intact CD45 D2.

**DISCUSSION**

We now demonstrate that CD45 D2 plays a crucial role in IL-2 production not accounted for by its PTPase activity. To our knowledge, these findings constitute the first in vivo demonstration of the critical importance of D2 for the function of any transmembrane PTPase. Furthermore, we show that CD45(0) interacts with TCR-\( \zeta \) in vivo and that this is dependent on CD45 D2. In our study, the 21- and 23-kDa phosphorylated species of \( \zeta \) preferentially associated with CD45(0), but only when CD45 D1 was inactive. This suggests that the binding of \( \zeta \) to CD45 is phosphotyrosine dependent. Furthermore, inactivation of CD45 D1, or inability of \( \zeta \) to interact with active CD45 through mutation of D2, are both associated with \( \zeta \) hyperphos-
phorylation. Taken together, our findings provide strong evidence that ζ is a direct substrate for CD45 in vivo. The highly regulated phosphorylation of the ζ ITAMs (42) and loss of IL-2 secretion when interaction between CD45 and ζ is prevented, strongly suggest that this substrate interaction is of physiologic importance.

As CD45 lacks SH2 domains, phosphotyrosine-dependent binding of ζ to CS mutants suggests interaction with CD45 through the substrate binding pocket of at least one of the PTPase domains. This is supported by our observation that increasing the concentration of competitive PTPase inhibitors (such as vanadate or phenyl arsenic oxide) in the lysis buffer, is associated with decreased co-precipitation of ζ with CD45 (data not shown). Given that CS PTPase mutants exhibit substrate trapping (19, 25, 43), and that CD45 D1 is required for ζ binding in vitro (26), we favor the hypothesis that phospho-ζ binds to CD45 through an interaction at least with the D1 binding pocket. Cleavage of phosphate from the bound phosphotyrosine residue would effect release of the substrate molecule.

The D2 catalytic center may also be involved in substrate binding, but because it already lacks catalytic activity, CS mutation is not required for substrate trapping to occur. In this model, the binding pocket of inactive D2 (wt or CS mutation) would bind particular phosphotyrosyl residues of ζ for subsequent dephosphorylation (at that or other phosphotyrosyl residues) by active CD45 D1. However, given substitutions at conserved Arg and Asp residues involved in substrate interaction and catalysis by PTPases (33, 44), it is not certain whether the “active site” of CD45 D2 is capable of binding substrates. Furthermore, it is unclear whether the large CD45 cytoplasmic domains are folded in a manner that would allow cooperative binding and dephosphorylation of closely spaced phosphotyrosine residues such as those found on ζ. Resolution of the CD45 crystal structure is likely to clarify these issues.

While CD45 is known to play an important role in the regulation of Lck and Fyn activity, we now show that ζ also serves as an in vivo CD45 substrate. Phosphorylation of ζ by Lck and/or Fyn leads to recruitment and subsequent phosphorylation and activation of ZAP-70, critical steps in T cell activation (41). Thus, the regulation of ζ phosphorylation and dephosphorylation by CD45 makes its role in signaling more complex than previously envisaged. Indeed, Neumeister Kersh et al. (42) have recently shown that phosphorylation of the six tyrosine residues within the ζ ITAMs is highly ordered and exquisitely sensitive to the exact nature of the activation stimulus. A priori, dephosphorylation of ζ by active CD45 might be expected to prevent or reduce ZAP-70 binding and decrease its accessibility to Lck and Fyn within the TCR complex. This might raise the “threshold” for T cell activation or might be involved in terminating the activation event and allowing the TCR complex to “reset.” In addition, our data suggest that recruitment of ζ by CD45 D2 plays a crucial positive role in TCR-mediated IL-2 production.

One explanation for this finding is that dysregulation of the precise pattern of phosphorylation within the ζ ITAMs in the setting of CD45 deficiency, alters the interaction of ζ with ZAP-70 and other associated signaling molecules in a manner that inhibits effective downstream signaling. Thus, the hyperphosphorylated ζ seen in transfectants expressing mutant CD45, may not be functionally equivalent to the phosphorylation of ζ seen in cells expressing wt CD45(0). Comparison of the actual sites of ζ phosphorylation in these transfectants may be revealing.

In addition to hyperphosphorylation of ζ, the specific loss of CD45 expression in our model is associated with deficient phosphorylation of ZAP-70. The dissociation of ζ and ZAP-70 phosphorylation in this setting could result from differential requirements for the phosphorylation and dephosphorylation of these critical signaling molecules. For example, unopposed constitutive PTK activity in CD45- cells may be sufficient to hyperphosphorylate TCR-ζ when normal dephosphorylation of ζ by CD45 is absent. While TCR-mediated activation results in further accumulation of phosphotyrosine on ζ, in the face of CD45 deficiency, residual PTK activity appears insufficient to normally phosphorylate associated ZAP-70. This suggests that a “signaling threshold” exists for the phosphorylation of ZAP-70 and propagation of downstream signaling. Suboptimal signals would allow phosphorylation of ζ but not of ZAP-70. This notion is supported by studies examining the response of murine T cell clones to altered peptide ligands. For example, Neumeister Kersh et al. (42) have shown that strong antagonist ligands can induce significant phosphorylation of 23-kDa forms of phospho-ζ (albeit, still lacking phosphorylation of one tyrosine residue), whereas ZAP-70 phosphorylation was only achieved after stimulation with optimal (agonist) ligand.

T cells expressing active CD45 D1 plus LAR D2 exhibit a pattern of ζ hyperphosphorylation similar to that described above. Unexpectedly, the inability of phospho-ζ to interact with active CD45 D1 in these cells is also associated with deficient phosphorylation of ZAP-70. Thus, in the context of CD45 D2 replacement, the efficiency of signal transduction is insufficient to activate ZAP-70. Based on these results, we speculate that CD45 may play a scaffolding role required for juxtaposition of ζ-bound ZAP-70 with active Lck or Fyn. This would facilitate subsequent phosphorylation and activation of ZAP-70. Alternatively, as suggested above, dysregulation of the specific sites and/or kinetics of phosphorylation and dephosphorylation of these molecules may occur when ζ and CD45 no longer interact, resulting in ineffective TCR-mediated signal transduction.

An alternative interpretation of our findings is that replacement of CD45 D2 with that of LAR results in abnormal distribution of CD45. For example, fodrin has been shown to bind to the membrane-proximal portion of CD45 D2 (45), whereas, LAR D2 has been shown to interact with several cytoplasmic proteins, TRIO and LIP, which are expressed in T cells (46, 47). It is conceivable that loss of fodrin binding and/or acquisition of interactions with TRIO and LIP, alters the distribution of CD45 and interferes with its ability to interact with its substrates, including ζ. Thus, it could be argued that the critical role of PTPase D2 is in localization and not substrate binding per se. However, this would not explain the specific requirement for CD45 D2 for the binding of ζ by glutathione S-transferase fusion proteins in vivo (26). Furthermore, this interpretation still supports the conclusion that the interaction between ζ and CD45 depends on PTPase D2 and plays a critical role regulating signal transduction.

Stimulation of murine T cell clones with partial agonists/antagonists has been associated with a relative increase in the phosphorylation of lower ζ-specific species of phospho-ζ (21 kDa) and a relative decrease in the larger (more heavily phosphorylated) species migrating at 23 kDa (48, 49). However, both the 21- and 23-kDa bands are comprised of multiple ζ phospho-species and significant phosphorylation in the absence of CD45 may result from appropriately strong alternative ligands (42). Moreover, to our knowledge, such alterations in ζ phosphorylation have not been established in human T cells. Nonetheless, the recruitment of ZAP-70 to phospho-ζ and absence of ZAP-70 phosphorylation seen after murine T cells are stimulated with partial agonists (42, 48, 49), does resemble the signaling pattern seen in our model in the face of “ineffective” CD45. This raises the possibility that CD45, which is intimately involved in
regulating these signals, may be involved in the generation of anergy. Whether this underlies the ability of certain anti-CD45 mAbs to induce tolerance in animal models of renal and islet cell transplantation (50, 51), will require further investigation.

Acknowledgments—We thank Drs. Haruo Saito and Michel Streuli for CD45 cDNA constructs, and Drs. Kim Bottomly, David Leitenberg, and Arthur Weiss for kindly providing antibodies. We additionally thank Drs. Haruo Saito, Christopher Rudd, and Tomas Mustelin for critical review and helpful discussions.

REFERENCES

1. Ralph, S. J., Thomas, M. L., Morton, C. C. & Trowbridge, I. S. (1987) EMBO J. 6, 1251–1257
2. Streuli, M., Hall, L. R., Saga, Y., Schlossman, S. F. & Saito, H. (1987) J. Exp. Med. 166, 1548–1556
3. Pingel, J. T. & Thomas, M. L. (1989) Cell 58, 1055–1065
4. Koretzky, G. A., Pircs, J., Thomas, M. L. & Weiss, A. (1990) Nature 346, 66–68
5. Östergaard, H. L., Shackelford, D. A., Hurley, T. R., Johnson, P., Hyman, R., Sefton, B. M. & Trowbridge, I. S. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 8895–8963
6. Mustelin, T., Coggeshall, K. M. & Altman, A. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 6302–6306
7. Hurley, T. R., Hyman, R. & Sefton, B. (1993) Mol. Cell Biol. 13, 1651–1656
8. McFarland, E. D., Hurley, T. R., Pingel, J. T., Sefton, B. M., Shaw, A. & Thomas, M. L. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 1402–1406
9. Burns, C. M., Sakaguchi, K., Appella, E. & Ashwell, J. D. (1994) J. Biol. Chem. 269, 13594–13600
10. D’Orso, U., Sakaguchi, K., Appella, E. & Ashwell, J. (1996) Mol. Cell. Biol. 16, 4996–5003
11. Mauro, L. J. & Dixon, J. E. (1994) Trends Biochem. Sci. 19, 151–155
12. Leichtleider, R., Sugimoto, S., Bennett, A., Kashishian, A., Cooper JA, et al. (1989) J. Immunol. 142, 909–912
13. Pei, D., Lorenz, U., Klingmüller, U., Neel, B. & CT, W. (1994) Biochemistry 33, 15483–15493
14. Novak, T., Farber, D. L., Leitenberg, D., Hong, S.-C., Johnson, J. & Bottomly, K. (1994) Immunol. 1, 109–119
15. McKenney, D. W., Onodera, H., Gorman, L., Mimura, T. & Rothstein, D. M. (1995) J. Biol. Chem. 270, 24949–24954
16. Onodera, H., Motto, D. G., Koretzky, G. A. & Rothstein, D. M. (1996) J. Biol. Chem. 271, 22225–22230
17. Majeti, R., Biilves, A., Noel, J., Hunter, T. & Weiss, A. (1998) Science 279, 88–91
18. Streuli, M., Krueger, N. X., Hall, L., Schlossman, S. & Saito, H. (1988) J. Exp. Med. 168, 1553–1562
19. Tonks, N. K. & Neel, B. G. (1996) Cell 87, 365–368
20. Krueger, N., Streuli, M. & Saito, H. (1990) EMBO J. 9, 3241–3252
21. Krueger, N. & Saito, H. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 7417–7421
22. Streuli, M., Krueger, N. X., Thai, T., Tang, M. & Saito, H. (1990) EMBO J. 9, 2239–2247
23. Johnson, P., Östergaard, H. L., Wasden, C. & Trowbridge, I. S. (1992) J. Biol. Chem. 267, 8035–8041
24. Tan, X., Stover, D. R. & Walsh, K. A. (1993) J. Biol. Chem. 268, 6835–6838
The Second Domain of the CD45 Protein Tyrosine Phosphatase Is Critical for Interleukin-2 Secretion and Substrate Recruitment of TCR-ζ in Vivo
Nobuyuki Kashio, Wataru Matsumoto, Sirlester Parker and David M. Rothstein

J. Biol. Chem. 1998, 273:33856-33863.
doi: 10.1074/jbc.273.50.33856

Access the most updated version of this article at http://www.jbc.org/content/273/50/33856

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 50 references, 32 of which can be accessed free at http://www.jbc.org/content/273/50/33856.full.html#ref-list-1