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Regulation of the Calcium/NF-AT T Cell Activation Pathway by the D2 Domain of CD45

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CD45 contains tandem repeated protein tyrosine phosphatase (PTP) domains and is essential for the initiation of the earliest activation events resulting from Ag ligation of the TCR. The second PTP domain (D2) contains four CK2 phosphorylation sites in a unique 19-aa insert, which are targets of CK2 phosphorylation. This study was designed to evaluate the roles of these Ser residues in T cell activation. Transient transfection of the CD45− T cell line, J45.01, with CD45 cDNA incorporating four Ser to Ala (S/A) mutations in the 19-aa insert did not affect the magnitude of NF-AT activation resulting from TCR ligation. However, the basal level of NF-AT activity in unstimulated cells expressing the CD45 S/A mutation was elevated 9- to 10-fold. Increased basal NF-AT was dependent on extracellular Ca2+ stores as judged by EGTA treatment. In additional experiments, isolation of stable clones derived from transfection of the CD45 S/A mutant into CD45− H45.01 cells showed sustained calcium flux after TCR engagement. The sustained calcium flux returned to baseline levels after addition of EGTA, suggesting that the expression of the CD45 S/A mutant may have prevented deactivation of plasma membrane calcium channels. Consideration of both transient and stable transfection systems suggests that in addition to being essential for initial events in T cell triggering, the intact CD45 D2, 19-aa insert is necessary for regulation of TCR-mediated calcium signaling pathways. The Journal of Immunology, 2000, 164: 2557–2564.

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D45 is a transmembrane protein tyrosine phosphatase (PTP) required for the initiation of Ag receptor-mediated signal transduction in T and B lymphocytes. CD45 contains two tandem PTP domains, designated D1 and D2. The membrane-proximal domain (D1) possesses all the PTP activity, while the second domain (D2) is catalytically inactive. The sequences of the D1 and D2 are highly homologous to the large family of PTPs containing the CX5R motif (1), and both CD45 domains are highly conserved from shark to human (2). The CD45 D2 domain also contains a highly conserved, 19-aa insert in the PTP sequence that is only found in the D2 of CD45 and not in any other PTP (2). This insert is highly acidic and is a target for casein kinase 2 (CK2) phosphorylation in T cells (3). CD45 has previously been shown to be a target for both in vitro and in vivo CK2 phosphorylation (4). Despite the high conservation of the CD45 D2, its function in T cell activation remains unclear. The precise conformational relationship between the D1 and the D2 domains has been demonstrated by experiments showing the loss of D1 PTP activity after the deletion of all or even small portions of the D2 PTP domain (5, 6).

T cell activation proceeds through at least three parallel and interacting pathways (7). The process of T cell activation is initiated by ligation of the TCR and CD4 (or CD8) by an MHC peptide Ag complex displayed on the surface of an APC. The first steps leading to activation involve a wave of tyrosine phosphorylation of the CD3 component of the TCR by the tyrosine kinase Lck (7). Other tyrosine kinases (such as ZAP-70) and adaptor molecules (such as LAT (8) and SLP-76 (9)) are rapidly recruited to the TCR, and each becomes further phosphorylated (7, 10). Subsequently, the MAPK pathway is activated via Grb2, SOS, and Ras. The JNK pathway is activated via small GTPases (such as Rac/CDC42) and requires costimulation through CD28 (11). A third major pathway in T cell activation proceeds through activation of PLCγ and PKC and the release of Ca2+ from the endoplasmic reticulum stores (reviewed in Ref. 12). TCR engagement results in the activation of phosphatidylinositol PLCγ1, which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate and diacylglycerol. Inositol 1,4,5-trisphosphate stimulates the release of calcium from intracellular stores, and cytoplasmic Ca2+ triggers the opening of plasma membrane calcium channel (CRAC), causing an influx of extracellular calcium (12). It is the extracellular Ca2+ entering through the CRAC channel that is largely responsible for the lengthy, sustained cytoplasmic Ca2+ levels observed after TCR stimulation (12, 13). At the end of the pathway, cytoplasmic calcium binds to the calcium-dependent regulatory protein, calmodulin, and the complex activates the phosphatase calcineurin, which activates NF-AT by dephosphorylation (12). Efficient T cell responses to Ags also require a costimual by CD28 receptor on T cells and B7 ligand on APC. CD28-B7 interaction, in conjunction with TCR stimulation, increases the duration of the response and augments the production of lymphokines, whereas TCR stimulation in the absence of CD28 ligation leads to anergy (14).

The critical role of CD45 in the TCR signaling process is in the initial tyrosine phosphorylation events involving the tyrosine kinase Lck. CD45 dephosphorylates the C-terminal, inhibitory site at position Tyr505 and thus keeps Lck in an active state (10, 15,

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3 Abbreviations used in this paper: PTP, protein tyrosine phosphatase; PTK, protein tyrosine kinase; PVDF, polyvinylidene difluoride; CsA, cyclosporin A; CD2, casein kinase 2; CRAC channels, calcium release-activated calcium channels; ER, endoplasmic reticulum; Lck, p56Lck; MAPK, mitogen-activated protein kinase; PLCγ, phospholipase Cγ; PKC, protein kinase C.
16). CD45-negative cells have lowered Lck activity in the membrane-associated pool of the PTK (17). This paradigm predicts that the loss of CD45 results in the loss of all signaling activity because of the lack of sufficient Lck in an activated state. Further support for the idea that Lck is a primary substrate for CD45 has been provided by the observation that F, hybrids between CD45 knockout mice and mice expressing an activated form of Lck regain the ability to be stimulated by Ag (18). Lymphocytes isolated from CD45 knockout mice cannot be stimulated through the TCR (19). Alternate views have been proposed that CD45 may also exert a negative regulatory effect on Lck (20). In addition to Lck, CD45 is likely to act on other substrates, such as TCRζ and ZAP-70 (21, 22).

In T cells CD45 is the most abundant membrane protein, comprising about 10% of the T cell membrane protein and >90% of the membrane-associated PTP activity (23).

The unique nature and high phylogenetic conservation of the 19-aa acidic region in the CD45 D2 domain led us to hypothesize that this insert serves as a regulatory module in lymphocyte activation. We have addressed the question of the role of the CD45 D2 in downstream signaling in T cells by performing mutational analysis of certain highly conserved sites in the 19-aa, acidic insert in the D2. The effect of mutation was determined by reconstitution of CD45- cells followed by functional analysis of various signaling pathways. A potential role for the D2 acidic insert has been identified in the regulation of the Ca²⁺/NF-AT pathway in resting and activated T cells.

Materials and Methods

Cells, Abs, and reagents

Jurkat cells (clone E6-1) (a human acute T cell leukemia line) and CD45-deficient Jurkat cells (clone J45.01) were obtained from Dr. Gary Koretzky (University of Iowa). H45.01, a CD45-deficient variant of HPB-ALL (24), was obtained from Dr. Arthur Weiss (University of California, San Francisco, CA). The cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 100 U/ml streptomycin/penicillin (Life Technologies, Gaithersburg, MD), and 50 mM 2-ME (Sigma, St. Louis, MO). Cyclosporin A (CsA) was purchased from Alexis Biochemicals (San Diego, CA). HRP-conjugated goat anti-rabbit and goat anti-mouse secondary Abs were purchased from Bio-Rad (Hercules, CA). CHO deficient in western blotting detection reagents were purchased from Amersham (Arlington Heights, IL).

Monoclonal anti-CD3 clone 235 (IgM type) and anti-CD28 clone N6E1 (IgG type) Abs were provided by Dr. Shu Man Fu (University of Virginia, Charlottesville, VA). Anti-Lck Ab was obtained from Dr. Bart Selton (The Salk Institute, La Jolla, CA). Phospho-p44/42 MAPK Ab was purchased from New England Biolabs (Beverley, MA); MAPK Ab was purchased from Sigma. Anti-phosphotyrosine Ab (4G10) was purchased from Upstate Biotechnology (Lake Placid, NY). CD45 Ab for Western blotting was purchased from Transduction Laboratories (Lexington, KY), and CD45 Ab for immunoprecipitation was purified from 9.4 hybridoma (American Type Culture Collection, Manassas, VA). Fluorescent CD45 Ab, PE anti-human CD45, was purchased from PharMingen (San Diego, CA). Anti-ZAP 70 Ab and anti-PLC Ab were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

DNA constructs and site-directed mutagenesis

The expression vector hLCA-NEO 3, which contained the intact wild-type CD45 cDNA under the control of SFFV promoter, was provided by Dr. Arthur Weiss. The construct expresses full-length CD45 with the low m.w. CD45 cDNA under the control of SFFV promoter, was provided by Dr. Arthur Weiss. The construct expresses full-length CD45 with the low m.w.

The expression vector hLCA-NEO 3, which contained the intact wild-type CD45 cDNA under the control of SFFV promoter, was provided by Dr. Arthur Weiss. The construct expresses full-length CD45 with the low m.w.
and subjected to FACS analysis as described above. Cells stained with secondary Ab alone were used as negative controls. FACS was performed with a Becton Dickinson Vantage FACS (San Jose, CA) at the Michigan State University Flow Cytometry Facility.

PTP assay

Stable transfected cells (5 × 10⁵) were washed twice with 20 mM Tris (pH 8.0) and 137 mM NaCl and lysed for 30 min at 4°C in Nonidet P-40 lysis buffer containing 1% Nonidet P-40 (Pierce, Rockford, IL), 20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 5 mM EDTA, 2 mM PMSF, 0.23 U/ml aprotinin, 0.5 µg/ml leupeptin, 0.7 µg/ml pepstatin A, and 10 µg/ml DNase I. After removal of nuclei by centrifugation, the lysate supernatant was incubated with anti-CD45 (clone 94, American Type Culture Collection) for 30 min followed by addition and incubation with GammaBind Plus Sepharose (Pharmacia Biotech, Piscataway, NJ) for another 30 min at 4°C. The immunoprecipitates were washed once with 20 mM Tris (pH 8.0), once with LiCl, once with 20 mM Tris (pH 8.0), and once with 1× PTP buffer (25 mM HEPES (pH 7.3), 5 mM EDTA, and 10 mM DTT) and were resuspended in 40 µl of 1× PTP. The PTP assay (Promega) was conducted at 30°C, and each assay contained 5 µl of tyrosine phosphopeptide substrate END(pY)INASL. After incubation for 30 min, the supernatant was added to 96-well plates, and the reaction was stopped by a mixture of malachite green and molybdate. The amount of released free phosphate was measured by the absorbance of the dye-phosphate complex at 600 nm wavelength using a microtiter plate reader (Molecular Devices, Eugene, OR), and the data were analyzed using SoftMaxPro (version 2.1.0) software. The Sepharose G-bound CD45 was boiled in SDS sample buffer, loaded in 10% SDS-polyacrylamide gel, and transferred to nitrocellulose membrane after electrophoresis. Western blotting was performed to confirm equal amounts of CD45 in the immunoprecipitates.

TCR stimulation and detection of MAPK activation

Cells were washed twice with PBS, resuspended into RPMI 1640 medium at 1 × 10⁶/ml, and incubated at 37°C for 5 min. Anti-CD3 mAb (clone 235, 1/500) and anti-CD28 Abs for 8 h, and luciferase activity was determined using a EPICS Elite software. The Sepharose G-bound CD45 was boiled in SDS sample buffer, loaded in 10% SDS-polyacrylamide gel, and transferred to nitrocellulose membrane after electrophoresis. Western blotting was performed to confirm equal amounts of CD45 in the immunoprecipitates.

Calvin flux analysis

Cells (1 × 10⁵) were washed with cell loading medium (RPMI 1640, 2% FCS, and 25 mM HEPES, pH 7.4) and incubated with 1.5 µM indo-1/AM (Molecular Probes) at 37°C for 1 h in the dark. The loaded cells were washed twice with DMEM (Life Technologies) plus 2% FCS and resuspended in a 10 ml of cell loading medium. Aliquots of 1 ml of loaded cells were analyzed relative to time by flow cytometry using an EPICS Elite flow cytometer (Coulter, Hialeah, FL). The ratio of fluorescence at 420 nm to that at 510 nm was used to indicate changes in calcium flux. Some samples were treated with 1 µM ionomycin or 8 mM EGTA, and stimulated with anti-CD3 and/or anti-CD28 Abs (1 µg each/10⁶ cells/ml). The flow cytometry data were analyzed using WinMDI software written by Joe Trotter (The Salk Institute, Flow Cytometry Laboratory).

Results

Mutations in the CD45 D2 domain acidic insert

We have shown previously that Ser phosphorylation of the D2 domain acidic insert by CK2 was blocked by mutation of Ser at positions 815, 818, 819, and 823. To study the role of the acidic insert Ser residues in CD45-mediated lymphocyte signal transduction, mutations were incorporated into expression vector hLCA.neo as shown in Fig. 1. These mutants were designated: S/A, containing the simultaneous replacement of four Ser at positions 815, 818, 819, and 823 to Ala; S/E, containing the simultaneous replacement of the four Ser to Glu residues; Δ19, CD45 mutant with the 19-aa acidic insert of D2 domain deleted; C⁶⁶⁷S, PTP inactive CD45 construct with Cys to Ser mutation in the D1 domain catalytic center. Some transfections yielded no signal, whereas others yielded wild-type signals. These results indicated that efficient CD45 expression was required for TCR-mediated NF-AT activation (Fig. 2, vector only). Introduction of wild-type CD45 into J45.01 cells restored the capacity of the cells to activate NF-AT-luciferase reporter upon TCR engagement (Fig. 2, wt), whereas introduction of the PTP inactive form of CD45 did not rescue signaling (Fig. 2, C⁶⁶⁷S). When CD45 with S/A mutations in the D2 domain acidic region was transiently expressed, NF-AT activity was not increased. Mutant CD45 cDNA was transiently expressed into CD45-deficient Jurkat T cells (Jurkat) together with NF-AT-luciferase or AP-1-luciferase reporter constructs. βgal reporter DNA was also cotransfected to normalize for transfection efficiency. Two days post-transfection, cells were incubated with anti-CD3 and anti-CD28 Abs for 8 h, and luciferase activity was determined using a luminometer (Fig. 2). Stimulation of Jurkat cells with anti-CD3/CD28 Abs did not result in NF-AT luciferase activation, confirming that efficient CD45 expression was required for TCR-mediated T cell activation (Fig. 2, vector only). Introduction of wild-type CD45 into J45.01 cells restored the capacity of the cells to activate NF-AT-luciferase reporter upon TCR engagement (Fig. 2, wt), whereas introduction of the PTP inactive form of CD45 did not rescue signaling (Fig. 2, C⁶⁶⁷S). When CD45 with S/A mutations in the D2 domain acidic region was transiently expressed, NF-AT activity was not increased.
transfection (Fig. 3). This result indicates that the effect of S/A mutant was over-

Luciferase was activated by the anti-CD3/CD28 stimulation to a similar extent as the wild type transfectant (Fig. 2, S/A). However, an elevated basal level of NF-AT activity was observed in S/A transfectants. A smaller, but reproducible, basal elevation of NF-AT activity was also observed for S/E and Δ19 transfectants, without alteration in the overall magnitude of response to anti-CD3/CD28 stimulation (Fig. 2, S/E and Δ19). The S/A mutant exhibited an average 9- to 10-fold increase in basal activity, while intermediate values were observed for the S/E mutant and Δ19 mutant (5- and 3-fold increases, respectively; Fig. 2B). The success of transfection for each mutant was judged to be at the same efficiency, because all the mutant constructs exhibited comparable overall stimulation with anti-CD3/CD28 (Fig. 2A), and comparable β-galactosidase activity was detected in each transfectant (data not shown). Although the cells were clearly functionally reconstituted, FACS analysis using PE-anti-CD45 Ab staining of the transiently transfected populations indicated CD45 expression in only about 5–8% of cells, compared with empty vector transfection (data not shown). Parallel experiments performed using an AP-1-luciferase demonstrated only a small activation after stimulation (Fig. 3A). The basal level of NF-AT activity was measured in J45.01 cells after transfection as shown above. The average and SD of three experiments are shown.

Stable transfectants expressing mutant CD45

The experiments described above suggested that the alterations caused by mutation of CD45 were primarily in the Ca^{2+}/NF-AT pathway. However, this was difficult to study further in a Jurkat transient transfection system because the overall expression of CD45 was quite low. We therefore endeavored to develop a system in which stable cell lines expressing mutated CD45 could be evaluated for multiple TCR signaling pathways and for Ca^{2+} influx under various experimental conditions.

CD45-negative H45.01 cells were transfected with mutant CD45 constructs by electroporation, and single, stable transfectants were selected by limiting dilution and G418 resistance. Selected clones were screened by immunoblotting with anti-CD45, and CD45 expression was confirmed by flow cytometric analysis of PE-anti-CD45-labeled cells (Fig. 5A). For each CD45 mutant construct, at least three individual clones expressing comparable location into the nucleus. CsA (100 ng/ml), an inhibitor of calcineurin, completely blocked the activation of NF-AT by TCR engagement, and it also abolished the elevated basal NF-AT activity in CD45 S/A transfectants (Fig. 3C). Thus, the elevation of NF-AT in CD45 S/A transfected resting T cells depended on the function of the Ca^{2+} pathway and calcineurin.

Cytoplasmic calcium flux after T cell activation is dependent on both intracellular and extracellular stores (12). Because EGTA chelates extracellular Ca^{2+} stores (12), we treated the CD45 S/A transfected cells with EGTA (8 mM) to determine whether the elevation of NF-AT depended on Ca^{2+} influx. EGTA completely abrogated the activation of NF-AT by TCR cross-linking, indicating that sustained calcium influx was required for activation of the transcription factor (Fig. 3D). At the same time, the basal elevation of NF-AT activity in CD45 S/A transfected decreased to background levels, suggesting that the effect of CD45 S/A expression was due to a loss of regulation of calcium influx.
levels of CD45 protein were chosen for functional analysis. CD45-expressing H45.01 clones were also stained with anti-CD3 (235 mAb) and FITC-goat anti-mouse Ab and subjected to flow cytometric analysis. Each stable transfectant used was shown to express essentially the same level of CD3 (Fig. 4B). As further confirmation of CD45 expression, the CD45 PTP activity of stably transfected cells was compared (Fig. 5). Wild-type and mutant CD45 were immunoprecipitated from individual transfected clones (5 × 10^7 cells) and subjected to tyrosine phosphatase assay using tyrosine phosphopeptide END(pY)INASL as substrate. Immunoprecipitates obtained from CD45^−/− H45.01 cells were used as negative controls. The acidic insert mutants were shown to have comparable PTP activity as wild-type transfectants (Fig. 5), while the C^667S mutant lacked PTP activity. The data presented here are representative of different individual clones.

**TCR stimulation of clones containing stably expressed CD45 mutants**

One of the earliest signaling events upon TCR stimulation is the activation of TCR-associated PTKs. The activated PTKs phosphorylate diverse downstream substrates, which leads to activation of the MAPK pathway and the Ca^{2+}/NF-AT pathway. To determine the effect of the CD45 mutation we compared the autophosphorylation and kinase activities of Lck and ZAP-70 after TCR stimulation. All the acidic insert mutations (S/A, S/E, and Δ19) exhibited similar activation patterns for both kinases, which peaked at 5 min after stimulation and returned to basal level by 30 min (data not shown). In addition, the overall tyrosine phosphorylation patterns of the CD45 mutant clones after stimulation were not detectably different from that of wild-type CD45 (data not shown). We next examined the activation of p44/42 MAPK in the mutant transfectants (Fig. 6). TCR-mediated activation of MAPK was detected by a phospho-MAPK-specific Ab that recognizes catalytically activated Erk1 and Erk2. Minutes after TCR cross-linking, phospho-MAPK was detected in wild-type CD45 transfectants, while CD45^−/− H45.01 cells failed to induce the phosphorylated form of MAPK (Fig. 6A). All three mutants of the D2 acidic region appeared to activate p44/42 MAPK to approximately the same extent as wild-type CD45 after stimulation for 5 min, followed by a decrease after 30 min of stimulation. We concluded, therefore, that mutations in the D2 acidic region did not detectably affect the MAPK pathway.

The Ca^{2+}/NF-AT pathway was then evaluated in mutant transfectants of H45.01 cells by Ca^{2+} flux analysis after TCR stimulation (anti-CD3/CD28). Stable transfectants were loaded with cell-permeable indo-1/AM, and cytoplasmic Ca^{2+} mobilization was determined by flow cytometry (Fig. 7). CD45^−/− H45.01 cells

**FIGURE 4.** CD45 and CD3 expression after stable transfection of H45.01 cells. The expression of CD45 (A) and CD3 (B) was determined by FACS analysis. The results shown are representative for selected clones used in the study. For CD45, positive fluorescence was compared with that of H45.01 cells (CD45 negative). CD3-positive fluorescence was compared with that obtained with second Ab alone (right panel, light peak) and to H45.01 (right panel, dark peak). The dashed line in each panel indicates the approximate demarcation of positive and negative signals.

**FIGURE 5.** PTP activity of wild-type and mutant CD45 in stable transfectants. Wild-type and mutant forms of CD45 were immunoprecipitated from equivalent amount of CD45-transfected H45.01 clones and subjected to in vitro PTP assays using tyrosine phosphopeptide END(pY)INASL as substrate. The average PTP activity of multiple clones is shown (average and SD). Immunoprecipitate from H45.01 cells was used as a negative control. PTP activity is expressed as nanomoles of free phosphate released per 10 min per CD45 immunoprecipitated from 5 × 10^7 cells.

**FIGURE 6.** Activation and phosphorylation of MAPK after TCR ligation of H45.01 cells expressing different forms of CD45. H45.01-transfected clones were activated with anti-CD3/CD28 and subjected to lysis and separation by SDS-gel electrophoresis. A, Phosphorylation of MAPK was detected by immunoblotting with anti-phospho-MAPK Ab. Immunoblottings with anti-MAPK (B) and anti-CD45 (C) were performed to verify expression levels.
did not exhibit any detectable Ca\(^{2+}\) flux upon stimulation (Fig. 7C). The intracellular calcium concentration of the wild-type CD45 transfectant increased within a few minutes after TCR crosslinking, peaked at about 5–6 min, and then gradually decreased to the baseline (Fig. 7A). When the CD45 S/A mutant transfectants were stimulated by anti-TCR, the calcium flux observed was of the same magnitude as that seen for cells expressing the wild type CD45 (Fig. 7B). However, the calcium flux in the S/A mutant appeared more sustained than that observed for the wild-type transfectant, and after longer incubation (up to 2 h) it was observed that the CD45 S/A mutant did not recover to the baseline (Fig. 7B plus additional clones shown in Fig. 7E). Ca\(^{2+}\) flux in cells expressing wild-type CD45 returned to baseline during this incubation (Fig. 7A and additional clones shown in Fig. 7D). When EGTA was added to the medium containing the CD45 S/A clone, the sustained calcium signal returned to baseline immediately (Fig. 7E), indicating that the abnormally sustained Ca\(^{2+}\) level required extracellular Ca\(^{2+}\). This result suggests that the elevated calcium level in CD45 S/A mutant cells results from the loss of regulation of a plasma membrane calcium channel. Interestingly, analysis of the CD45 S/E and Δ19 mutants showed that these clones exhibited a calcium flux that was intermediate and close to the wild-type pattern (data not shown).

Discussion

The cytoplasmic portion of most of the receptor-type PTPs contain two tandemly arranged PTP domains. The membrane-proximal domain (D1) exhibits PTP activity, while the membrane-distal domain (D2) is usually enzymatically inactive despite having high primary and secondary structural similarity to the PTP family of proteins. Although the role of the D2 domain in CD45 function remains unknown, it has been proposed that the D2 may have a distinct specificity for unusual substrates, that it may serve as a regulatory domain, or that it may serve as a docking module for signaling proteins or adaptor molecules. We have focused our study on the 19-aa acidic insert sequence in the D2 because 1) among PTPs, the acidic insert is unique to CD45; 2) the insert is highly conserved among different species (2); and 3) the insert is a target of post-translational modification by CK2 (3). Based on a comparison of CD45 to the three-dimensional crystal structure of the PTP family of phosphatases, we concluded that the loop containing the 19-aa insert would lie at the opening of the substrate binding cleft of the inactive D2 enzyme (1). The loop is probably exposed on the outside of the molecule, because it is available for CK2 phosphorylation (3). Phosphorylation of the acidic insert by CK2 has been proposed to modulate the PTP activity of CD45 for certain substrates (3). In the present study we have addressed the question of the function of the D2 domain of CD45 by mutation of four serines of the acidic insert that are the target of post-translational modification by CK2 (3).

The CD45 acidic insert mutant proteins supported TCR-mediated activation. The only mutant that did not support activation was the C\(^{667}\)S mutant that lacked PTP activity. For the stable mutants, the activations of Lck, Zap-70, and MAPK were similar regardless of whether the cells expressed wild-type CD45 or the mutated CD45 S/A. For these signaling molecules, the expression of CD45 S/A did not affect the magnitude or the duration of activation. In addition, the overall patterns of tyrosine-phosphorylated proteins after stimulation were similar regardless of the CD45 form expressed. Examination of the Ca\(^{2+}\) pathway, however, revealed that the CD45 S/A mutant significantly altered the down-regulation of extracellular Ca\(^{2+}\) influx through membrane channels. Cells expressing CD45 S/A exhibited a sustained Ca\(^{2+}\) flux after activation that lasted long beyond the point when the Ca\(^{2+}\) levels of wild-type CD45-expressing cells returned to basal levels. The sustained Ca\(^{2+}\) level in CD45 S/A-expressing cells

![FIGURE 7. Calcium flux after TCR stimulation of HPB 45.1 (CD45 \(^{-}\)) cells expressing wild-type or mutant forms of CD45. A, Wild-type; B, S/A; C, empty vector. Cells (1 \(\times\) 10\(^{6}\)) were loaded with 1.5 \(\mu\)M indo-1/AM, stimulated with anti-CD3/CD28 (designated ωTCR, arrow), and subjected to flow cytometric analysis. The ratio of fluorescence at 420 nm to that at 510 nm was used to indicate changes in calcium flux. D, Two clones of H45.01 cells expressing wild-type CD45 were stimulated with anti-CD3/CD28, and intracellular calcium levels were measured for about 10–15 min and then 2 h later (x-axis scale break). E, Three independent clones of H45.01 cells expressing CD45 S/A were stimulated, and intracellular calcium levels were measured for 15 min and then 2 h later. Additions of anti-CD3/CD28 Abs (ωTCR) and 8 mM EGTA are indicated by arrows.](http://www.jimmunol.org/)

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immediately returned to normal when EGTA was added to the medium. Because EGTA chelates external Ca\(^{2+}\) and does not enter cells, we conclude that the extended flux was due to a loss of ability to down-regulate Ca\(^{2+}\) influx and not to the release of internal stores of Ca\(^{2+}\), which are usually depleted quickly after TCR activation. CD45 could act directly on CRAC channels to prevent their inactivation, or there could be other intermediates involved (such as Lck). Previous evidence supports the idea that inactivation of Ca\(^{2+}\) channels is a regulated event. For example, the inactivation of L-type channels is regulated by Ca\(^{2+}\)/calmodulin and depends on specific short sequences in the channel cytoplasmic tail (27). Because little is known about the physical nature of CRAC channels in lymphocytes, the interaction with CD45 described in this report may provide novel approaches to the study of Ca\(^{2+}\) flux in T cells.

C corroboration of our results with the H45.01 stable clones was provided by the observation of a similar deregulation of the Ca\(^{2+}\)/NF-AT pathway by an independent, transient transfection system. In this system we transiently transfected a CD45\(^{-}\)/Jurkat line, J45.01, with the acidic insert mutants, and the effect on downstream pathways was evaluated. The Ca\(^{2+}\)/NF-AT pathway was deregulated in a way that led to aberrantly high basal levels of active NF-AT in resting cells. The elevated basal level was completely abrogated by treatment with CsA, showing that the calmodulin/calciuretin pathway was involved in generation of the observed NF-AT activation. In addition, treatment of the cells with EGTA, which chelates external Ca\(^{2+}\), completely suppressed the high basal activity, suggesting that an influx of external Ca\(^{2+}\) is necessary to maintain the aberrant NF-AT levels. Increased levels of Ca\(^{2+}\) were not detectable in the transient system due to the low number of transfected cells.

The results obtained using the two model systems described in this report support each other, but the same measurements were not possible with both transfection systems. In addition, the cell lines used may also differ somewhat with regard to signaling pathways. For example, NF-AT activity measurements in transiently transfected H45.01 cells might not have been successful due to the low expression of NF-AT in these cells (28). Other reports have suggested that the HPB cell line may exhibit low activation of PLC and PKC upon TCR stimulation (13, 29). Despite these differences, the results we obtained were quite complementary, and both transfection systems supported the conclusion that CD45 plays a role in the regulation of Ca\(^{2+}\)/NF-AT signaling pathways.

This conclusion is also supported by the observations of others concerning the role of CD45 in the activation of T cells. For example, Leitenberg and Bottomly, who examined the effect of anti-CD45 Abs on Ca\(^{2+}\) flux during TCR activation, concluded that CD45 played a role in the regulation of influx of extracellular Ca\(^{2+}\) (30). In addition, experiments in which the D2 domain of CD45 was replaced with the homologous D2 of LAR PTP suggested that the CD45 D2 domain was required for Ca\(^{2+}\)/NF-AT-dependent IL-2 secretion (31). The observation that the D2 domain of LAR (which does not contain the 19-aa insert region) could not substitute the D2 domain of CD45 suggests that the insert was essential for CD45 function.

The D2 domain of CD45 is also necessary for the full expression of D1 activity. Mutagenesis studies have revealed that the intact D2 domain was required for the enzymatic activity of CD45 expressed in rabbit reticulocyte in vitro transcription/translation system (5). Our studies have also shown that the intact D2 domain was essential for CD45 expression in H45.01 cells used in the current study (unpublished data). Most studies of the expression of the cytoplasmic domain of CD45 have shown that the D2 domain was essential for the expression of D1 PTP activity, and deletion of the D2 19-aa insert reduced activity of the D1 (5). More recently, recombinant CD45 D1 domain alone was expressed in a bacterial system and was shown to have enzymatic activity, but the presence of the D2 domain was found to increase the thermostability of D1 domain (32). Taken together these studies and the current report show that the D2 domain is important in the expression and function of CD45.

Further work will be needed to determine the exact mechanism by which the intact acidic insert is required for maintaining Ca\(^{2+}\) homeostasis in a basal state. Ser to Ala mutation exhibited the largest effect in our studies, suggesting the hydroyl groups of the Ser residues in the wild-type protein allowed CD45 to regulate the levels of Ca\(^{2+}\). Such regulation could occur by interaction of CD45 with the CRAC channel or by altering substrate selectivity of CD45. The recently reported crystal structure of LAR (33) suggests that the acidic insert could extend to the region of the D1, where it could alter the accessibility of substrates to the active site. Introduction of CD45 S/A in J45.1 cells could have resulted in higher Ca\(^{2+}\)/NF-AT levels by not down-regulating the Ca\(^{2+}\) levels after fluctuations during normal activity (such as the cell cycle or stimulated growth after addition of serum). A similar mechanism could function to produce sustained Ca\(^{2+}\) levels after TCR ligation in HPB cells. Because the physical nature of the Ca\(^{2+}\) channels is unknown, it will be important to direct future experiments toward elucidating the mechanism of interaction of CD45 with Ca\(^{2+}\) regulation in the cell.

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References


