Phosphorylation of CD45 by Casein Kinase 2
MODULATION OF ACTIVITY AND MUTATIONAL ANALYSIS*

(Received for publication, July 27, 1998, and in revised form, January 6, 1999)

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CD45 is a receptor-type protein-tyrosine phosphatase (PTP) that is required for antigen-specific stimulation and proliferation in lymphocytes. This study was designed to determine the nature of specific kinases in lymphocytes that phosphorylate CD45 and to determine the effect of phosphorylation on CD45 PTP activity. A major cytoplasmic lymphocyte kinase that phosphorylated CD45 was identified as casein kinase 2 (CK2) by use of an in-gel kinase assay in combination with immunoprecipitation, immunodepletion, and specific inhibition. Mutational analysis of CK2 consensus sites showed that the target for CK2 was in an acidic insert of 19 amino acids in the D2 domain, and Ser to Ala mutations at this site reduced CK2 phosphorylation of CD45. CK2 phosphorylation increased CD45 activity 3-fold toward phosphorylated myelin basic protein, and this increase was reversible by PP2A treatment. Mutation of Ser to Glu at the CK2 sites had the same effect as phosphorylation and also tripled the V_max of CD45. CD45 isolated in vivo was highly phosphorylated and could not be phosphorylated by CK2 without prior dephosphorylation with phosphatase PP2A.

We conclude that CK2 is a major lymphocyte kinase that is responsible for in vivo phosphorylation of CD45, and phosphorylation at specific CK2 sites regulates CD45 PTP activity.

The role of CD45 protein-tyrosine phosphatase (PTP)\(^1\) in lymphocyte signaling has been the subject of extensive investigation (1–4). CD45 is a transmembrane PTP of hematopoietic cells composed of 1268 total amino acids with an external domain containing alternately used exons, which leads to the lymphocyte-specific expression of at least eight different isoforms (1, 5, 6). The cytoplasmic domain consists of 702 amino acids and contains two tandem repeated PTP domains designated D1 and D2 (1). The membrane-proximal PTP domain (D1) is constitutively active, and the second PTP domain (D2) is considered to be inactive (7). The catalytic activity of the D1 but not the D2 domain is required for TCR signal transduction in CD45-deficient cell lines (8). The role of CD45 in the antigen-specific activation of B and T cells has been documented by demonstrating that T cells and B cells lacking CD45 fail to respond to antigen stimulation (9, 10). This observation has been confirmed in CD45 knockout mice in which the antigen signaling capacity of T and B cells was severely diminished and the transition of thymocytes to maturity was impaired (11, 12). CD45 is believed to activate the Src family protein-tyrosine kinases by dephosphorylating the regulatory Tyr(P) near the C terminus of T cell receptor or B cell receptor-associated Src family kinases (13–16). However, recently it has become clear that the regulation of Src family kinases is likely to be more complex since the discovery that the activating tyrosine phosphorylation site in the kinase domain is also dephosphorylated by CD45 (17). The importance of the CD45 PTP activity in the activation of T cells has been demonstrated by showing that chimeric proteins containing only the cytoplasmic domain of CD45 were capable of restoring normal T cell receptor activation (18–20). Despite previous research there is still much to be learned about the range of natural substrates of CD45 as well as about the nature of other proteins that may interact with CD45.

Phosphorylation of CD45 may play an essential role in the function of CD45 and may regulate PTP activity, substrate specificity, subcellular localization, and/or docking with other signaling molecules. Decreased PTP activity of CD45 was found to correlate with decreased serine phosphorylation after calcium ionophore treatment of T cells (21), and serine residues on CD45 have been shown to be phosphorylated in response to T cell treatment with phorbol esters (22) and after IL-2 treatment of CTLL-2.4 cells (23). Little or no modulation in CD45 PTP activity was observed after phosphorylation in these reports. In other studies, serine phosphorylation was observed after lectin treatment of T cells, and tyrosine phosphorylation of CD45 has been reported in phenylarsine oxide-treated T cells (24, 25).

CD45 was phosphorylated after in vitro treatment with casein kinase 2 (CK2) and other serine/threonine kinases such as protein kinase C and glycogen synthase kinase (26). Increased CD45 PTP activity was found after phosphorylation with p50^CSK tyrosine kinase (24) and after sequential tyrosine phosphorylation by v-Ab1 kinase (using ATPyS) followed by serine phosphorylation with CK2 (25).

Using two-dimensional TLC, HPLC, and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS), we have identified several in vivo phosphorylation sites of CD45 (27). One phosphorylation site identified in that study, Ser^309, is in a putative substrate-binding loop of the inactive D2 PTP domain. Three other sites, Ser^304, Thr^1246, and Ser^1246 are in the C-terminal tail of the molecule. Another multiply phosphorylated region was tentatively localized to the 19-amino acid acidic insert in the D2 domain (27). The importance of CD45...
phosphorylation sites to PTP activity or to T cell activation remains unknown. The present study was designed to evaluate the relationship of precise CD45 phosphorylation events to the functional role of CD45. In this study, we have identified CK2 as a lymphocyte kinase that targets CD45 and that is responsible for phosphorylation of CD45 in the 19-amino acid acidic region of the D2 domain of CD45. This region is a unique insert in the D2 domain that is not found in the CD45 D1 domain or in any other PTP D1 or D2 domain. Investigation of the relationship of the CK2 phosphorylation sites to the PTP activity of CD45 showed that phosphorylation of the D2 acidic region by CK2 increased CD45 activity 3-fold toward phosphorylated myelin basic protein.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture—CTLL-2 (murine cytolytic T cell line), DO-11.10 (murine T cell hybridoma) and 702/3.12 (murine pre-B lymphocyte cell line) were obtained from ATCC (Rockville, MD). Jurkat (clone E6–1) (human acute T cell leukemia cell line), and CD45-deficient Jurkat clone (J45.01) were obtained from Dr. Gary Koretzky (University of Iowa). The cells were cultured in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies), streptomycin/penicillin (100 units/ml; Life Technologies), and 50 mM β-mercaptoethanol (Sigma). Recombinant IL-2 (Cetus Corp.) was added to the CTLL-2 cultures at 7 units/ml. BW5147 (murine T lymphoma), WEHI274.1 (murine monocye), P815 (murine mastocytoma), and NIH3T3 (murine fibroblast) were obtained from ATCC and were grown in Dulbecco’s modified Eagle’s medium (Life Technologies) containing 10% heat-inactivated fetal bovine serum, streptomycin/penicillin (100 units/ml), and 50 mM β-mercaptoethanol. Cells were maintained in an exponential growth state (0.1–5.0 × 10^6 cells/ml), and cultures with viability greater than 97% were harvested for experimental use.

Site-directed Mutagenesis—The bacterial expression vector pET3p-His,CD45, which expresses the cytoplasmic domain of murine CD45 with a His tag introduced to the amino terminus, was kindly provided by Dr. Pauline Johnson (University of British Columbia, Vancouver, Canada) (28). Multiple point mutations in the acidic insert of the D2 domain of CD45 were made using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Desired mutations (underlined) were incorporated into a pair of oligonucleotide primers, each complementary to opposite strands of the parental DNA template. The primers used for Ser to Glu (965, 968, 969, and 973) mutagenesis were as follows: 5′-CTTCTGACTCATCGTCAGGCTCACAAGAG-3′ and 5′-GCTGACTCAGAAG-3′. The primers used for Ser to Ala (965, 968, 969, and 973) mutagenesis were as follows: 5′-CTTCTGACTCATCGTCAGGCTCACAAGAG-3′ and 5′-GCTGACTCAGAAG-3′.

The primers were extended by EcoRV DNA polymerase during a short temperature cycling (95 °C for 30 s, 55 °C for 1 min, 65 °C for 13.5 min (2 min/kb of plasmid length), 18 cycles), and the parental DNA template was then digested by DpnI endonuclease. Mutants were selected after the synthesized DNA was transformed into Escherichia coli XL1-Blue and later verified by sequencing.

Purification of Recombinant CD45 Proteins and In-gel Kinase Assay—Recombinant wild type and mutant cytoplasmic domain CD45 (designated His6-cytCD45) was purified from E. coli BL21(DE3) as described (28). The size of the purified proteins was determined on 10% SDS-polyacrylamide gel, and the concentration was determined by the Bio-Rad protein assay. The in-gel kinase assay was adapted from a previously described method (29). Briefly, 10% polyacrylamide gel was cross-linked with 50 µg/ml of His6-cytCD45 substrate, while the stacking gel was prepared without the substrate. Cell lysates (1 × 10^6 cells) or immunoprecipitates were loaded onto the gel for electrophoresis. The gel was washed thoroughly with 2% 2-propanol to remove SDS, and the protein kinases in the gel were then denatured with two incubations with 6 M guanidine HCl (Life Technologies) and renatured with five changes of 0.04% Tween 40 (Sigma) at 4 °C. The gel was preincubated in the kinase assay buffer (40 mM HEPES, pH 8.0, 2 mM DTT, 0.1 mM EDTA, 5 mM Mg(OAc)2, 0.2 mM CaCl2) for 30 min at room temperature.

The kinase reaction was started by incubating the gel in the kinase assay buffer containing 5 µCi [γ-32P]ATP (3000 Ci/mmol, NEN Life Science Products) for 1 h at 30 °C. After the reaction, the gel was washed four to five times with 5% (v/v) trichloroacetic acid solution containing 1% sodium pyrophosphate, until the radioactivity of the solution approached background. The gel was dried on 3MM Whatman paper and subjected to PhosphorImager analysis (Molecular Dynamics Inc., Sunnyvale, CA).

Immunoprecipitation and Immunoblotting—Cells were washed twice with ice-cold phosphate-buffered saline (137 mM NaCl, 3 mM KCl, 8 mM Na2HPO4, 1 mM KH2PO4, pH 7.4) and then lysed in an appropriate volume (5 × 10^7 cells/ml) of lysis buffer (1% Nonidet P-40 (Pierce), 20 mM Tris, pH 8.0, 137 mM NaCl, 10% glycerol, 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 0.23 units/ml aprotinin, 0.5 µg/ml leupeptin, 0.7 µg/ml pepstatin A, 10 µg/ml Dnase I, 1 µM okadaic acid, 6 mM sodium fluoride, 2 mM sodium orthovanadate, and 4 mM sodium molybdate) for 30 min on ice. Cell nuclei were pelleted by centrifugation at 12,000 rpm at 4 °C for 15 min, and the supernatants were incubated with the antisera to CK2α, CK2β', or CKβ kindly provided by Dr. David Litchfield (University of Western Ontario, London, Canada) (30) or with antibodies to CD45 (clone 9.4, ATCC) for 2 h. GammaBind Plus Sepharose (Amersham Pharmacia Biotech) was added, followed by rocking at 4 °C for 1 h. Immune complexes were washed sequentially with 1% Nonidet P-40 lysis buffer, phosphate-buffered saline (pH 7.4), and 0.5 M NaCl, 0.5% Nonidet P-40.
Fig. 3. Inhibition of in-gel kinase activity with inhibitors of CK2. CTLL-2 lysates (lanes 1–5) were subjected to in-gel kinase assay with 50 μg/ml His6-cytCD45 as substrate (lanes 2–5) in the presence of inhibitors of CK2. CTLL-2 lysates were also analyzed with no in-gel substrate (lane 1). Recombinant human CK2α was also subjected to in-gel kinase assay as a control (lanes 6–9). The inhibitors used were heparin (lanes 3 and 7), GTP (lanes 4 and 8) and poly-Glu-Tyr (lanes 5 and 9). The positions of the CD45-targeted in-gel kinase bands are indicated with arrows at 45 and 40 kDa. Protein size is in kDa.

Fig. 4. Immunoprecipitation of the CD45-targeted kinase with anti-CK2 antiserum. A, CTLL-2 cell lysates were subjected to immunoprecipitation with anti-CK2α (lane 1), anti-CK2α' (lane 2), and anti-CK2β (lane 3) and separated with a gel containing His6-cytCD45 as substrate. Control "precipitation" using beads alone is shown in lane 4. Immunoprecipitates were also analyzed without in-gel substrate (bottom). The positions of CK2α and CK2α' are indicated with arrows at 45 and 40 kDa, respectively. B, CK2 was depleted from CTLL-2 lysates with anti-CK2α (lane 2), anti-CK2α' (lane 3), and anti-CK2β (lane 4), and the depleted lysates were subjected to in-gel kinase assay using His6-cytCD45 as substrate. The starting lysate was analyzed in lane 1, and control depletion using beads alone is shown in lane 5. Only one round of depletion was performed.

**PTP Assay and Kinetic Analysis**—The PTP activity of CD45 was determined as described previously (31) using 32P-labeled myelin basic protein (MBP) and MBP as substrates. Briefly, 50 μg of Enhanced Raytide (Calbiochem) or 250 μg of MBP (Sigma) was labeled with 50 Ci of [γ-32P]ATP by incubation with 500 ng of recombinant Src tyrosine kinase (obtained from Dr. J. Dixon, University of Michigan) for 1 h at 30 °C in 160 μl of reaction mixture containing 500 μM ATP, 10 mM MgCl2, 16 mM HEPES, pH 7.5, 0.03 mM EDTA, 0.07% β-mercaptoethanol. Labeled 32P-Raytide or MBP was added to 100 μl of 5 mg/ml bovine serum albumin and 70 μl of 50% cold trichloroacetic acid followed by centrifugation at 12,000 rpm for 15 min. The pellet was then washed twice with 10% cold trichloroacetic acid and resuspended in 200 μl of 200 mM Tris, pH 8.0. PTP assays were carried out at 30 °C, and each assay contained 5 μl of 10X PTP buffer (250 mM HEPES, pH 7.3, 50 mM EDTA, 100 mM DTT), 35 μl of H2O, 5 μl of 32P-labeled MBP, and 5 μl of sample to be assayed. After incubation for various times, aliquots of the reaction mixture were taken out and added to 0.75 ml of acidic charcoal suspension (0.9 M HCl, 90 mM Na2HPO4, 2 mM NaH2PO4, and 4% (w/v) active charcoal (Sigma)) to stop the reaction. After centrifugation, the amount of released 32P in the supernatant was measured in a scintillation counter. For the His6-cytCD45 kinetics, specific activity is expressed as nmol/min/mg of protein and was plotted against substrate concentration. Kinetic parameters were calculated by nonlinear curve fitting of the data to the Michaelis-Menten equation using Microcal Origin software (Microcal Software Inc., Northampton, MA). The kinetics parameters of CK2 (Boehringer Mannheim) with His6-cytCD45 as substrate were determined with a CK2 assay using Whatman P81 phosphocellulose paper squares as described previously (26).

**CK2 Phosphorylation of His6-cytCD45**—Wild type and mutant His6-cytCD45 protein (1 μg) was either mock-treated or treated with 0.04 milliliters of recombinant CK2 (Boehringer Mannheim) at 30 °C for 30 min in the presence of 20 mM Tris, pH 7.5, 5 mM MgCl2, 1 mM DTT, and 5 μM ATP. PP2A treatment was performed as follows. His6-cytCD45 was phosphorylated as described above, followed by the addition of sufficient heparin (10 μg/ml) to inhibit CK2 without inhibiting CD45 (determined by titration of heparin with each enzyme). The phosphorylated CD45 was then mock-treated or incubated with PP2A (0.5 units; Promega) for an additional 30 min at 30 °C. The mixture was then
RESULTS

Identification of CD45 Kinase by In-gel Kinase Assay—In-gel kinase assays were used in an effort to identify kinases from T cell lines derived from various tissues. Candidate serine/threonine kinases in this molecular weight range included MAP kinase and CK2. Examination of the CD45 cytoplasmic domain sequence showed that CD45 had several consensus sites for CK2 phosphorylation, while there were few conserved serine or threonine residues that could serve as MAP kinase substrates. Immunoblotting with a mixture of anti-CK2α and anti-CK2α' showed that CK2 was widely expressed in these cell types and precisely overlapped with the in-gel kinase activity (Fig. 2B). We then addressed the question of the identity of the CD45 kinase by use of CK2-specific inhibitors.

In Vitro Kinase Labeling of CD45 and PP2A Treatment—Immuno-precipitated CD45 from Jurkat T cells (1 × 10⁶ cells for each sample) were either mock-treated or treated with PP2A (Promega) for 1 h at 30 °C in 40 μl of reaction mixture containing 20 mM MgCl₂, 50 mM Tris, pH 7.5, 1 mM DTT, 1 μl of PP2A (0.5 units/μl). Treated immunoprecipitates were then washed with phosphate-buffered saline (pH 7.4), 0.5 M LiCl (pH 7.4), and 20 mM Tris (pH 7.4). The in vitro kinase labeling by CK2 was performed at 30 °C. Each reaction contained 4 μl of 10× kinase buffer (200 mM Tris, pH 7.5, 50 mM MgCl₂, 10 mM DTT), 20 μl of immunoprecipitated CD45, 1 μl of recombinant CK2 (0.2 milliunits/μl), 1 μl of 0.1 mM ATP, 10 μCi of [γ-³²P]ATP (3000 Ci/mmol; NEN Life Science Products), and H₂O to 40 μl. The reaction was incubated for 30 min and then terminated by the addition of SDS sample buffer at 100 °C. Labeled CD45 immunoprecipitates were loaded onto 7.5% SDS-polyacrylamide gel, followed by electrophoresis and PhosphoImager analysis.

FPLC Analysis—FPLC analysis was performed using a Mono-Q anion exchange column (Amersham Pharmacia Biotech) using three different buffer systems described previously (28) and a NaCl gradient from 150 to 600 mM. Eluted proteins were assayed by the release of phosphate using pNPP (Sigma) as substrate. CK2 phosphorylation of His₆-cytCD45 for FPLC analysis was performed as described above.

Tryptsin Digestion and Mass Spectrometry—CD45 tryptic digestion and mass spectrometry were performed as described previously (27). Briefly, SDS-PAGE-purified, ³²P-labeled CD45 was transfected to polyvinylidene difluoride membrane, excised, and subjected to tryptic digestion with 10 μg of trypsin (Promega) at 37 °C. Tryptic peptides were recovered and subjected to HPLC fractionation using a microbore reverse-phase HPLC system (Microm BioResources, Inc., CA) (27). The hydrophilic, multiply phosphorylated HPLC fraction 4 (27) was subjected to MALDI-MS (Voyager Elite time-of-flight; PerSeptive Biosystems, Framingham, MA) exactly as described previously (27). A computer program, MSU MapMass (32), was used to calculate the average masses of all possible peptide and phosphopeptide fragments from CD45.

Distribution of CD45 In-gel Kinase Activity—The CD45 in-gel kinase activity was found to be broadly distributed among a panel of murine cell lines, including T cell lines (CTLL-2, DO-11.10, BW5147), a B cell line (70Z/3.12), myeloid cells (WEHI264.1, P815), a fibroblast line (NIH3T3), and a human T cell line (Jurkat) (Fig. 2A). Each cell type exhibited two bands resulting from a CD45-selective kinase at 40 and 45 kDa. This indicated that the CD45 kinase was ubiquitously expressed in cell lines derived from various tissues. Candidate serine/threonine kinases in this molecular weight range included MAP kinase and CK2. The position of the beginning of the β1-strand (INAS) found in the family of PTP molecules is shown by the arrow. The standard single-letter amino acid codes are used. The accession numbers for the sequences are as follows: mouse, P06860; rat, P04417; human, P08575; chicken, Z21886; and shark, U34750.

40 and 45 kDa was selective for CD45 because the incorporation into other substrates such as MBP at 50 μg/ml or even at 500 μg/ml only resulted in a basal level of phosphorylation at the same position (Fig. 1A, lanes 3 and 4).

The insert is boxed, and the approximate lengths of all possible peptide and phosphopeptide fragments from CD45 are indicated in Fig. 3. The active portion of CK2 catalytic subunit that phosphorylated CD45 trypsin digestion into other substrates such as MBP at 50 μg/ml only resulted in a basal level of phosphorylation at the same position (Fig. 1A, lanes 3 and 4).

CD45 sequence homology in the region of the 19-amino acid D2 insert. Comparison of the interspecies homology of the CD45 D2 region containing the 19-amino acid acidic insert unique to CD45. The insert is boxed, and the approximate lengths of all possible peptide and phosphopeptide fragments from CD45 are indicated in Fig. 3. The active portion of CK2 catalytic subunit that phosphorylated CD45 trypsin digestion into other substrates such as MBP at 50 μg/ml only resulted in a basal level of phosphorylation at the same position (Fig. 1A, lanes 3 and 4).

CD45 Phosphorylation by a Kinase Doublet. Digestion and phosphoamino acid analysis of both phosphorylated bands showed that the proteins were exclusively phosphorylated on serine residues (not shown). The enhanced labeling at 40 and 45 kDa was selective for CD45 because the incorporation into other substrates such as MBP at 50 μg/ml or even at 500 μg/ml only resulted in a basal level of phosphorylation at the same position (Fig. 1A, lanes 3 and 4).
FIG. 6. In-gel kinase analysis of CK2 immunoprecipitates using mutant CD45 as substrate. Anti-CK2α (lane 1), anti-CK2α' (lane 2), and anti-CK2β (lane 3) immunoprecipitates from CTLL-2 cells were subjected to an in-gel assay system containing the following substrates. A, His₆-cytCD45 with four Ser to Ala mutations at residues 965, 968, 969, and 973; B, wild-type His₆-cytCD45; C, no substrate. Control "precipitation" using beads alone is shown in each lane 4. The size of SDS-PAGE markers is shown in kDa.

**Table 1** Kinetic parameters of the CK2 site-mutated His₆-cytCD45 with ³²P-MBP as substrate

| CD45 form       | $K_m$ (µM) | $V_{max}$ (µmol/min/mg) |
|-----------------|------------|-------------------------|
| Wild type       | 0.94 ± 0.47 | 24.2 ± 2.4              |
| S/A             | 1.18 ± 0.42 | 33.3 ± 2.7              |
| S/E             | 1.95 ± 0.29 | 85.1 ± 3.5              |
| Wild type       | 0.94 ± 0.47 | 24.2 ± 2.4              |
| S/A             | 1.18 ± 0.42 | 33.3 ± 2.7              |
| S/E             | 1.95 ± 0.29 | 85.1 ± 3.5              |

$K_m$ and $V_{max}$ were calculated using nonlinear curve fitting with Microcal Origin software.

CD45 in these cells.

**CD45 as a Substrate for CK2**—In order to further characterize the nature of CK2 phosphorylation of CD45, a kinetic analysis was performed. The $K_m$ was 0.51 µM, and the $V_{max}$ was 35.5 nmol/min/mg with CD45 as a substrate of CK2. These parameters were comparable with reports of CK2 kinetics with other protein substrates (for example, $K_m = 1.1$ µM and $V_{max} = 82.5$ nmol/min/mg with eIF-2 (36)). With a $K_m$ in the submicromolar range, we conclude that CD45 is an excellent substrate for CK2 (37).

**Mutation of the CK2 Consensus Phosphorylation Sites Blocks Phosphorylation by CD45-targeted In-gel Kinase Activity**—The 19-amino acid acidic insert of the D2 domain (Fig. 5, boxed) was compared for different species and four highly conserved CK2 phosphorylation sites consisting of the consensus sequence Ser-X-X-acidic group (38) were noted (Fig. 5, shaded residues). All four CK2 consensus-site serines at positions 965, 968, 969, and 973 were mutated to alanines in His₆-cytCD45 to preclude potential phosphorylation. The mutated protein was then incorporated into an SDS-polyacrylamide gel at 50 µg/ml gel, and an in-gel kinase assay was performed using immunoprecipitates of anti-CK2α, anti-CK2α', and anti-CK2β (Fig. 6A, lanes 1, 2, and 3, respectively). Control experiments used the same immunoprecipitates with wild type His₆-cytCD45 as substrate (Fig. 6B) and with no substrate (Fig. 6C). The reduction of labeling with the serine to alanine mutated form of His₆-cytCD45 was essentially complete, showing that these sites represented the major CK2 phosphorylation sites in the CD45 cytoplasmic domain.

**Serine Phosphorylation of the Acidic Insert in the D2 Domain Increases CD45 Activity**—The high conservation of the CK2 phosphorylation sites in the acidic insert region of CD45 led us to hypothesize that phosphorylation (or introduction of additional acidic residues) at this site would modulate CD45 activity. To test this hypothesis, we compared the kinetics of the His₆-cytCD45 mutant forms using ³²P-MBP as a substrate (Fig. 7A). MBP was used because it is an excellent substrate for CD45 (26) and because it was necessary to prepare it in sufficient quantity to perform repeated kinetic analysis at substrate saturating levels. The kinetics of wild type His₆-cytCD45 are shown in Table I and are comparable with previous reports (26). Ser to Ala mutations only slightly altered the basic kinetic parameters of His₆-cytCD45, while the introduction of acidic residues (Glu) into the Ser CK2 sites resulted in a 3-fold increase in $V_{max}$ and a small increase in $K_m$ (Table I).

We then evaluated the effect of CK2 phosphorylation on CD45 activity at single substrate concentrations (8 µM) as determined from Fig. 7A. Wild type His₆-cytCD45 was phosphorylated with CK2 followed by comparison of the PTP activity with the unphosphorylated form of the protein. For ³²P-MBP, wild type CD45 activity was enhanced after CK2 phosphorylation by about 3-fold (Fig. 7B, wt; gray bar). Mutation of the four consensus Ser residues (965, 968, 969, and 973) to Ala (S/A) did not increase the activity of CD45, and phosphorylation of the Ser to Ala mutant did not exhibit an increase in activity (Fig. 7B, gray bar). Mutation of the four Ser residues to Glu (to mimic phosphorylation) resulted in a 3-fold activity increase toward MBP (Fig. 7B, S/E, white bar), and there was no change in the activity of the Ser to Glu mutant after phosphorylation with CK2 (Fig. 7B, S/E, gray bar). Interestingly, the large increase in activity upon phosphorylation of the acidic domain was only observed with MBP and not with two other small substrates, for example ³²P-Raytide (Fig. 7C) and pNPP (data not shown). Only a small increase in activity toward ³²P-Raytide was observed after phosphorylation of wild-type His₆-cytCD45 with CK2 (Fig. 7C, wt). Further, neither Ser to Ala or Ser to Glu mutation nor CK2 phosphorylation had significant effect on the activity of CD45 with Raytide as substrate (Fig. 7C, S/A and S/E).

We then determined the activity of CK2-phosphorylated CD45 after removal of phosphate with PP2A (Fig. 7D). After the CK2 phosphorylation, we added heparin to inhibit CK2 activity and then incubated with PP2A. The amount of heparin added (10 µg/ml) was determined by titration to achieve a balance in which the heparin inhibited the CK2 without inhibiting CD45 or PP2A (data not shown). Using this protocol, we were able to show, in the same set of experiments, that CK2 increased the activity of CD45 and that subsequent dephosphorylation with PP2A reversed the activation. Complete reversal may not have been achieved during the short incubation, possibly due to residual CK2 activity and the presence of excessive ATP.

In order to verify that treatment with CK2 resulted in the phosphorylation of all the CD45 present, we subjected the CK2-treated, activated His₆-cytCD45 from Fig. 7B to analytical FPLC separation on a Mono-Q anion exchange column (Fig. 8). Mock-treated (ATP without added CK2) and CK2-phosphorylated wild-type His₆-cytCD45 were separated using NaCl gradient elution from 150 to 600 mM. Wild type His₆-cytCD45 eluted in about 14 ml at 240 mM NaCl (active fractions determined by pNPP hydrolysis were shaded), and a large peak of ATP eluted at about 180 mM (about 10 ml) (Fig. 8A). Analysis of CK2 phosphorylated His₆-cytCD45 indicated that the active fractions were retained by the column and required from 340 to...
360 mM NaCl for elution (about 19 ml) (Fig. 8B). The phosphorylated CD45 appeared as two peaks probably resulting from differently phosphorylated forms. One of these CD45 forms eluted at the same salt concentration as the Ser to Glu mutant (340 mM NaCl), which contains four additional negative charges out of 715 amino acids (shown in Fig. 8E). The FPLC elution profiles of wild-type and the Ser to Ala mutant are also shown for comparison (both eluted at 240 mM NaCl) (Fig. 8, C and D). Consideration of the FPLC data suggests that the two peaks represent CD45 forms containing different numbers of
phosphates. In parallel experiments, the stoichiometry of phosphorylation of His6-cytCD45 was estimated at 2.5 mol of phosphate/mole of protein by quantitation of the incorporation of $^{32}$P of known specific activity into CD45 (data not shown).

**CK2 Targeted Sites in CD45 Are Phosphorylated in Vivo**—We found that CK2 immunoprecipitates from Jurkat T cells had constitutively high activity toward CD45, and the overall activity of CK2 did not change after stimulation of Jurkat cells with anti-TCR, phorbol 12-myristate 13-acetate, or ionomycin (data not shown). This result suggested that cytoplasmic CK2 maintained a high level of phosphorylation of CD45 in the acidic insert. To address the question of whether CD45 CK2 sites were phosphorylated in vivo, immunoaffinity-purified CD45 from Jurkat T cells was subjected to in vitro kinase labeling by recombinant CK2α and [γ-$^{32}$P]ATP (Fig. 9). It was found that immunoprecipitated CD45 from Jurkat T cells could not be labeled easily by exogenous CK2 (Fig. 9A, lane 1). Since phosphorylation at the CK2 sites could have blocked the addition of further phosphates, CD45 immunoprecipitates were pretreated with PP2A phosphatase before the kinase labeling. PP2A treatment converted CD45 to a form that could be successively phosphorylated with CK2 (Fig. 9A, lane 2). Immunoprecipitates from a CD45-deficient Jurkat clone (J45.01) were used as controls (Fig. 9, lanes 3 and 4). Further evidence for the existence of in vivo multiple phosphorylations at the CD45 CK2 sites was obtained by MALDI-MS analysis of tryptic peptides obtained from in vivo $^{32}$P-labeled CD45 (Fig. 9B). The hydrophilic CD45 tryptic peptides from HPLC fraction 4 (27) were subjected to MALDI-MS analysis exactly as described previously (27). Although these hydrophilic peptides typically presented weak signals (not necessarily reflective of abundance), the MALDI-MS analysis revealed a tryptic peptide at (M + H)$^+$ of 2542, which correlated with the CD45 tryptic peptide from the acidic insert with three phosphate residues (predicted (M + H)$^+$ of 2545; expected error of about 0.1%). Taken together, these results strongly support the notion that the CD45 CK2 sites are multiply phosphorylated in vivo.

**DISCUSSION**

In this report, we have positively identified the D2 acidic insert as containing the sites for CK2 phosphorylation and have shown that phosphorylation at those sites leads to a large increase in the $V_{\text{max}}$ of CD45. This increase in $V_{\text{max}}$ could lead to a major alteration of the signaling capacity of CD45 in the initiation of antigen stimulation in lymphocytes. Phosphorylation of CD45 at acidic domain CK2 sites increased the PTP activity of CD45 about 3-fold using $^{32}$P-MBP as substrate. This activation was not apparent with other substrates, suggesting substrate selectivity for phosphorylated CD45. A kinetic analysis of His6-cytCD45 found its $V_{\text{max}}$ and $K_m$ to be in general agreement with previously determined values (26, 39). By use of analytical FPLC separation, we verified that all of the His6-cytCD45 was phosphorylated after CK2 treatment as indicated by increased retention on an anion exchange column (Fig. SB). The fact that two FPLC peaks were observed after CK2 phosphorylation indicated the presence of multiple phosphorylated forms. Importantly, although there are other potential CK2 sites in CD45, the Ser to Ala mutation of the D2 acidic insert blocked phosphorylation by CK2 and also increased activity after such treatment. The acidic insert contains four CK2 phosphorylation sites that are conserved in all species examined, and mutation of those four serine sites (965, 968, 969, and 973) abolished greater than 95% of the ability of CK2 to phosphorylate CD45.

The increase in CD45 activity after phosphorylation is consistent with a previous report in which decreased PTP activity of CD45 was accompanied by a decrease in serine phosphorylation (21). In other studies, PTP activity of CD45 was not modulated by CK2 phosphorylation, possibly because the CD45 utilized in these studies was already highly phosphorylated and therefore could not be further activated (25, 26). The modulation of serine phosphorylation of CD45 has been demonstrated under various stimuli (21–23). Some studies have failed to detect a relationship between phosphorylation and PTP activity (22, 23), while others have shown modulation of CD45 activity upon phosphorylation (21, 24, 25). The discrepancy between these studies may have stemmed from the difficulty of isolating and assaying CD45 immediately after stimulation and from the lack of precise knowledge of in vivo phosphorylation states. CK2 is a ubiquitous serine/threonine kinase that is expressed in virtually all cell types (35, 40). CK2 has been reported to be highly expressed in some transformed and proliferating cells, and, when overexpressed in transgenic mice, the CK2 gene acts as an oncogene in cooperation with myc (41, 42). CK2 exists as a tetramer composed of two catalytic $\alpha$-chains (aa, $\alpha_\alpha$, or $\alpha'_\alpha$) and two regulatory $\beta$-chains (40). The $\beta$-chain is almost identical among various mammalian species, and the CK2$\alpha$ and CK2$\alpha'$ chains are highly homologous to each other as well as highly conserved (40). CK2 is found in both the nucleus and the cytoplasm, and it phosphorylates a number of signaling proteins in both compartments (e.g. Jun, Myc, Myb, Rb, and p53) (35) at consensus phosphorylation sites composed of Ser/Thr-X-X-Glu/Asp or Ser/Thr-X-X-acidic group (38). Although there has been much work performed on the nature and activity of CK2, a definitive role in signal transduction is still somewhat obscure (40).

In this report, we showed that analysis of CK2 may be performed with an in-gel kinase method using CD45 as a substrate. CD45 was an excellent substrate for CK2 and became highly phosphorylated with only 50 $\mu$g of substrate/ml of gel, while most other in-gel kinase methods have used from 500 $\mu$g/ml to 1 mg/ml of substrate (43). The kinase that phosphorylates CD45 was identified as CK2 by a combination of immunoprecipitation, immunodepletion, specific inhibition, and mutation of CK2 consensus sites. Our data demonstrated that while both the $\alpha$- and $\alpha'$-chains of CK2 phosphorylate CD45, CK2$\alpha'$ is the most active form on CD45. In addition, the relative ability of anti-CK2$\alpha'$ to deplete CK2 from CTLL-2 lysates suggested that CK2$\alpha'$ was the predominant form of CK2 that phosphorylated CD45 in these cells. In the in-gel kinase assay, the CK2$\beta$ chain was separated from the CK2 catalytic subunit and thus was not necessary for CD45 phosphorylation. Immunoprecipitated CK2 in its native state (containing $\alpha$, $\alpha'$- , and $\beta$-chains) also efficiently phosphorylated His6-cytCD45 (data not shown). Our results have extended previous reports that predicted that CD45 would be a substrate of CK2 and that CK2 was able to phosphorylate CD45 in vitro (25, 26, 44).

The insert that contains the CK2 phosphorylation sites is a conserved, highly acidic sequence of 19 amino acids that exists only in the D2 domain of CD45 and not in other PTPs. Alignment of the D2 sequence of CD45 to the x-ray crystal structure of other PTPs showed that the D2 acidic insert lies just N-terminal to the highly conserved YINAS sequence that forms the $\beta$1-helix (45). This would place the acidic insert of 19 amino acids in a loop near the opening of the inactive D2 catalytic cleft. Phosphorylation of this insert could interfere with interdomain duplex formation postulated to involve the binding of the N-terminal wedge of one PTP domain to the catalytic cleft of a second PTP domain (7, 46, 47). This could make the catalytic site of the D1 domain more accessible to substrate and thus increase activity. It is also possible that the phosphorylation state could directly influence catalytic activity by affect-
ing interactions between the D1 and D2 domains. The observation that CK2 phosphorylation increased the activity of CD45 is consistent with either hypothesis.

Future work will focus on functional analysis of the 19-amino acid acidic insert in vivo. It will be of great interest to find out whether or not this unique insert serves as a docking site for substrates or signaling molecules. Yet another question to be addressed is why CD45 is endogenously phosphorylated to a high level in this already very acidic region. Further investigation will be directed at the physiological relevance of the insert and the phosphorylation sites. It is expected that phosphorylation and/or dephosphorylation of the acidic insert might play a role in the activation and/or desensitization of the T cell receptor complex.

Acknowledgments—We thank Dr. David Litchfield for providing the anti-CK2 antisera and Dr. Pauline Johnson for providing the expression construct containing His-cytoplasmic domain of CD45. We thank Drs. S. Kang, P. Liao, and D. Gage (Michigan State University) for performing the mass spectral analysis and Dr. Richard Shwartz (Michigan State University) for many helpful discussions during the preparation of this manuscript. We especially thank Dr. Honggao Yan (Michigan State University) for assistance in the execution and interpretation of kinetics experiments.

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