Expression of CD45 Lacking the Catalytic Protein Tyrosine Phosphatase Domain Modulates Lck Phosphorylation and T Cell Activation*

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Received for publication, November 24, 2004, and in revised form, January 26, 2005
Published, JBC Papers in Press, February 1, 2005, DOI 10.1074/jbc.M413265200

The function of the second protein tyrosine phosphatase domain (D2) in two-domain protein tyrosine phosphatases (PTP) is not well understood. In CD45, D2 can interact with the catalytic domain (D1) and stabilize its activity. Although D2 itself has no detectable catalytic activity, it can bind substrate and may influence the substrate specificity of CD45. To further explore the function of D2 in T cells, a full-length construct of CD45 lacking the D1 catalytic domain (CD45RABC-D2) was expressed in CD45+/− and CD45− Jurkat T cells. In CD45− Jurkat T cells, CD45RABC-D2 associated with Lck but, unlike its active counterpart CD45RABC, did not restore the induction of tyrosine phosphorylation or CD69 expression upon T cell receptor (TCR) stimulation. Expression of CD45RABC-D2 in CD45+/− Jurkat T cells resulted in its association with Lck, increased the phosphorylation state of Lck, and reduced T cell activation. TCR-induced tyrosine phosphorylation was delayed, and although MAPK phosphorylation and CD69 expression were not significantly affected, the calcium signal and IL2 production were severely reduced. This indicates that the non-catalytic domains of CD45 can interact with Lck in T cells. CD45RABC-D2 acts as a dominant negative resulting in an increase in Lck phosphorylation and a preferential loss of the calcium signaling pathway, but not the MAPK pathway, upon TCR signaling. This finding suggests that, in addition to their established roles in the initiation of TCR signaling, CD45 and Lck may also influence the type of TCR signal generated.

CD45 is a transmembrane two-domain protein tyrosine phosphatase (PTP) expressed exclusively in leukocytes (reviewed in Refs. 1–4). There are 21 transmembrane PTP in the human genome, and 12 have two domains (5). The catalytic activity of CD45 and other transmembrane two-domain PTP, such as PTPα and LAR, resides primarily in the first membrane proximal PTP with little or no activity attributed to the second PTP domain, D2 (6). In the case of CD45, the majority of data indicate that D2 is inactive (7–9). It lacks critical catalytic residues and cannot readily bind phosphotyrosine (10). Two functions have been proposed for CD45-D2: 1) to interact with and stabilize the catalytic D1 domain of CD45 (7, 11) and 2) to bind substrate and facilitate substrate recruitment (10, 12).

CD45 is required for T cell activation by constitutively dephosphorylating the negative regulatory tyrosine of Lck, Tyr505 (reviewed in Refs. 4 and 13–15). The restoration of T cell signaling by activated Lck in CD45− T cells (16) and restoration of T cell development in the CD45 null mouse by expression of Lck Y505F (17) support this finding. Dephosphorylation at Tyr505 creates a “primed” Lck molecule that can become activated upon TCR encounter with antigen, which initiates the signal transduction cascade by phosphorylating downstream substrates such as CD3ζ and ZAP-70 and leads to the activation of PLCγ-1, inositol 1,4,5-trisphosphate (IP3) generation, an increase in intracellular calcium, CD69 expression, and IL2 production (18, 19). However, in CD45−/- thymocytes and in some CD45− T cell lines, Lck is hyperphosphorylated at both the negative and autophosphorylation sites, suggesting that CD45 may also down-regulate Lck activity by dephosphorylating the autophosphorylation site (reviewed in Refs. 20 and 21). CD45 has also been implicated in down-regulating Src family kinase activity by negatively regulating integrin and CD44-mediated T cell adhesion (22, 23). Although Lck and, to a lesser extent, Fyn are considered major substrates for CD45 in T cells (reviewed in Refs. 24 and 25), other CD45 substrates may also exist such as TCRζ, ZAP-70 and Jak kinases (26–28).

Although CD45-D2 has no catalytic activity, it has been implicated in regulating TCR signaling. CD45, but not a CD45-LAR-D2 chimera, decreased CD3ζ phosphorylation levels and induced ZAP-70 phosphorylation and IL2 production upon TCR stimulation, suggesting that CD45-D2 was important for directing CD3ζ dephosphorylation and ZAP-70 phosphorylation (12). Mutation of residues within the unique acidic 19 amino acid insert in CD45-D2 resulted in increased calcium levels in one T cell line and sustained calcium influx after TCR stimulation in another cell line (29). However, in another study, calcium levels and MAPK activation were reduced when cells expressing the 19 amino acid deletion mutant were stimulated via the TCR (30).

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CD45 can associate with Lck in T cells (31, 32), and in vitro studies indicate that there is a complex interaction occurring between both the catalytic and non-catalytic regions of Lck and CD45 (33). CD45-D2 binds to the kinase domain of Lck in a phosphotyrosine-independent manner, and the region in Lck responsible for mediating this interaction, the subdomain X region of Lck, is implicated in facilitating substrate binding and promoting dephosphorylation (10). Here, we wanted to evaluate the interaction between the noncatalytic domains of CD45 and Lck in T cells.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Transfectants**—The human Jurkat leukemia T cell lines, clone E6.1 (CD45 positive, J1), and clone 45.01 (CD45 negative, J2), were purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 1 mM sodium pyruvate, and 100 μg/ml streptomycin-penicillin. J1 and J2 cell lines were transfected by electroporation with constructs encoding full-length mouse CD45RABC, mouse CD45RABC lacking residues 649–939 (mouse CD45RABC-D2), or the pBCMGNeo vector (34) as a control. Deletion of residues 649–939 essentially removes the catalytic D1 and PTP domain and the spacer region (7). Thus, CD45RABC-D2 expresses the extracellular RABC isoform, the transmembrane region, the membrane-proximal region, the inactive second PTP domain, and carboxy tail (residues 1–648 and 940–1268). Cells were selected using 0.5–1 mg/ml active G418 (Invitrogen), and mouse CD45 expression was analyzed by flow cytometry. Following four rounds of sorting on the FACSVantage (BD Biosciences) for high and comparable expression of mouse CD45, two independent clones from J1 and J2 cells were selected for further analysis, clones J1D2.1 and J1D2.2 and clones J2D1 and J2D2.

**Antibodies**—The monoclonal Abs T293/3 (mouse IgG, anti-human CD45) and I3/2 (rat IgG2b, anti-mouse CD45) were gifts from I. S. Trowbridge (35). OKT3 (mouse IgG1, anti-human CD3), C305 (mouse IgM, anti-human TCR β subunit), and MB4B4 (rat IgG2a, anti-mouse CD45, B exon) were from ATCC. Antibodies were purified from tissue culture supernatants using protein A- or G-Sepharose 4B beads according to standard procedures. Anti-Lck (rabbit anti-Lck antisera, R54 3B) and anti-CD45 (rabbit anti-catalytic domain of CD45, R54 2B) and anti-CD45 (rabbit anti-catalytic domain of CD45 antisera, R202 2) were generated as previously described (36). Anti-phospho-p44/42 MAPK, E10 (mouse anti-human IgG1), was purchased from Cell Signaling (Beverly, MA). Polyclonal goat anti-rat IgG coupled to horseradish peroxidase (HRP) or fluorescein isothiocyanate (FITC) was purchased from Jackson Immunoresearch Laboratories (Bar Harbor, ME). Polyclonal goat anti-mouse IgG conjugated with FITC or HRP was purchased from Caltag Laboratories (Burlingame, CA) and Southern Biotech (Birmingham, AL) respectively. Phycocerythrin-coupled anti-human CD45RB mAb, MB4B4 (1:5), and mouse and human CD45 were characterized by Western blotting with tissue culture supernatants containing anti-CD45 mAb, MB4B4 (1:5), and mouse and human CD45 were detected by rabbit anti-CD45 cytoplasmic domain antiserum, R202 2 (1: 5000), and Lck was detected with rabbit R54 3B antisera (1:5000) along with the appropriate HRP-conjugated secondary Ab (1:5000). For analysis of the phosphorylation state of Lck, 4G10 (1:5000) was used with protein A-conjugated HRP. Blots were developed using ECL (Amer sham Biosciences) and exposed to BioMax Film from Eastman Kodak Co. In co-immunoprecipitation experiments, Lck blots were stripped and reprobed with CD45R antibody.

**Measurement of Calcium Mobilization**—Calcium (Ca2+) mobilization was assayed in response to TCR cross-linking as described previously (37). 5 × 106 cells in 1 ml of Buffer A (Dulbecco’s modified Eagle medium containing 10 mM HEPE (pH 7.0)) were loaded with the Ca2+ indicator dye Indo-1/AM (Molecular Probes, Eugene, OR) at a concentration of 5 μM and incubated at 37 °C for 30 min. 1 ml of Buffer B (Dulbecco’s modified Eagle medium containing 10 mM HEPE and 5% fetal calf serum (pH 7.4)) was added, and the sample was kept at 37 °C for another 30 min. The cells were washed with Buffer C (Dulbecco’s modified Eagle’s medium containing 10 mM HEPE and 5% fetal calf serum (pH 7.2)) once and resuspended in Buffer C at a final concentration of 1 × 106/ml. Once a base line was taken, the analysis was paused, anti-TCR C305 was added to 50 μM, and analysis was immediately resumed. Ionomycin (1 μM final concentration) was used as a contrast to confirm that cells responded equally to the influx of calcium. A FACS Vantage cell sortor (BD Biosciences) was used to measure the fluorescence emissions from free and Ca2+-bound Indo-1/AM. Fluorescence emissions were separated by a 505-nm short pass beam splitter into component emissions by passage through 424- and 530-nm-centered 10-nm bandpass filters to detect violet and blue, respectively. Ratios of the emissions were calculated and plotted against time.

**IL2 Secretion**—Cells (1 × 105) in 200 μl of RPMI 1640 medium containing 10% fetal calf serum were stimulated in flat-bottom 96-well tissue culture plates with C305 (5–50 μg/ml) plus phorbol 12-myristate 13-acetate (PMA) (10 ng/ml, Sigma). IL2 secretion was determined after 12 h by enzyme-linked immunosorbent assay (BD Biosciences) performed in duplicate or triplicate.

**Cell Activation**—For analysis of tyrosine and MAPK phosphorylation, 1–2 × 106 cells (in 20-μl volume) were stimulated with C305 (10 μg/sample) from 0 to 60 min at 37 °C. Unstimulated control samples were treated with C305 and lysed immediately. At various time points, reactions were placed on ice and 6 μl of 5% Triton X-100 lysis buffer added. Cell lysates were centrifuged at 14,000 × g for 10 min at 4 °C. The supernatant was mixed with 3× SDS-PAGE reducing sample buffer, boiled, and centrifuged briefly. 2 × 105 cell equivalents were analyzed on 10% acrylamide gels and transferred to PVDF. The membranes were incubated with either anti-phosphotyrosine (4G10, 1:5000) or anti-phospho-ERK1/2 (E10, 1:1000), washed, and then incubated with goat anti-mouse-conjugated HRP (1:5000) and visualized using ECL.

For analysis of CD69 expression after TCR stimulation, cells (5 × 105/sample) were suspended in 20 μl of media and stimulated with C305 (10 μg/sample) at 37 °C for 30 min or left untreated. Up to 1 ml of fresh media was added into each sample, and cells were incubated at 37 °C from 0 to 8 h. Cells were incubated with anti-human CD69-coupled phycocerythrin and analyzed by flow cytometry.

**RESULTS**

**Expression of CD45RABC-D2 in CD45−Jurkat T Cells Does Not Restore TCR Signaling Events**—To determine whether CD45-D2 has functions that are independent of the catalytic domain, a construct expressing full-length mouse CD45RABC lacking the catalytic D1 domain (amino acids 649–939) was transfected into CD45− Jurkat T cells (see “Experimental Procedures” for details). Stable transfectants were sorted for high expression of mouse CD45 and, if required, also sorted to ensure comparable levels of TCR/CD3 expression among the untransfected cells, vector alone control, and the CD45RABC-D2 transfected cells. CD45RABC-D2-transfected...
cells were then cloned, and at least two clones were chosen for subsequent analysis (J' D2.1 and J' D2.2) (Fig. 1, A and B). Full-length CD45RABC was also transfected into CD45 Jurkat T cells. The levels of expression of mouse CD45, TCR, and CD3 were checked routinely by FACS analysis to ensure comparable levels of expression (Fig. 1A). In addition, the presence of human CD45 was monitored by FACS analysis as low levels of human CD45 had previously been reported in the J45.01 cells (38) and were observed in this case (Fig. 1A). Fig. 1B demonstrates the expression of CD45RABC-D2 and CD45RABC in J- cells after immunoprecipitation of mouse CD45, separation on SDS-PAGE, transfer to PVDF membrane, and then immunoblotting with an anti-mouse CD45RB-specific antibody.

In the CD45 Jurkat cells, Lck is hyperphosphorylated at the negative regulatory site and TCR stimulation does not occur (reviewed in Refs. 1, 2, and 4). Transfection of the full-length CD45RABC, but not CD45RABC-D2, restored the initial induction of tyrosine phosphorylation upon TCR stimulation (Fig. 1C). CD45RABC also restored the induction of expression of CD69 monitored from 2 to 8 h, whereas the expression of CD45RABC-D2 had no effect on CD69 expression, even when stimulation was prolonged to 24 h (data not shown). This is consistent with CD45RABC-D2 lacking protein tyrosine phosphatase activity.

CD45RABC-D2 Associates with Lck in CD45- and CD45 Jurkat T Cells and Results in Increased Lck Phosphorylation in the CD45 Jurkat T Cells—Mouse CD45RABC-D2 was transfected into CD45 (JE6.1) Jurkat T cells (J’), and cells were sorted for high levels of expression of CD45RABC-D2 and approximately equivalent levels of expression of human CD45, TCR, and CD3 compared with the levels of the parental line (J”). Flow cytometry indicates the expression of these molecules (Fig. 2A). Specific expression of transfected mouse CD45RABC-D2 was also confirmed by bloting for anti-mouse CD45RB in J’ cell lysates (Fig. 2B, upper panel). Cell lysates were also blotted with anti-CD45 cytoplasmic domain antisera that recognize both mouse and human CD45.2 This result shows the presence of endogenous human CD45 in J’ cells and J+ cells transfected with vector alone (J’ V) and both human

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and transfected mouse CD45RABC-D2 in J⁺ D2.1 and D2.2 cells (Fig. 2B, lower panel). The antisera detect primarily two CD45 isoforms in J⁺ and J⁺ V cells that correspond to the lower molecular mass isoforms of CD45 (39). Coincidently, the transfected CD45RABC-D2 lacking D1 but expressing all three alternatively spliced exons (ABC) migrated to a similar position as the lower molecular mass isoform of endogenous CD45.

Mouse CD45 was immunoprecipitated from 1% Triton-X-100-soluble and 1% Brij-58 detergent-soluble lysates of CD45⁺ and CD45⁻ cells, subjected to SDS-PAGE, and then immunoblotted for Lck. Fig. 3, A and B, shows that Lck co-immunoprecipitated with CD45RABC and CD45RABC-D2 in both the CD45⁺ and CD45⁻ T cells. This demonstrates that CD45RABC-D2 can associate with Lck in the absence of endogenous CD45. It also suggests that the interaction occurs independently of Lck phosphorylation, because Lck is hyperphosphorylated in CD45⁻ but not in the CD45⁺ cells (Fig. 3C). In addition, this shows that the association can occur in the absence of the active PTP domain, CD45-D1.

CD45RABC-D2 Disrupts T Cell Activation

To determine whether the presence of CD45RABC-D2 affected TCR signaling and T cell activation, the cells were stimulated with soluble TCR antibody. Cells expressing CD45RABC-D2 showed a slower induction of tyrosine-phosphorylated proteins (Fig. 4A). Maximum phosphorylation was observed in the parental CD45⁺ cells after 1 min, whereas comparable phosphorylation was not observed in the CD45RABC-D2-transfected cells until 5 min.

CD45RABC-D2 Does Not Affect MAPK Activation or CD69 Expression

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To determine whether this change translated to any changes in downstream effects, MAPK (ERK1/2) phosphorylation and CD69 expression levels were monitored. Fig. 4B showed that, whereas MAPK phosphorylation was absent in the CD45^− T cells and induced at 5 min in the CD45^+ T cells, the presence of CD45RABC-D2 did not significantly affect the induction time or intensity of ERK1/2 phosphorylation. Because CD69 expression is downstream of MAPK activation (40), we examined the induction of CD69 expression after TCR stimulation. Fig. 4C showed that the basal level of CD69 was slightly decreased in CD45RABC-D2-expressing cells. There was also a slight delay of CD69 induction observed at 1 h, but by 2 h, CD69 induction was comparable. This observation suggests that the slight delay in the induction of tyrosine phosphorylation is insufficient to cause significant differences downstream in the MAPK pathway leading to CD69 expression.

CD45RABC-D2 Prevents Calcium Flux and IL2 Production—To determine whether CD45RABC-D2 affected the other major signaling pathway in TCR signaling, the calcium pathway, cells were loaded with the fluorescent Ca^{2+} indicator dye, Indo-1/AM. Changes in intracellular calcium levels were monitored on the FACS Vantage, and after a base line had been established, TCR antibody was added to the cells. Fig. 5A (upper panel) indicated that, although the CD45^− cells had a robust increase in intracellular calcium, cells expressing CD45RABC-D2 had virtually no calcium response. Their response was similar to Jurkat cells expressing no CD45 (J^−). The addition of ionomycin (Fig. 5A, lower panel) led to similar increases in intracellular calcium, indicating that all of the cells were capable of responding to calcium.

TCR signaling pathways in Jurkat T cells converge to stimulate IL2 gene transcription. To determine the outcome of TCR signaling in the CD45RABC-D2-transfected CD45^− T cells, cells were stimulated with TCR antibody together with PMA. The amount of IL2 secreted after 12 h of stimulation was measured by enzyme-linked immunosorbent assay. Fig. 5B indicated that the expression of CD45RABC-D2 dramatically reduced IL2 production in CD45^+ Jurkat cells. A similar effect was also seen when the CD3 antibody, OKT3, and PMA were used for stimulation (data not shown). IL2 was not produced in the absence of CD45 or by PMA alone. CD45RABC-D2-transfected cells did not secrete significant levels of IL2, even after 48 h of stimulation (data not shown).

DISCUSSION

This study indicates that a CD45 protein lacking the catalytic domain of CD45 can associate with Lck in T cells, independently of the phosphorylation state of Lck. Although the precise region of CD45 mediating this interaction was not determined, in vitro data indicate that the second PTP domain of CD45, D2, can bind directly to Lck in a phosphotyrosine-independent manner (10). Furthermore, data from this study show that expression of CD45RABC-D2 results in an increase in Lck phosphorylation in CD45^+ T cells, suggesting that, in the absence of an active D1, CD45RABC-D2 binds to Lck and prevents its dephosphorylation by endogenous CD45. This is also consistent with in vitro data showing a role for D2 in binding substrate and facilitating substrate dephosphorylation (10). In addition, when CD45 D2 was replaced with LAR-D2 and the chimera was expressed in T cells, there was no decrease in CD3ζ phosphorylation, leading Kashio et al. (12) to conclude that CD45-D2 has a role in binding substrate. The data presented here are consistent with this conclusion as we show that CD45RABC-D2 binds to the major CD45 substrate in T cells, Lck. However, it is also possible that other regions of CD45 may facilitate an indirect interaction with Lck. For example, the extracellular domain of CD45, primarily CD45R0, has been reported to associate with CD4, which in turn binds Lck (41, 42). Although the transmembrane region of CD45 is not required for its association with Lck, it does mediate an interaction with CD45AP (43), which also binds to Lck (44). Although CD45RABC-D2 can associate with Lck in the absence of CD45, CD45RABC-D2 may also bind to CD45 because a small amount co-immunoprecipitated with endogenous CD45 (data not shown). This may also contribute to the reduced effectiveness of CD45, as it has been suggested that the membrane-proximal region of one CD45 molecule may bind the catalytic domain of another and down-regulate its function (45, 46).

In Jurkat T cells, CD45 is required for the following TCR signaling events: the induction of tyrosine phosphorylation; PLCγ-1 activation; production of IP_3; and increase in intracellular calcium levels (38, 47). This positive effect on TCR signaling has been attributed to the ability of CD45 to dephosphorylate the negative regulatory tyrosine, Tyr505, in Lck, which generates a primed Lck that can be activated when the TCR...
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Recognizes antigen (reviewed in Refs. 4, 15, and 18). In this study, CD45RABC-D2 disrupted the ability of endogenous CD45 to efficiently dephosphorylate Lck and resulted in aberrant TCR signaling. It caused a slight delay in the induction of tyrosine-phosphorylated proteins, but did not affect the tyrosine phosphorylation of MAPK or the induction of CD69 expression. However, it did have a dramatic effect on calcium flux and IL2 production. In other studies, deletion or mutation of the unique acidic region in CD45-D2 generated defects in calcium signaling and, in one case, MAPK activation in T cells (29, 30) and when CD45-D2 was replaced with LAR-D2, TCR-induced IL2 production was reduced (12). Together, these data point toward a role for the noncatalytic D2 domain in regulating TCR signaling.

Jurkat T cells and its associated T cell signaling mutants have been invaluable in identifying the components of the TCR signaling pathway (48). However, one caveat to the use of the Jurkat T cell line is that it is defective in the expression of two lipid phosphatases, SHIP and PTEN, that results in the constitutive activation of the phosphatidylinositol 3-kinase pathway. However, how this impacts TCR signaling events in this and other studies is not clear as the importance of phosphatidylinositol 3-kinase in TCR signaling is still poorly understood. TCR signaling activates at least two major signaling pathways leading to IL2 production, the Ras-MAPK pathway and the calcium pathway, and CD45 and Lck were thought to be required for both pathways. Indeed, CD45 is required for the activation and induction of tyrosine phosphorylation of PLCγ-1 (38, 47), which generates diacylglycerol and IP3, and then IP3 triggers the release of calcium from intracellular stores (reviewed in Ref. 49). In addition, TCR-induced phosphorylation of MAPK was dependent on Src family kinase activation as 1) via PLCγ/H9253 and IL2 production. In other studies, deletion or mutation of the cells and help with the calcium assays. We thank Dr. Jackie Felberg, Jacqueline Lai, Brian Ruffell, and Jennifer L. Cross for reading the manuscript.

Acknowledgments—We thank Dr. Ian Trowbridge for the gift of antibodies and Andy Johnson at the University FACs facility for sorting the cells and help with the calcium assays. We thank Dr. Jackie Felberg, Jacqueline Lai, Brian Ruffell, and Jennifer L. Cross for reading the manuscript.

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