

# The Extracellular Domain of CD45 Controls Association with the CD4-T Cell Receptor Complex and the Response to Antigen-specific Stimulation

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## Summary

The CD45 tyrosine phosphatase plays an important role in regulating T lymphocyte activation, but the function of the different isoforms of CD45 is not known. T cell transfectants have been prepared that express individual CD45 isoforms in cells with a well-defined T cell receptor (TCR) from the D10 T helper 2 clone. We find that cells bearing low molecular weight CD45 isoforms are far more efficient in responding to stimulation with peptide and antigen-presenting cells compared with cells bearing high molecular weight CD45 isoforms. One hypothesis for the preferential activation of cells that express low molecular weight CD45 isoforms is that they interact with other cell surface antigens important in TCR signaling, altering their phosphorylation status and affecting the character of the signal transduction pathway. In this report, using cells expressing single isoforms, we demonstrate that low molecular weight isoforms of CD45 preferentially associate with CD4 and the TCR complex compared with high molecular weight isoforms. The molecular basis for this interaction was further examined using a glycosyl phosphatidyl inositol (GPI)-linked form of CD45Null (lacking tyrosine phosphatase domains), which preferentially associated with CD4 compared with GPI-linked CD45ABC, and cytoplasmic tail mutants of CD4, which retained the ability to coassociate. Using this panel of transfectants, it is clear that the interaction between CD4 and CD45 does not require the cytoplasmic domains of CD45, but is dependent on the specific external domain of the various isoforms: low molecular weight species were more likely to associate with the CD4-TCR complex than the higher molecular weight isoforms, and their ability to coassociate correlated with the magnitude of the response to specific antigen.

CD45 is a transmembrane tyrosine phosphatase composed of a large heavily glycosylated extracellular portion that can exist as at least nine different isoforms generated by alternative RNA splicing of exons 4–7. The expression of exons 4–6 are designated on the protein as A, B, and C, respectively, whereas the null and exon-1 forms of CD45 do not contain exons 4–6 (CD45Null) or exons 4–7 (CD45Ex-1). These isoforms range in molecular mass from ~180 to 220 kD, and their expression is regulated during hematopoietic development and on different cell lineages (1, 2). The cytoplasmic portion of CD45 functions as a protein tyrosine phosphatase (PTPase)<sup>1</sup> and is essential for the generation of biochemical signaling events after stimulation of the TCR complex (3–6); however, the role of the different CD45 isoforms in T cell activation is unknown. In

T lymphocytes, the expression of different isoforms of CD45 is tightly regulated during thymic development (7, 8), as well as during the generation and maturation of an immune response in the periphery (9–12). Lower molecular weight CD45 isoforms are preferentially expressed on T cells with a memory phenotype as defined by enhanced response to recall antigen, distinct patterns of lymphokine secretion, and the ability to provide help for B cell Ig production (9–12). In addition, distinct patterns of CD45 isoform expression are found on Th1 and Th2 clones, which are defined by different patterns of cytokine production (13). The correlation of different CD45 isoform expression with different stages of T cell maturation and different effector functions, as well as the known activity of CD45 in signal transduction pathways, raise the hypothesis that different isoforms of CD45 may selectively regulate the diverse outcomes possible after recognition of antigen by the TCR.

To investigate the role of the different CD45 isoforms

<sup>1</sup>Abbreviations used in this paper: GPI, glycosyl phosphatidyl inositol; PI-PLC, phosphatidyl inositol-specific phospholipase C treatment; PTPase, protein tyrosine phosphatase.

after antigen-specific stimulation of TCR, a novel model system has been developed in which individual isoform-specific cDNAs have been transfected into a CD45<sup>-</sup> mutant of the BW5147 thymoma. These cells have been constructed to express a TCR of known specificity, and their response to peptide and allogeneic stimulation was investigated (14). These studies showed that the cells expressing the CD45Null and CD45C isoforms were preferentially induced to secrete IL-2 after antigen-specific stimulation compared with cells expressing CD45ABC and CD45BC. These data are similar to independent results from Ong et al. (15) using transgenic mice that overexpress CD45Null or CD45ABC. Thymocytes that constitutively expressed a CD45Null transgene demonstrated an increased sensitivity to antigen-specific negative selection in the thymus and TCR-mediated apoptosis compared with thymocytes that overexpressed the ABC isoform. It therefore appears that distinct isoforms of CD45 can differentially affect T cell development and T cell activation.

One model to explain CD45 isoform-specific regulation of T cell activation is that different isoforms of CD45 may preferentially coassociate *in cis* with other molecules on the T cell surface, which in turn may directly or indirectly modulate the activity of the cytoplasmic tyrosine phosphatase domains or their access to different substrates. CD45 has been described to associate with a variety of other molecules on the surface of the T cell, including CD2, CD3, CD4, Thy-1, and CD26 (16, 22). The association with CD4 is of particular interest since the CD4-associated tyrosine kinase lck has been shown to be a potential substrate for the CD45 tyrosine phosphatase *in vitro* and *in vivo* (24–26). The association of specific CD45 isoforms with CD4-lck could increase lck activity by maintaining the dephosphorylation of the regulatory COOH-terminal tyrosine 505. Alternatively, an association of CD4 with CD45 before TCR signaling may prevent the access of lck to the TCR complex and prevent the phosphorylation of TCR components (e.g.,  $\zeta$  chain), thus modifying the T cell activation cascade. Previously published data from our laboratory have suggested a preferential association of CD45 and CD4 after capping with anti-CD4 antibodies on normal mouse T cells and with Th clones (17). Since these cells express a mixture of CD45 isoforms, a precise determination of which isoforms interact with CD4 was not possible.

In this report, we use an extensive panel of cell lines expressing individual CD45 isoforms to directly examine the effect of specific CD45 isoforms on the response to antigen stimulation and on association with CD4 and the TCR. These results indicate that responsiveness to antigen-specific stimulation is directly correlated with the size of the CD45 isoform and that this directly parallels the ability of specific CD45 isoforms to coassociate with CD4 and the TCR. The molecular basis for CD4-CD45 interaction, as well as the requirement for CD45 PTPase activity and CD4-lck association for antigen-specific stimulation, was further examined using truncated forms of CD45 lacking the cytoplasmic tyrosine phosphatase domains and CD4

mutants that do not bind to lck. Using this panel of transfectants, it is clear that the interaction between CD4 and CD45 does not require the cytoplasmic domains of CD45, but is dependent on the specific external domain of the various isoforms, with low molecular weight species more likely to coassociate with CD4 than the higher molecular weight isoforms.

## Materials and Methods

**Generation of CD45 Transfectants.** A CD45<sup>-</sup> mutant of the BW5147 AKR thymoma was generously provided by Dr. R. Hyman (The Salk Institute, La Jolla, CA). Before transfection with CD45, this cell line was reconstituted with the CD3  $\delta$  and  $\zeta$  chains and the T cell receptor  $\alpha$  and  $\beta$  chains cloned from the D10.G4.1 Th2 cell clone, and also transfected with wild-type murine CD4 as previously described (14). This cell line (BWTCR/CD4) was then either transfected with intact CD45 cDNAs or transfected with glycosyl phosphatidyl inositol (GPI)-linked CD45. The generation of the CD45ABC, CD45BC, CD45C, and CD45Null transfectants have been described previously (14). cDNA encoding for the CD45B and CD45Ex-1 isoforms was obtained by PCR amplification of BALB.k spleen cDNA using a forward primer 5'-CGCAGTGCATGTAGCTAGCAACTGGTTTG-3' encoding nucleotides 1–29 of CD45 mRNA (exon 1b form), and a reverse primer 5'-CTGTGAGCATTCTTC-TAGATTATTAAACACG-3' encoding nucleotides 880–850 of the ABC form of CD45. PCR products were digested with NheI and XbaI and separated on a 2% low-melt agarose gel (Seaplaque; FMC Corp., Rockland, ME). Fragments corresponding to single-exon forms and the Ex(-1) form were excised from the gel and cloned into XbaI-generated expression vector pARV-1(H) (14). Insert orientation was determined by restriction analysis. The identity of single-exon forms was determined by restriction analysis of PCR products generated using the internal primers P1 (in exon 2) and P2 (in exon 7) (13). Properly oriented B and Ex(-1) fragments were sequenced to verify their identity and ensure their correctness. Clones with the correct sequence were digested with XbaI and ligated to a 3-kb XbaI fragment containing the remainder of the CD45 coding region and a portion of the 3' untranslated region.

The GPI-linked construct was prepared by insertion of the extracellular domain of CD45 into the Aval site of the pFRSV plasmid containing the Thy-1 GPI linkage described by Slanetz and Bothwell (27). The extracellular coding portion of CD45ABC and CD45Null was obtained by PCR amplification of the intact cDNA contained in the pARV-1 expression vector (28), using primers that bracket the entire extracellular coding region of CD45 in the sequence described by Thomas et al. (29) (residues 23–46, 5'-GCTCGAGTGTTCTTAGGGTAAGAGAG-3'; and residues 1801–1823, 5'-GCCCGAGGGTTTAGCATTAAA-ATTTGT-3'). This construct was linearized by digestion with SalI and ligated to the pMCFR plasmid (linearized with XhoI) containing the gene encoding resistance to hygromycin B. All transfections were performed by electroporation using a gene pulser (Bio-Rad Laboratories, Hercules, CA) as previously described. The CD45 transfectants were selected by resistance to hygromycin and examined for CD45 expression and sensitivity to phosphatidyl inositol-specific phospholipase C treatment (PI-PLC) by flow cytometric analysis.

**Generation of CD4 and CD8 Cell Lines.** CD4-mutant retroviral constructs in the pMV7 vector were generously provided by

D. Littman (New York University) and were used to infect CD4<sup>-</sup> BWtCR cells that had previously been transfected with CD45Null. The following mutant CD4 constructs were used: CD4Ti (cytoplasmic tail deletion) (30), MCA 1/2 (Cys to Ala mutation in cytoplasmic tail) (30), and 4/8/4 (CD8 transmembrane region) (Littman, D.R., unpublished data). Retroviral infection using the Ψ2 packing line and selection of CD4<sup>+</sup> cells by magnetic beads was carried out as described by Xu and Littman (31). Cells transfected with CD8α were obtained by electroporation using the pSRα vector (14) and was selected with puromycin with cDNA generously provided by P. Kavathas (Yale University).

**PI-PLC Treatment.** 10<sup>6</sup> cells were suspended in 100 μl of RPMI 1640 supplemented with 10 mM Hepes and incubated with 100 mU of PI-PLC (Boehringer Mannheim Corp., Indianapolis, IN) for 1 h at 37°C. A parallel sample of cells was treated identically, except in the absence of PI-PLC. The cells were then labeled with anti-CD3, -CD4, and -CD45 antibodies and were analyzed by flow cytometry to evaluate specific removal of the GPI-linked CD45.

**Flow Cytometric Analysis.** The following anti-CD45 mAbs were used in this study: M1/9.3.4.HL.2, pan-specific anti-CD45 (TIB122; American Type Culture Collection [ATCC], Rockville, MD); 14.8, anti-CD45A (TIB164; ATCC); C363.16A, anti-CD45B (11); C455.14F, anti-CD45C (Bottomly, K., unpublished data). In addition, anti-CD4 (GK1.5) (32) and an anti-clonotypic antibody for the D10 TCR (3D3) (33) were also used. All antibodies were purified from culture supernatants on protein G columns and dialyzed against PBS before use. The cells were labeled with saturating amounts of the indicated antibody diluted in PBS supplemented with 1% FCS and 0.02% sodium azide (staining buffer) and incubated on ice for 30 min. The cells were then washed and incubated with FITC-conjugated goat anti-rat IgG or goat anti-mouse IgG (Hyclone Laboratories Inc., Logan, UT) for an additional 30 min on ice, and then washed and fixed in PBS with 1% paraformaldehyde. Fluorescence analysis was done using a flow cytometer (FACScan®; Becton Dickinson & Co., Mountain View, CA), and the data were analyzed using the LYSYS II software package (Becton Dickinson & Co.).

**Cell Culture and IL-2 Production.** All cell lines were maintained in Eagle's high amino acid medium (Click's medium; Irvine Scientific, Santa Ana, CA) supplemented with 9% FCS, 5 × 10<sup>-5</sup> M 2-ME, 2 mM L-glutamine, 10 mM Hepes, and antibiotics. BW transfectants in stable growth phase were stimulated for 2 d in culture in 96-well flat-bottom microtiter wells precoated with anti-clonotypic TCR mAb (3D3). The cells were coated with different dilutions of antibody in PBS for 4 h, washed, and 1–2 × 10<sup>5</sup> BW cells in 250 μl of media were added. In parallel, an equal number of BW transfectants were stimulated with conalbumin peptide (HRGAIEWEGIESG) plus 10<sup>6</sup> T cell-depleted splenocytes from B10.BR mice in round-bottom microtiter wells. After 48 h, the supernatants were harvested and stored frozen at -20°C. IL-2 production was determined using the IL-2-dependent CTLL-2 cell line. 10<sup>4</sup> CTLL cells were cultured in twofold dilutions of supernatant, and 48 h later the cells were pulsed with 1 μCi [<sup>3</sup>H]thymidine. After 16–24 h the cells were harvested, and thymidine incorporation was determined by liquid scintillation counting.

**Cocapping Assay.** 10<sup>6</sup> BW cells were induced to cap by labeling with either biotinylated anti-CD4 (GK1.5) or anti-TCR (3D3) for 30 min in a 37°C water bath. The cells were then washed once and resuspended in streptavidin-PE (CALTAG Laboratories, South San Francisco, CA) and incubated at 37°C for

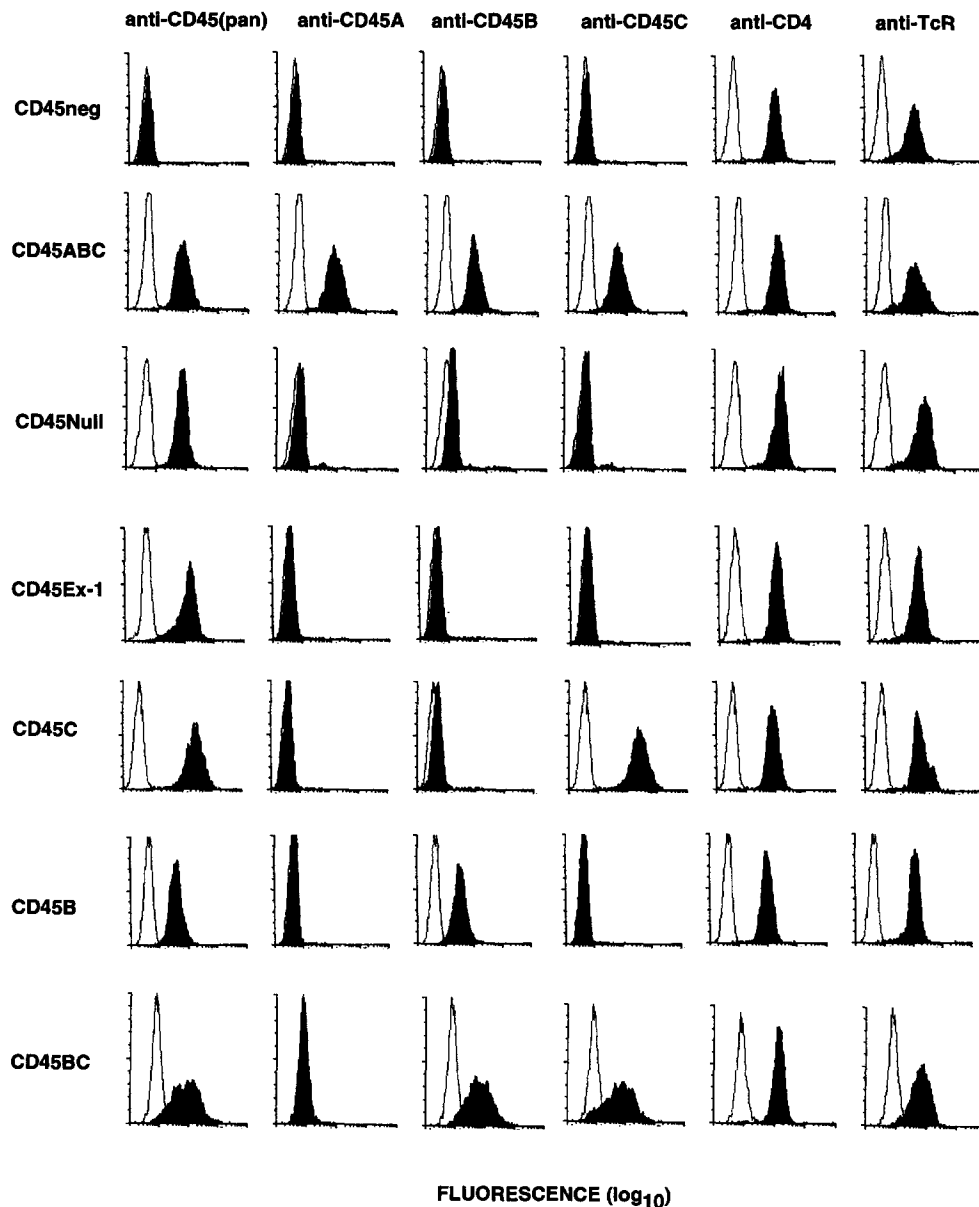
30 min for CD4 capping or 15 min for TCR capping. The cells were then washed with ice-cold staining buffer and labeled with fluorescein-conjugated anti-CD45 or anti-CD3ε antibodies (PharMingen, San Diego, CA), and then fixed with 1% paraformaldehyde in PBS. Control cells (labeled with PE or FITC alone) were always prepared simultaneously for each cell line to set compensation parameters.

Fluorescence localization was determined using a scanning video laser cytometer (ACAS 570; Meridian Instruments, Inc., Okemos, MI) equipped with a 200-mW argon laser. FITC and PE fluorescence was simultaneously detected using a 575-nm short-pass dichroic filter followed by a 530-nm band-pass filter to detect FITC fluorescence and 605-nm long-pass (or in some experiments 575-nm band-pass) filter to detect PE fluorescence. Fluorescence cross-over was corrected for each cell line using the X-OVER compensation software package (Meridian Instruments, Inc.) using capped cells singly labeled with PE or uncapped cells labeled with FITC-conjugated antibodies. For each experimental group, 100 capped cells were counted, and the percentage of capped cells with coassociated CD45, CD3, or CD4 was determined.

## Results

**Immunophenotype of CD45-transfected Cell Lines.** The CD45<sup>-</sup> mutant of the BW5147 thymoma has been used to generate an extensive panel of T cell lines that express individual CD45 isoforms, the CD4 coreceptor, and an MHC class II-restricted TCR specific for a conalbumin peptide. The cells are routinely sorted and subcloned to express similar levels of TCR, CD4, and CD45. The immunophenotype of the different cell lines is illustrated in Fig. 1, including surface labeling with antibodies specific for the CD45-variable exons A, B, or C. Cell lines expressing six different CD45 isoforms (CD45ABC, CD45BC, CD45B, CD45C, CD45Null, CD45Ex-1) have been generated, as well as cells bearing truncated and mutated forms of CD45 and CD4. Differential isoform expression was confirmed by immunoprecipitation and Western blot analysis (14, Bottomly, unpublished data) in addition to the flow cytometric data presented in Fig. 1.

**CD45 Isoform Regulation of Antigen-induced IL-2 Production.** Cells expressing different CD45 isoforms were compared for their ability to secrete IL-2 after peptide plus APC stimulation and compared with maximal IL-2 production after stimulation with plate-bound anti-TCR antibody (Fig. 2). Fig. 2 A illustrates representative results from cells expressing CD45ABC and CD45Null. As we have previously described, both cell lines are able to produce similar levels of IL-2 after stimulation with plate-bound anti-TCR mAb, which is independent of the expression of CD4 or CD45 (14). Although no difference was seen in response to plate-bound antibody, cells expressing the null isoform of CD45 were preferentially stimulated after exposure to peptide and APC. These results have been consistently observed after testing in multiple independently transfected cell lines (14). To determine if CD45 isoform-specific regulation is correlated with the size of the isoform, or if cells expressing isoforms of similar size show differential IL-2 production after

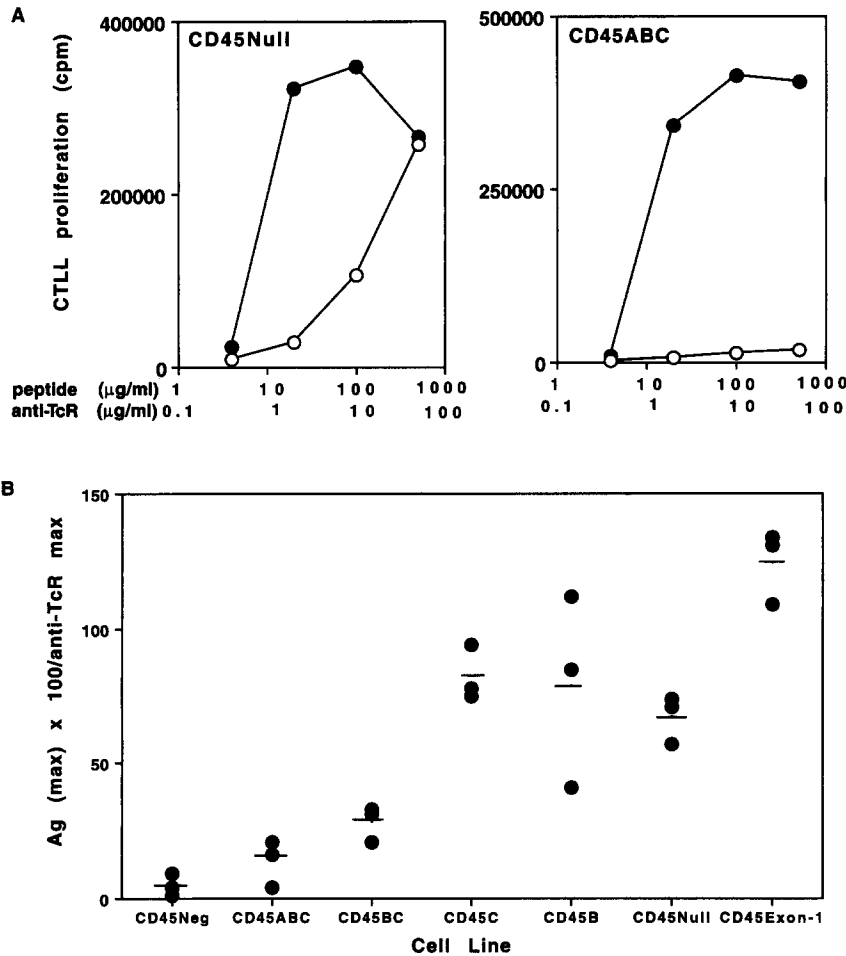


**Figure 1.** Immunophenotype of CD45 transfectants. The panel of BW transfectants was labeled with antibodies to different CD45 isoforms, CD4, and the D10 TCR, and analyzed by flow cytometry. Specific staining is indicated by the solid histogram, background by the open histogram.

antigen exposure, we have extended our analysis with a panel of six different CD45 transfectants (Fig. 2 B). These results suggest that the ability to produce IL-2 after antigen stimulation is directly correlated with the size of the CD45 isoform. Both of the single-exon forms (CD45B, CD45C), CD45Null, and CD45Ex-1, respond relatively well to antigen stimulation compared with the cells expressing higher molecular weight CD45 isoforms (CD45ABC, CD45BC).

*CD45 Isoform-specific Association with CD4 and the TCR.* One possible mechanism for CD45 isoform-specific regulation after antigen stimulation is that different CD45 isoforms preferentially associate with other molecules that are important in TCR signaling. Of particular interest is an association with CD4, since the CD4-associated lck kinase has been identified as a potential substrate for the CD45 tyrosine phosphatase. In addition, we have previously found

using Th cell clones or naive and memory CD4 T cell subsets that CD45 isoforms reactive with an antibody specific for the B exon did not associate with CD4, whereas “B-negative” isoforms did associate (17). These studies were difficult to interpret because both Th clones and naive and memory CD4 T cells each express multiple CD45 isoforms, and the presence of the B exon does not discriminate between single-, double-, or triple-exon-expressing isoforms. To precisely identify any preferential CD45 isoform association with CD4, we have developed a cocapping assay using a video laser cytometer able to simultaneously analyze cells labeled with both FITC- and PE-conjugated antibodies. CD4 expressed on cells transfected with the different CD45 isoforms was induced to cap by treatment with biotinylated anti-CD4 and PE-streptavidin, and the presence of CD45 (or CD3 as a positive control) associated



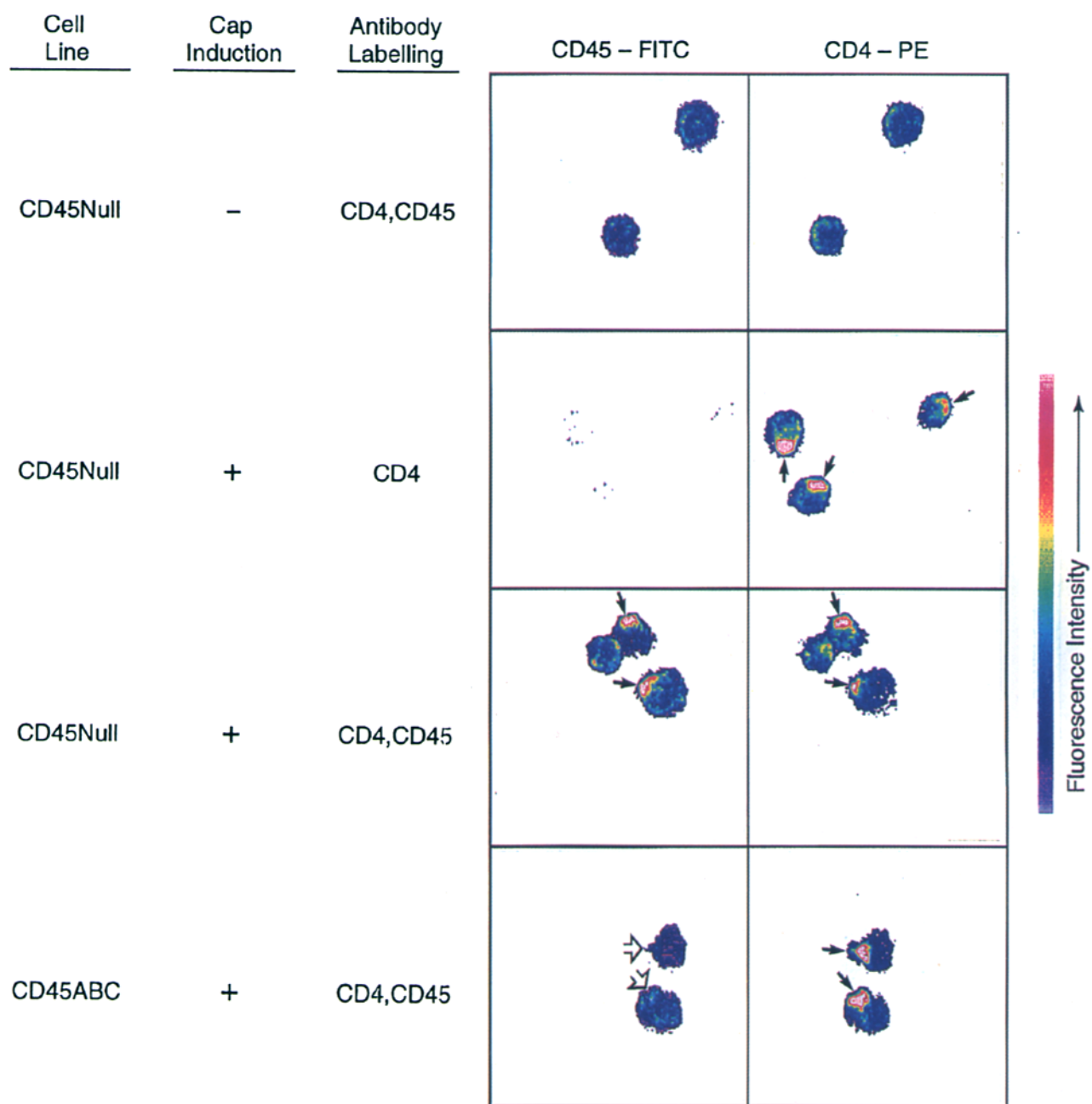
**Figure 2.** The ability to respond to antigen-specific stimulation directly correlates with the size of the CD45 isoform. (A) Cells expressing either CD45Null or CD45ABC were stimulated with CA peptide plus mitomycin C-treated T depleted splenocytes (open circles) or with plate-bound anticolonotypic TCR (solid circles). After 48 h, the supernatants were collected and tested for the presence of IL-2 using the IL-2-dependent cell line CTLL. (B) Summary of antigen responsiveness of BW cells transfected with different CD45 isoforms. The maximum response to antigen stimulation is presented as a percent of the maximal response to plate-bound anti-TCR antibody for each individual cell line. The results from three independent experiments (solid circles) are shown for each cell line.

with the CD4 “cap” was detected by labeling with fluorescein-labeled anti-CD45 or anti-CD3 antibodies. As illustrated in Figs. 3 and 4, the association of CD45 with CD4 was preferentially detected on cells expressing CD45Null, whereas CD45ABC was rarely found associated with CD4. Fig. 3 is a representative image obtained using the video laser cytometer where fluorescence intensity is depicted on a pseudocolor scale ranging from dark blue (low fluorescence) to white (high fluorescence). The panel on the left represents the level of fluorescein labeling, and the panel on the right represents PE labeling of the same cells. Controls were included to show the cells in which capping is not induced, and cells were labeled with a single fluorochrome to ensure that PE fluorescence (in this case) was not detected by the fluorescein detector. The bottom half of the figure demonstrates association of CD45 with the cap of CD4 in cells bearing CD45Null and lack of association in cells bearing the CD45ABC isoform. These results are representative of 11 independent experiments. When the entire panel of cells transfected with different CD45 isoforms was analyzed for colocalization in a quantitative manner (Fig. 4), we found that the association of CD45 and CD4 was directly correlated with the size of the CD45 isoform (Fig. 4 A), in parallel with their ability to respond to antigen-

specific stimulation. As an internal control, all the cell lines were equivalent in the ability of CD3 to associate with CD4, which was independent of the CD45 isoform (Fig. 4 B) and occurred in CD45<sup>-</sup> cells (latter data not shown).

Since CD3 is found associated with capped CD4, it seems likely that cells bearing low molecular weight CD45 isoforms would demonstrate association with the TCR in addition to CD4. Therefore, the association of CD45 isoforms with the TCR complex was also examined (Fig. 5). The TCR complex of BW cells expressing either CD45Null or CD45ABC was induced to cap, and as seen with CD4, the low molecular weight CD45 isoform (CD45Null) was found to preferentially associate with the TCR complex compared with the higher molecular weight CD45 isoform (CD45ABC). These results suggest that cells expressing low molecular weight isoforms can more readily form a trimolecular complex of CD3, CD4, and CD45, and that this may enhance the efficiency of the TCR signaling complex.

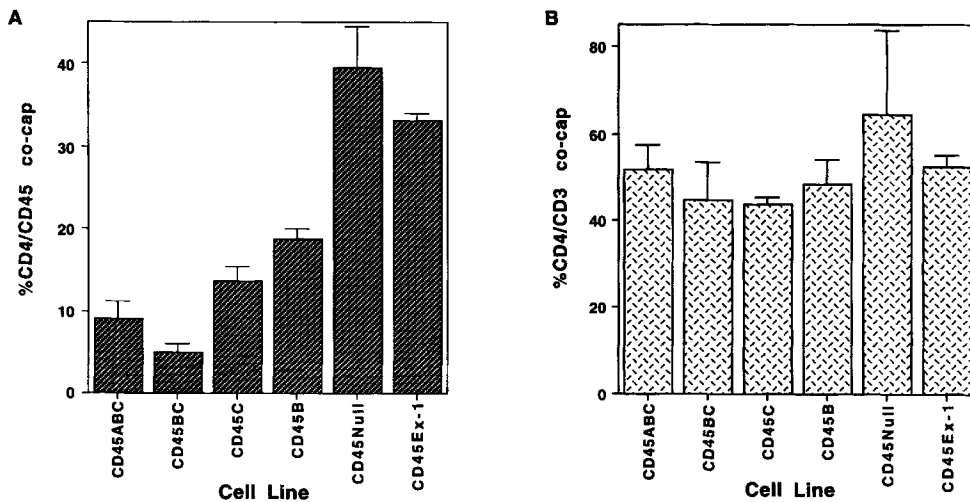
*CD4-CD45 Association Is Independent of the CD45 and CD4 Cytoplasmic Domains.* Since CD45 has been shown to be associated with the cytoskeleton (34), it is important to determine the role of the cytoplasmic domains of CD45 in facilitating cocapping with CD4. To evaluate the requirement for the cytoplasmic portion of CD45 for CD4



**Figure 3.** CD45 isoform-specific association with CD4. CD4 was induced to cap on BW cells transfected with CD45ABC or with CD45Null by incubation with biotinylated anti-CD4 (GK1.5) for 30 min followed by PE-streptavidin for an additional 30 min at 37°C. The cells were then washed in cold PBS with azide, incubated on ice with fluoresceinated anti-CD45 for 30 min, and fixed with 1% paraformaldehyde. Analysis was done using an ACAS 570 video laser cytometer equipped with a 200-mW argon laser. Red and green fluorescence were detected using a dichroic 575-nm short-pass filter followed by 605-nm long-pass filter for PE detection and a 530-nm band-pass filter for FITC detection. Compensation parameters were determined with cells labeled with a single fluorochrome. The data are presented as a pseudocolor image with gradations in color indicating changes in fluorescent intensity. The arrows indicate the regions on the cell where capping occurred.

coassociation to occur, or to determine if the extracellular domains are sufficient to mediate isoform-specific association, additional experiments were done with heterochimeric forms of CD45 in which the extracellular portion of CD45 was ligated with the GPI anchorage of Thy-1 (GPI-linked CD45) (Fig. 6 A). As shown in Fig. 6 B, these ex-

periments demonstrate that the ability of CD45 to colocalize is primarily dictated by the extracellular domains of CD45, since the GPI-linked null isoform cocapped with CD4 as well as the wild-type transmembrane CD45Null, whereas the GPI-linked ABC isoform, like its wild-type counterpart, did not demonstrate significant cocapping.



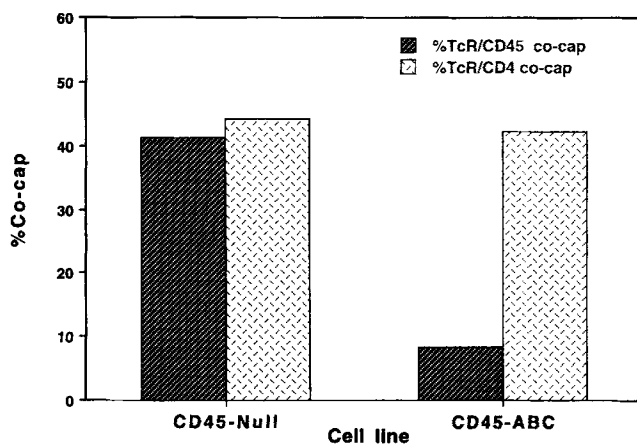
**Figure 4.** The ability of CD45 to associate with CD4 is directly correlated with the size of the variable external domain of CD45. The BW transfectants were analyzed for CD45 (A) or CD3 association (B) with capped CD4 using the video laser cytometer as described in Fig. 3. The percentage of capped cells that demonstrated CD45 or CD3 association was determined. The data represent the average plus SD of two independent experiments in which at least 50 capped cells were analyzed for each cell line.

Despite association between CD45 and CD4 on the GPI-linked null transfectant, this cell line did not respond to antigen stimulation (Fig. 6 C). These data emphasize the importance of the CD45 tyrosine phosphatase domains in addition to the presence of CD45 low molecular weight isoforms in generating efficient TCR signal transduction after physiologic stimulation with peptide and APC.

Since CD45 has also been demonstrated to associate with lck (35), we have done similar cocapping analysis on CD45Null BW cells that express mutant and truncated forms of CD4 (MCA, cytoplasmic Cys-Ala mutation, which does not allow lck association; CD4-Ti, cytoplasmic tail deletion) to determine the influence of CD4-associated lck on the CD45-CD4 association (Fig. 7 A). These experiments showed that CD4-CD45 association occurred independently of CD4-lck association and in the absence of the

cytoplasmic domain of CD4. In addition, we determined if the transmembrane domain of CD4 was required for colocalization using the 4/8/4 mutant (transmembrane portion of CD8 in place of CD4). This cell line also demonstrated cocapping with CD45 similar to the wild-type CD4 cell line (Fig. 7 A), providing further evidence that CD4-CD45 coassociation is regulated by the extracellular domains of both CD4 and CD45. Control experiments using cells transfected with CD8 $\alpha$  instead of CD4 demonstrate the specificity of the CD4-CD45Null interaction, since capped CD8 $\alpha$  failed to significantly associate with CD45-Null (data not shown).

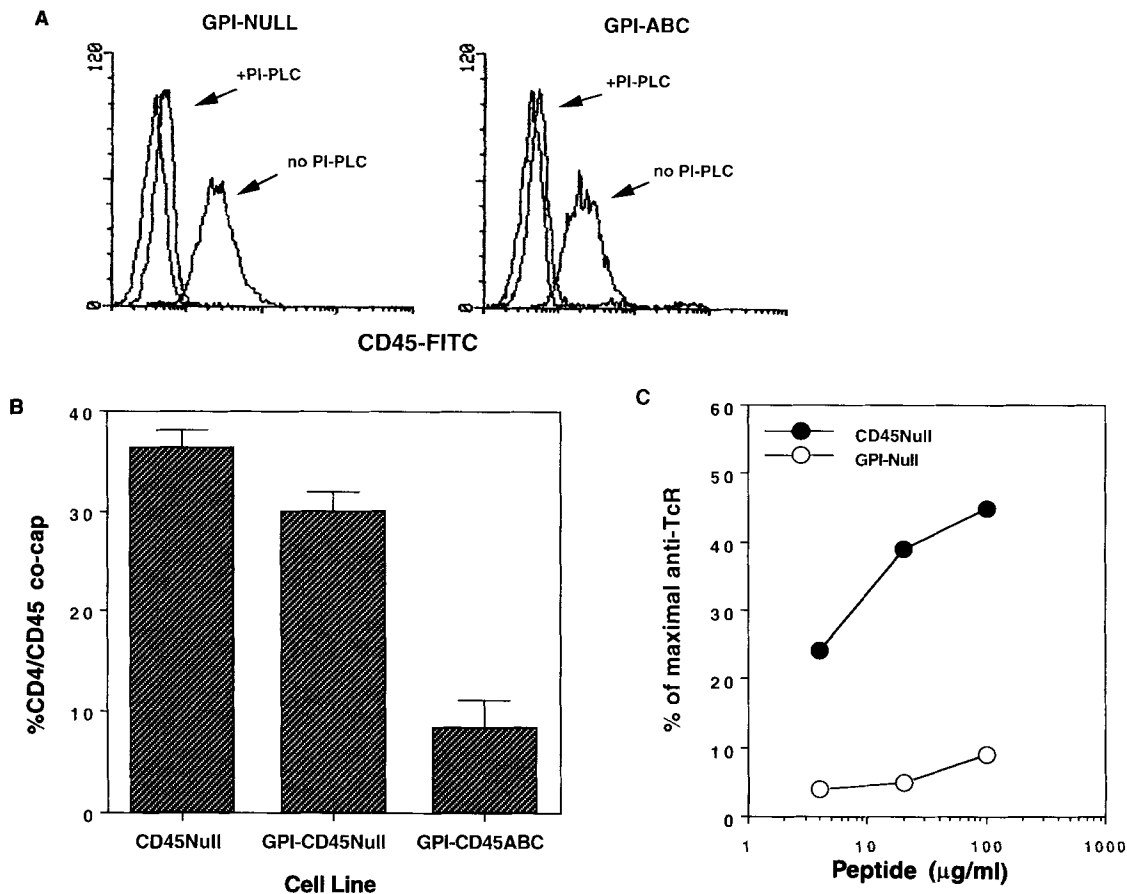
We have also used the CD4 mutants to examine the requirements of CD4-associated lck for IL-2 production after antigen-specific stimulation. As shown in Fig. 7 B, the lck-binding mutant of CD4 (MCA mutant) is not able to reconstitute antigen-specific signaling in the BW cells compared with wild-type CD4. This is consistent with the model that association of CD4 and CD45 may enhance access of CD4-associated lck to the tyrosine phosphatase domains of CD45, which in turn may dephosphorylate the COOH-terminal negative regulatory site of lck, thus increasing kinase activity.



**Figure 5.** CD45Null preferentially colocalizes with the TCR complex. The TCR complex was induced to cap on BW cells transfected with CD45ABC or CD45Null with biotinylated clonotypic antibody to the D10 TCR (3D3) for 30 min followed by PE-SA for an additional 15 min. The cells were then labeled with fluorescein-labeled anti-CD45 or anti-CD3 and analyzed for CD45 or CD4 association with the TCR cap as described in Fig. 3.

## Discussion

Although it is clear that the tyrosine phosphatase domains of CD45 play an important role in T cell development and activation, the potential role of the variable ectodomains of CD45 in the regulation of T cell activation has been elusive. In this report we provide evidence that CD45 isoform-specific regulation of CD4 T cell activation is determined by the size of the variable extracellular domain of CD45 and correlates with the ability of CD45 to colocalize with CD4 and the TCR complex. The association of CD45 with other molecules important in TCR signaling may be important for the generation of optimal signal transduction. Other investigators have shown that heterochimeric forms of CD45 that contain the cytoplas-



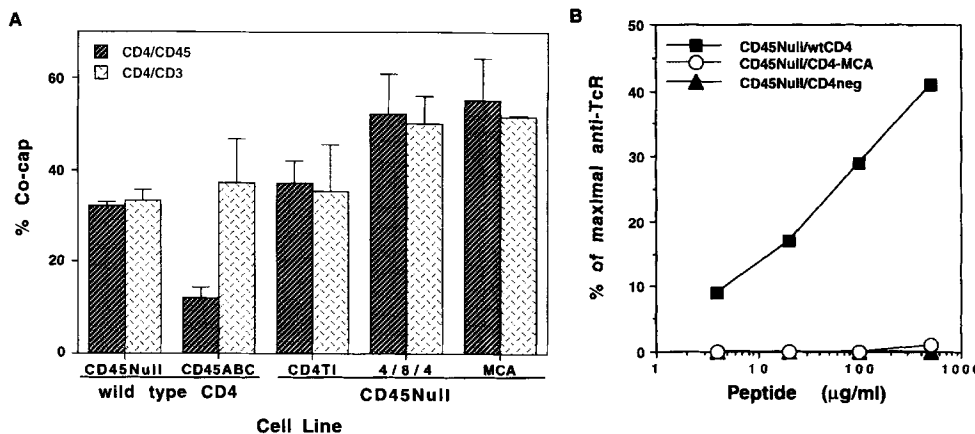
**Figure 6.** Preferential cocapping of the CD45Null isoform with CD4 is independent of the cytoplasmic domain of CD45, although the PTPase domains are required for IL-2 production after antigen stimulation. (A) GPI-linked CD45Null and CD45ABC transfectants were labeled with anti-CD45 antibodies before and after treatment with PI-PLC, and CD45 expression was determined by flow cytometry. (B) GPI-linked CD45Null and ABC transfectants were analyzed for CD4 cocapping compared with the intact CD45Null transfectant as described in Fig. 3. The results represent the average and SD of the percentage of cocapped cells from two independent experiments. (C) The GPI-Null transfectant was analyzed for antigen-specific stimulation compared with the wild-type CD45Null cell line. The results are normalized for the maximal IL-2 production of each cell line by plate-bound anti-TCR antibody as described in Fig. 2.

mic CD45 PTPase domains but express a different external domain are able to reconstitute TCR signaling in CD45<sup>-</sup> (3, 5). One interpretation of these studies is that the extracellular domains of CD45 are not required for TCR signaling. It should be noted, however, that T cell stimulation in these studies was done using anti-TCR antibodies, and we also do not find any isoform regulation of signaling using anti-TCR antibodies (Fig. 1, reference 14). CD45 isoform-specific regulation is only observed after stimulation with specific antigen, which is presumably a lower potency signal involving fewer antigen receptors than antibody-mediated TCR stimulation.

The ability of CD45Null and CD4 to associate was independent of both the cytoplasmic domains of CD4 and CD45, indicating that the association is primarily regulated by the extracellular domains of CD45; however, the structural basis for this interaction is not certain. Electron micrographs of purified CD45ABC suggest that it exists as an extended rodlike structure, whereas CD45Null may be

more globular in form, which may promote enhanced access to other surface antigens on the T cell (36). In BW cells transfected with CD8 $\alpha$  and CD45Null, minimal levels of association (15%) between CD45 and CD8 are seen, suggesting that CD45Null does not associate nonspecifically with other surface antigens and that the association with CD4 and the TCR is relatively specific. Previous studies from our laboratory also suggest that the association between CD4 and CD45 is relatively specific, since CD45 failed to associate with class I MHC and LFA-1 (17). However, we cannot rule out that CD45Null may associate with other molecules on the surface of the T cell in addition to CD4 and the TCR, nor can we exclude the possibility that the interaction with the CD4-TCR complex is mediated indirectly by other molecules. Finally, it should also be noted that although the experiments presented in this report focus on the association of CD45 *in cis* with other molecules on the surface of the T cell, they do not rule out the possibility that there are additional associations





**Figure 7.** Coassociation of CD4 with CD45Null does not require CD4-lck association or the CD4 transmembrane and cytoplasmic domains, although CD4-lck association is required for antigen-specific stimulation of the CD45Null transfectant. (A) CD45Null-bearing cells transfected with the CD4Ti- (cytoplasmic tail mutant), MCA- (lck-binding mutant), and 4/8/4- (contains CD8 transmembrane region) mutant forms of CD4 were analyzed for cocapping as in Fig. 3 compared with wild-type CD4 on CD45Null- and CD45ABC-bearing cells. The results represent the average and SD of the percentage of cocapped cells

from two independent experiments. (B) The CD45Null/CD4-MCA mutant was analyzed for antigen-specific stimulation compared with CD45Null/wild-type CD4 and CD45Null/CD4<sup>-</sup> cells as described in Fig. 2.

in *trans* with APC or other accessory cells that may further modify substrate access to CD45 and T cell activation.

Regardless of the structural basis for the coassociation of low molecular weight isoforms of CD45 with the TCR complex, these results support a model by which the CD45 extracellular domains can control the physical configuration of molecules around the TCR. This may result in differential substrate access to the CD45 PTPase domains depending on the CD45 isoform, thus altering proximal and downstream signaling events. In support of this hypothesis is the observation of Maroun et al. (37) that cross-linking with anti-CD45 antibodies has differential inhibitory effects on TCR signaling in CD4 T cells, which primarily express low molecular weight isoforms (single or null exon forms), versus CD8 T cells, which primarily express high molecular weight isoforms. Thus anti-CD45 cross-linking may prevent association with the TCR complex in CD4 T cells, but would not affect signaling in CD8 T cells in which CD45 and coreceptor-TCR complex are not usually associated.

Our results (this report and reference 14) and the data of Ong et al. (15) support the idea that low molecular weight isoforms of CD45 enhance the efficiency of TCR signaling, and we suggest that differential association of CD45 isoforms with the TCR complex may explain these results. The mechanism by which this occurs may be complex, but one model that is consistent with the data is that during CD4-dependent stimulation of the TCR, low molecular weight isoforms of CD45 may have preferential access to

the CD4-associated lck kinase, increasing kinase activity and enhancing the efficiency of TCR signal transduction. In addition to lck, several other molecules important in TCR signaling have been identified as potential substrates for CD45, including the tyrosine kinases fyn, ZAP-70, and the  $\zeta$  chain of the TCR (38-41). Similarly to lck, fyn is a src family tyrosine kinase with a negative regulatory COOH-terminal tyrosine phosphorylation site that can be dephosphorylated by CD45, thus increasing kinase activity, and like lck may have a role in enhancing TCR signaling *in vivo* when CD45 is part of the TCR complex. A complication of this model is that in contrast to lck and fyn, dephosphorylation of the  $\zeta$  chain by CD45 may have a negative effect on TCR signal transduction by preventing the association of ZAP-70, which appears to be an important step in T cell activation (42, 43). Interestingly, decreased phosphorylation of  $\zeta$  and lack of ZAP-70 association is characteristic of TCR signaling by altered peptide ligands (44, 45) and may reflect an alteration in the balance of the effect of CD45 on lck/fyn activation versus  $\zeta$  and ZAP-70. Although the precise mechanism that regulates these apparently contrasting effects of CD45 on T cell activation remains unclear, a model in which different isoforms of CD45 may affect the molecular composition of the TCR complex and may differentially affect signaling pathways provides a possible framework for understanding the differences in the downstream consequences of TCR signaling during maturation in the thymus, as well as during differentiation in the periphery after antigen stimulation.

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## References

1. Trowbridge, I.S., and M.L. Thomas. 1994. CD45: an emerging role as a protein tyrosine phosphatase required for lymphocyte activation and development. *Annu. Rev. Immunol.* 12:85–116.
2. Trowbridge, I.S., H.L. Ostergaard, and P. Johnson. 1991. CD45: a leukocyte-specific member of the protein tyrosine phosphatase family. *Biochim. Biophys. Acta.* 1095:46–56.
3. 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).* 260:541–544.
4. Desai, D.M., J. Sap, J. Schlessinger, and A. Weiss. 1993. Ligand-mediated negative regulation of a chimeric transmembrane receptor tyrosine phosphatase. *Cell.* 73:541–554.
5. Hovis, R.R., J.A. Donavan, 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).* 260:544–546.
6. Desai, D.M., J. Sap, O. Silvennoinen, J. Schlessinger, and A. Weiss. 1994. The catalytic activity of the CD45 membrane-proximal phosphatase domain is required for TCR signalling and regulation. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:4002–4010.
7. Chang, H.-L., L. Lefrancois, M.H. Zaroukian, and W.J. Esselman. 1991. Developmental expression of CD45 alternate exons in murine T cells. Evidence of additional exon use. *J. Immunol.* 147:1687–1693.
8. Hathcock, K.S., G. Laszlo, H.B. Dickler, S.O. Sharrow, P. Johnson, I.S. Trowbridge, and R.J. Hodes. 1992. Expression of variable exon A-, B-, and C-specific CD45 determinants on peripheral and thymic T cell populations. *J. Immunol.* 148:19–28.
9. Bottomly, K., M. Luqman, L. Greenbaum, S. Carding, J. West, T. Pasqualini, T., and D.B. Murphy. 1989. A monoclonal antibody to murine CD45R distinguishes CD4 T cell populations that produce different cytokines. *Eur. J. Immunol.* 19:617–623.
10. Lee, W.T., X.-M. Yin, and E.S. Vitetta. 1990. Functional and ontogenetic analysis of murine CD45Rhi and CD45Rlo CD4<sup>+</sup> T cells. *J. Immunol.* 144:3288–3295.
11. Akbar, A.M., L. Terry, A. Timms, P.C.L. Beverley, and G. Janossy. 1988. Loss of CD45R and gain of UCHL1 reactivity is a feature of primed T cells. *J. Immunol.* 140:2171–2178.
12. Sanders, M.E., M.W. Makgoba, and S. Shaw. 1988. Human naive and memory T cells: reinterpretation of helper-inducer and suppressor-inducer subsets. *Immunol. Today.* 9:195–199.
13. Luqman, M., P. Johnson, I.S. Trowbridge, and K. Bottomly. 1991. Differential expression of the alternatively spliced exons of murine CD45 in Th1 and Th2 cloned lines. *Eur. J. Immunol.* 21:17–23.
14. Novak, T., D. Farber, D. Leitenberg, S. Hong, P. Johnson, and K. Bottomly. 1994. Isoforms of the transmembrane tyrosine phosphatase CD45 differentially affect T cell recognition. *Immunity.* 1:109–119.
15. Ong, C.J., D. Chui, H.-S. Teh, and J.D. Marth. 1994. Thymic CD45 tyrosine phosphatase regulates apoptosis and MHC-restricted negative selection. *J. Immunol.* 152:3793–3805.
16. Mittler, R.S., B.M. Rankin, and P.A. Kiener. 1991. Physical associations between CD45 and CD4 or CD8 occur as late activation receptor-stimulated human T cells. *J. Immunol.* 147:3434–3440.
17. Dianzani, U., M. Luqman, J. Rojo, J. Yaga, J.L. Baron, A. Woods, C.A. Janeway, and K. Bottomly. 1990. Molecular associations on the T cell surface correlate with immunological memory. *Eur. J. Immunol.* 20:2249–2257.
18. Dianzani, U., V. Redoglia, F. Malavasi, M. Bragardo, C.A. Janeway, and K. Bottomly. 1992. Isoform-specific associations of CD45 with accessory molecules in human T lymphocytes. *Eur. J. Immunol.* 22:365–371.
19. Altevogt, P., J. Schreck, B. Schraven, S. Meuer, Y. Shirrmacher, and A. Mittsch. 1990. Association of CD2 and T200 (CD45) in mouse T lymphocytes. *Int. Immunol.* 2:353–360.
20. Schraven, B., Y. Samstag, P. Altevogt, and S.C. Meurer. 1990. Association of CD2 and CD45 on human T lymphocytes. *Nature (Lond.).* 345:71–74.
21. Volarevic, S., C.M. Burns, J.J. Sussman, and J.D. Ashwell. 1990. Intimate association of Thy-1 and the T-cell antigen receptor with the CD45 tyrosine phosphatase. *Proc. Natl. Acad. Sci. USA.* 87:7085–7089.
22. Torimoto, Y., N.H. Dang, E. Vivier, T. Tanaka, S.F. Schlossman, and C. Morimoto. 1991. Coassociation of CD26 (dipeptidyl peptidase IV) with CD45 on the surface of human T lymphocytes. *J. Immunol.* 147:2514–2517.
23. 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 kinase in murine lymphoma T-cell lines. *Proc. Natl. Acad. Sci. USA.* 86:8959–8963.
24. McFarland, E.D.C., T.M. 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.* 90:1402–1406.
25. Mustelin, T., K.M. Coggeshall, and A. Altman. 1989. Rapid activation of the T-cell tyrosine protein kinase pp56<sup>lck</sup> by the CD45 phosphotyrosine phosphatase. *Proc. Natl. Acad. Sci. USA.* 86:6302–6306.
26. Mustelin, T., and A. Altman. 1990. Dephosphorylation and activation of the T cell tyrosine kinase p56<sup>lck</sup> by the leukocyte common antigen (CD45). *Oncogene.* 5:809–813.
27. Slanetz, A.E., and A.L.M. Bothwell. 1991. Heterodimeric, disulfide linked  $\alpha/\beta$  T cell receptors in solution. *Eur. J. Immunol.* 21:179.
28. Johnson, P., L. Greenbaum, K. Bottomly, and I.S. Trowbridge. 1989. Identification of the alternative spliced exons of murine CD45 (T200) required for reactivity with B220 and other T200-restricted antibodies. *J. Exp. Med.* 169:1179–1184.
29. Thomas, M.L., P.J. Reynolds, A. Chain, Y. Ben-Neriah, and I.S. Trowbridge. 1987. B-cell variant of mouse T200 (Ly-5): evidence for alternative mRNA splicing. *Proc. Natl. Acad. Sci. USA.* 84:5360–5363.
30. Gleichhaus, N., N. Shastri, D.R. Littman, and J.M. Turner. 1991. Requirement for association of p56<sup>lck</sup> with CD4 in antigen-specific signal transduction in T cells. *Cell.* 64:511–520.
31. Xu, H., and D.R. Littman. 1993. A kinase-independent

- function of lck in potentiating antigen-specific T cell activation. *Cell*. 74:633–643.
32. Dialynas, D.P., D.B. Wilde, P. Marrack, A. Pierres, K.A. Wall, W. Havran, G. Otten, M.R. Loken, M. Pierres, J. Kappler, and F.W. Fitch. 1983. Characterization of the murine antigenic determinant, designated L3T4a, recognized by monoclonal antibody GK1.5: expression of L3T4a by functional T cell clones appears to correlate primarily with class II MHC antigen-reactivity. *Immunol. Rev.* 74:29–56.
  33. Kaye, J., S. Porcelli, J. Tite, B. Jones, and C.A. Janeway. 1983. Both a monoclonal antibody and antisera specific for determinants unique to individual cloned helper T cell lines can substitute for antigen and antigen presenting cells in the activation of T cells. *J. Exp. Med.* 158:836–856.
  34. Bourguignon, L.Y.W., S.J. Suchard, M.L. Nagpal, and J.R. Glenny. 1985. A T lymphoma transmembrane glycoprotein (gp180) is linked to the cytoskeletal protein fodrin. *J. Cell Biol.* 101:477–487.
  35. Koretzky, 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.* 268:8958–8964.
  36. McCall, M.N., D.M. Shotton, and A.N. Barclay. 1992. Expression of soluble of rat CD45. Analysis by electron microscopy and use in epitope mapping of anti-CD45R monoclonal antibodies. *Immunology*. 76:310–317.
  37. Maroun, C., and M. Julius. 1994. Distinct involvement of CD45 in antigen receptor signalling in CD4<sup>+</sup> and CD8<sup>+</sup> primary T cells. *Eur. J. Immunol.* 24:967–973.
  38. Mustelin, T., T. Pessa-Morikawa, M. Autero, M. Grassman, L.C. Andersson, C.G. Gahmberg, and P. Burn. 1992. Regulation of the p56<sup>lck</sup> protein tyrosine kinase by the CD45 phosphotyrosine phosphatase. *Eur. J. Immunol.* 22:1173–1178.
  39. Shiroo, M., L. Goff, M. Biffen, E. Shivnan, and D. Alexander. 1992. CD45 tyrosine phosphatase-activated p59<sup>lck</sup> couples the T cell antigen receptor to pathways of diacylglycerol production, protein kinase C activation and calcium influx. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:4887–4897.
  40. Furukawa, T., M. Itoh, N.X. Krueger, M. Streuli, and H. Saito. 1994. Specific interaction of the CD45 protein tyrosine phosphatase with tyrosine-phosphorylated CD3  $\zeta$  chain. *Proc. Natl. Acad. Sci. USA.* 91:10928–10929.
  41. Mustelin, T., S. Williams, P. Taylor, C. Couture, G. Zenner, P. Burn, J.D. Ashwell, and A. Altman. 1995. Regulation of the p70<sup>ZAP</sup> tyrosine protein kinase in T cells by the CD45 phosphotyrosine phosphatase. *Eur. J. Immunol.* 25:942–946.
  42. Chan, A.C., M. Iwashima, C.W. Turck, and A. Weiss. 1992. ZAP70: A 70 kd protein-tyrosine kinase that associated with the TCR  $\zeta$  chain. *Cell*. 71:649–662.
  43. Iwashima, M., B.A. Irving, N.S van Oers, A.C. Chan, and A. Weiss. 1994. Sequential interactions of the TCR with two distinct cytoplasmic tyrosine kinases. *Science (Wash. DC)*. 263:1136–1139.
  44. Sloan-Lancaster, J., A.S. Shaw, J.B. Rothbard, and P.M. Allen. 1994. Partial T cell signalling: altered phospho-zeta and lack of zap-70 recruitment in APL-induced T cell anergy. *Cell*. 79:913–922.
  45. Madrenas, J., R.L. Wange, J.L. Wang, N. Isakov, L.E. Samelson, and R.N. Germain. 1995.  $\zeta$  phosphorylation without ZAP-70 activation induced by TCR antagonists or partial agonists. *Science (Wash. DC)*. 267:515–518.