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*J Immunol* 2001; 166:7208-7218; doi: 10.4049/jimmunol.166.12.7208

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CD45 Function Is Regulated by an Acidic 19-Amino Acid Insert in Domain II That Serves as a Binding and Phosphoacceptor Site for Casein Kinase 2

Susanna F. Greer, Yan-ni Wang, Chander Raman, and Louis B. Justement

In this study experiments were conducted to elucidate the physical/functional relationship between CD45 and casein kinase 2 (CK2). Immunoprecipitation experiments demonstrated that CK2 associates with CD45 and that this interaction is inducible upon Ag receptor cross-linking in B and T cell lines as well as murine thymocytes and splenic B cells. However, yeast two-hybrid analysis failed to demonstrate a physical interaction between the individual CK2 α, α′, or β subunits and CD45. In contrast, a yeast three-hybrid assay in which either CK2 α and β or α′ and β subunits were coexpressed with the cytoplasmic domain of CD45, demonstrated that both CK2 subunits are necessary for the interaction with CD45. Experiments using the yeast three-hybrid assay also revealed that a 19-aa acidic insert in domain II of CD45 mediates the physical interaction between CK2 and CD45. Structure/function experiments in which wild-type or mutant CD45RA and CD45RO isoforms were expressed in CD45-deficient Jurkat cells revealed that the 19-aa insert is important for optimal CD45 function. The ability of both CD45RA and CD45RO to reconstitute CD3-mediated signaling based on measurement of calcium mobilization and mitogen-activated protein kinase activation was significantly decreased by deletion of the 19-aa insert. Mutation of four serine residues within the 19-aa insert to alanine affected CD45 function to a similar extent compared with that of the deletion mutants. These findings support the hypothesis that a physical interaction between the CD45 cytoplasmic domain and CK2 is important for post-translational modification of CD45, which, in turn, regulates its catalytic function.


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Received for publication November 10, 2000. Accepted for publication April 12, 2001.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported in part by National Institutes of Health Grant GM46524.
2 S.F.G. and Y.W. contributed equally to this work and should be considered as co-first authors.
3 Address correspondence and reprint requests to Dr. Louis B. Justement, 378 Wallace Tumor Institute, Division of Developmental and Clinical Immunology, University of Alabama, Birmingham, AL 35294-3300. E-mail address: louis.justement@ccc.uab.edu
4 Abbreviations used in this paper: PTK, protein tyrosine kinase; AgR, Ag receptor; PTP, protein tyrosine phosphatase; BCR, B cell Ag receptor; DI, CD45 protein tyrosine phosphatase domain I; DII, CD45 protein tyrosine phosphatase domain II; CK2, casein kinase 2; BD, GAL4 binding domain; AD, GAL4 activation domain; Erk1/2, extracellular signal-regulated kinase 1/2; Jnk, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase.
been shown to selectively alter the ability of CD45 to dephosphorylate artificial substrates in vitro (18–20). The 19-aa insert contains multiple casein kinase 2 (CK2) consensus sites, suggesting that the catalytic function of CD45 may be regulated by serine phosphorylation of DI. Indeed, studies have demonstrated that CD45 is phosphorylated by CK2 at a high stoichiometry (22) and that multiple residues within the 19-aa insert are phosphoacceptor sites for this kinase (23, 24). Phosphorylation of CD45 within the acidic insert has been shown to regulate both its substrate specificity as well as its activity in vitro (23, 24). Additionally, work has shown that decreased serine phosphorylation of CD45 is associated with a decrease in its catalytic function (24, 25). These findings demonstrate that the ability of DI to alter the substrate specificity and/or catalytic activity of DI resides in part within the unique 19-aa insert and is regulated by reversible serine phosphorylation (24, 25).

In this study experiments were conducted to further elucidate the functional/physical nature of the molecular interaction between CD45 and CK2. The results obtained demonstrate that CD45 and the αβ subunits of CK2 physically interact with one another via the 19-aa acidic insert in DI of CD45. Mutational analyses suggest that CD45 catalytic function is regulated by CK2-dependent binding to and/or phosphorylation of CD45 within the acidic insert.

Materials and Methods

Cells and cell culture

The B lymphoma cell line K46-17 μm (K46), provided by Dr. M. Roth (Max Plank Institut fur Immunologie, Freiburg, Germany), was maintained in IMDM supplemented with 5% FBS (HyClone, Logan, UT), 2 mM l-glutamine, 50 μM 2-ME, 100 μg/ml streptomycin-penicillin, and 50 μg/ml gentamicin (Sigma) at 37°C under 5% CO2. The Jurkat human leukemia-T cell line (clone E6-1) and the CD45-negative variant (J45.01) were purchased from American Type Culture Collection (Manassas, VA) and were used in these studies. Rabbit antiserum specific for CK2 (Upstate Biologicals, Lake Placid, NY), and anti-human CK2 (Transduction Laboratories, Lexington, KY) mAbs were purchased for these experiments. Protein A-agarose was obtained from Life Technologies.

CD45 was immunoprecipitated with protein G-Sepharose 4B Fast Flow (Amersham-Pharmacia Biotech, Piscataway, NJ) mAbs were purchased from BioSource (Camarillo, CA). Protein A-agarose was obtained from American Type Culture Collection (Manassas, VA) and were maintained in RPMI 1640 supplemented as described above. To obtain thymocytes and splenocytes, 6- to 8-wk-old C57BL/6 mice obtained from The Jackson Laboratory (Bar Harbor, ME) were sacrificed, and the thymus and spleens were removed. Single-cell suspensions were prepared, and the cells were resuspended in cold RPMI and then centrifuged at 1500 rpm for 5 min. Five milliliters of erythrocyte lysis buffer (150 mM NH4Cl, 10 mM KHCO3, and 10 μM Na2EDTA, pH 7.4) was added to the cell pellet, and the cells were resuspended by vortexing and then centrifuged at 1500 rpm for 5 min. To isolate spleen B cells, splenocytes were washed in RPMI and resuspended in tissue culture supernatant containing the mAbs T24 and HO.13-4 (mouse anti-Thy-1.2, 1 ml each) for 10 min on ice. Subsequently, low tox rabbit complement (Life Technologies, Grand Island, NY), 10 μg/ml DNase I (Sigma, St. Louis, MO), and 5 mM MgCl2 were added, and the cells were incubated at 37°C for 40 min. The splenic B cells were washed in RPMI 1640 and resuspended in complete RPMI 1640 for use in experiments.

Biological reagents

The mAbs used in these studies were OKT3 (mouse IgG, anti-human CD3), I3/2.5 (rat IgG2a, anti-mouse CD45), 145.2-C11 (hamster, anti-human CD3), MB23G2 (rat IgG2a, anti-mouse CD45, B exon), MB4B4 (rat IgG2a, anti-mouse CD45, B exon), T24 (anti-mouse Thy 1.2), and HO.13-4 (anti-mouse Thy 1.2). The mAbs were purified using protein A-agarose. CD45 was immunoprecipitated with protein G-Sepharose beads precoated with a saturating amount of the mAb I3/2.5 (anti-murine CD45). Immune complexes bound to beads were collected and washed five times with lysis buffer containing 0.2% Nonidet P-40. The beads were resuspended in 25 μl SDS-PAGE sample-reducing buffer, boiled for 4 min, and centrifuged at 15,000 × g for 5 min. After centrifugation, 20 μl supernatant from each immune complex sample and 5 μl from total lystate control samples were separated on 8–10% acrylamide gels by SDS-PAGE and transferred to Hybond-ECL nitrocellulose membranes (Amersham-Pharmacia Biotech). The membranes were blocked with 5% nonfat dry milk in TBST for 1 h at room temperature and were washed five times with TBST. The membranes were then incubated with anti-CK2 mAb or the polyclonal antiserum against CK2α for 1 h at room temperature and were washed five times with TBST. Next, the membranes were incubated with the appropriate secondary Ab coupled to HRP for 1 h at room temperature and washed five times with TBST. The CK2α band was visualized using ECL with Supersignal reagent (Pierce, Rockford, IL).

In parallel experiments thymocytes (2 × 107/sample) were resuspended in complete RPMI containing 5% FBS and rested at 37°C for 20 min. The cells were stimulated with mAb directed against CD3 (145.2-C11, 10 μg/ml) for 10 min at 37°C. Control samples received no stimulation, but were incubated at 37°C for 10 min. Reactions were stopped by the addition of ice-cold PBS, the cells were lysed as described above, and the lysates were precipitated with protein G-Sepharose and protein A-agarose. CD45 was immunoprecipitated using protein G-Sepharose beads that had been pre-coated first with the mAbs MB23G2 and MB4B4 (anti-murine CD45 B exon) and then with saturating amounts of the I3/2.5 mAb. Similarly, splenic B cells (2 × 107/sample) were resuspended in complete RPMI containing 5% FBS and were rested at 37°C for 20 min. The cells were stimulated with mAb directed against the BCR as described above. Control samples received no stimulation, but were incubated for 10 min at 37°C. Reactions were stopped by the addition of ice-cold PBS, the cells were lysed, and the lysates were precipitated with protein G-Sepharose and protein A-agarose. CD45 was immunoprecipitated with protein G-Sepharose beads pre-coated with a saturating amount of the mAb I3/2.5. Samples were analyzed by Western blotting as described above.

Analysis of CK2 association with wild-type CD45 and CD45 mutants transfected into Jurkat cells was performed using the solid phase immunoprecipitation technique. Ninety-six-well microtiter plates were coated with I3/2.3 (25 μg/ml in 100 μl) in PBS at 4°C overnight, after which the plates were washed five times with PBS. J45.01 transfectants expressing wild-type CD45 or mutant CD45 (2 × 105/sample) were lysed in 150 μl lysis buffer containing 1% Nonidet P-40 for 1 h on ice. The lysates were centrifuged at 13,000 × g for 15 min, and the detergent-soluble material (100 μl) was added to the wells of the precoated 96-well microtiter plate. The plates were incubated at 4°C overnight, then washed three times, and 38 μl SDS-PAGE sample buffer was added. The plates were incubated at 70°C for 10 min, and the samples were boiled in 0.5 ml lysis buffer and transferred to 1.5-ml microtube tubes. The samples were boiled for 5 min, and the immune complexes were separated by SDS-PAGE on 10% acrylamide gels. Coprecipitation of CK2 with CD45 was detected by Western blotting as described previously.

Yeast two-hybrid assay

To generate the GAL4 binding domain-CD45 cytoplasmic domain (BD-CD45) fusion, the entire 702-aa cytoplasmic domain of CD45 (Try244, Thr248) was amplified from the CD45α minigene/ECMVneo cDNA construct (a gift from Dr. M. Thomas, Washington University, St. Louis, MO) using PCR with sense (5'-TATAAAATCTATGCTGGC-3') and antisense (5'-CTGTGTCATGGCTGTT-3') primers. The PCR product encoding the cytoplasmic domain of CD45 was directionally cloned into the yeast expression vector pGBT9 (Clontech, Palo Alto, CA) and analyzed using fluorescent dye terminator sequencing (ABI PRISM; Perkin-Elmer,
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Branchburg, NJ) to confirm sequence accuracy. The sequenced BD-CD45 fusion construct was transformed into MAV203 yeast cells according to the manufacturer’s instructions (ProQuest Two-Hybrid System; Life Technologies), and the yeast was screened to rule out nonspecific activation of the GAL4 promoter. The BD-CD45 construct was then used in yeast two-hybrid screens to assay for interactions between the GAL4 promoter and GAL4 AD-Ck2 fusion proteins encoded by the AD-Ck2α, AD-Ck2β, and AD-Ck2β constructs that had previously been generated using the yeast expression vector pACTII (Matchmaker, Clontech). Growth on uracil-9 product encoding CD45 PTP DII was then directionally cloned into the yeast Schematic representation of BD-CD45 cytoplasmic domain was amplified from the AD-CK2. To generate the third construct necessary for the three-hybrid assay, CK2

Yeast three-hybrid assay

To generate the third construct necessary for the three-hybrid assay, CK2β was amplified from the AD-Ck2β construct by PCR with sense (5'-AGT

GGATCCTGACGTTAAGATGAGCA-3') and antisense (5'-AGT GTCGACTCTGGAAGGTTGGCAAA-3') primers. CK2β was cloned into the yeast expression vector p14 MET25 (a gift from Dr. K. S. Campbell, Fox Chase Cancer Center, Philadelphia, PA) and transformed into MAV203 yeast cells. The p14 MET25-CK2β construct was then screened for nonspecific activation of the GAL4 promoter. Yeasts that contained the p14 MET25-CK2β plasmid were used in yeast three-hybrid screens in which they were cotransformed with AD-Ck2α and BD-CD45, AD-Ck2α and BD-CD45 PTPDI, AD-Ck2α and BD-CD45 PTPDII, AD-Ck2β and BD-CD45 PTPDI, AD-Ck2β and BD-CD45 PTPDII, AD-Ck2α and BD-CD45 Δ958-973, AD-Ck2β and BD-CD45 Δ958-973, AD-Ck2β and BD-CD45 PTPDIΔ958-973, and AD-Ck2α and BD-CD45 PTPDIΔ958-973. Growth on uracil-deficient plates was assessed at 48 h following transfer of cotransformed yeast from tryptophan-deficient (Trp') and leucine-deficient (Leu') selection medium.

Reconstitution of CD45 expression in the J45.01 CD45-negative Jurkat cell line

The J45.01 CD45-negative cell line was used for structure/function studies of CD45 following electroporation with cDNA encoding wild-type or mutated forms of this PTP. The CD45 minigene expression constructs used for electroporation were provided by Dr. M. Thomas (Washington University). Two expression constructs were used; the CD45α minigene construct encodes the high m.w. isoform of mouse CD45 (CD45RA), and the CD45α minigene construct encodes the low m.w. isoform of mouse CD45 (CD45RO). The CD45α and -minigene constructs were mutagenized using the QuickChange mutagenesis kit from Stratagene to generate the acidic insert in DII (aa 958–973 and 808–826, respectively) resulting in the generation of cDNA minigene constructs encoding CD45RA;Δ 958–973 and CD45RO;808–826. Additional mutations were introduced into the CD45RA minigene construct, resulting in conversion of the serines contained within the acidic insert in DII (S965, 968, 969, and 973) to alanine (CD45RA;S965/968/969/973A). Electroporation was used to introduce the wild-type and mutant CD45 minigene constructs into J45.01 cells (1 × 10^7) were resuspended in 500 μl IMDM and were transduced with 10 μg of cDNA using a Becton Dickinson electroporator (San Jose, CA) with settings of 960 mF and 0.25 kV. After 48 h cells were selected in medium containing 1 mg/ml G418 (Life Technologies). Drug-resistant cells were analyzed by flow cytometry after staining with biotinylated I3/2.3 and PE-streptavidin to determine the surface expression of mouse CD45. Multiple rounds of FACS were used to isolate bulk populations of transected J45.01 cells that expressed comparable levels of wild-type and mutant CD45.

Measurement of calcium mobilization

Studies were performed with parental Jurkat cells (clone E6-1), J45.01 CD45-negative cells, and J45.01 transfectants in which Ca^{2+} mobilization was assayed in response to CD3 cross-linking as described previously (26). Cells were loaded with the Ca^{2+} indicator dye indo-1/AM (Molecular Probes, Eugene, OR) at a final concentration of 5 μM. Cells loaded with indo-1 were analyzed using a Becton Dickinson FACSVantage flow cytometer equipped with an Enterprise laser from Coherent (Santa Clara, CA) with settings of 960 mF and 0.25 kV. After 48 h cells were suspended in 500 μl IMDM and were transduced with 10 μg of cDNA using a Becton Dickinson electroporator (San Jose, CA) with settings of 960 mF and 0.25 kV. After 48 h cells were analyzed by flow cytometry after staining with biotinylated I3/2.3 and PE-streptavidin to determine the surface expression of mouse CD45. Multiple rounds of FACS were used to isolate bulk populations of transected J45.01 cells that expressed comparable levels of wild-type and mutant CD45.

Measurement of mitogen-activated protein kinase (MAPK) activation

Experiments were performed with parental Jurkat cells, J45.01 CD45-negative cells, and J45.01 transfectants to monitor CD3-mediated activation of the MAPK extracellular signal-regulated kinase 1/2 (Erk1/2) and c-Jun N-terminal kinase (Jnk). Cells (1 × 10^7/sample) were analyzed by flow cytometry to establish a baseline for the intracellular concentration of free Ca^{2+} ions. Experiments were performed with parental Jurkat cells (clone E6-1), J45.01 CD45-negative cells, and J45.01 transfectants in which Ca^{2+} mobilization was assayed in response to CD3 cross-linking as described previously (26). Cells were loaded with the Ca^{2+} indicator dye indo-1/AM (Molecular Probes, Eugene, OR) at a final concentration of 5 μM. Cells loaded with indo-1 were analyzed using a Becton Dickinson FACSVantage flow cytometer equipped with an Enterprise laser from Coherent (Santa Clara, CA) with settings of 960 mF and 0.25 kV. After 48 h cells were suspended in 500 μl IMDM and were transduced with 10 μg of cDNA using a Becton Dickinson electroporator (San Jose, CA) with settings of 960 mF and 0.25 kV. After 48 h cells were analyzed by flow cytometry after staining with biotinylated I3/2.3 and PE-streptavidin to determine the surface expression of mouse CD45. Multiple rounds of FACS were used to isolate bulk populations of transected J45.01 cells that expressed comparable levels of wild-type and mutant CD45.
washed twice and then lysed in 0.5 ml lysis buffer containing 1% Nonidet P-40. Cell lysates were incubated on ice for 1 h and then centrifuged at 13,000 x g for 15 min at 4°C. Detergent-soluble material (25 μl) was mixed with an equal volume of 2× SDS-PAGE sample reducing buffer, boiled, and centrifuged at 15,000 x g for 5 min. Twenty microliters from each sample were separated by SDS-PAGE on 10% acrylamide gels and transferred to Hybond-ECL nitrocellulose. The membranes were blocked and then incubated with either anti-phospho-Erk1/2 (Thr202, Tyr204) or anti-phospho-Jnk (Thr183, Tyr185) polyclonal Ab (New England Biolabs, Beverly, MA). Next, the membranes were washed and probed with goat anti-rabbit IgG coupled to HRP (BioSource). Phosphorylation of Erk1/2 and Jnk was visualized using ECL. To ensure equal loading of Erk1/2 or Jnk, the membranes were stripped by incubating them in buffer containing 10 mM Tris, pH 2.3, and 150 mM NaCl at 70°C for 1 h, after which they were washed extensively in TBST. The membranes were then blocked and probed with anti-Erk1/2 or anti-Jnk polyclonal Ab to detect the total amount of Erk1/2 or Jnk, respectively. The proteins were visualized by incubating the membranes with goat anti-rabbit IgG coupled to HRP, followed by ECL.

Results
CK2 associates with CD45 in B and T cell lines
Previous studies have demonstrated that changes in the phosphorylation status of CD45 play a role in regulating its substrate specificity and/or catalytic activity. It has been shown that tyrosine phosphorylation of CD45 followed by CK2-dependent serine phosphorylation results in increased activity based on dephosphorylation of artificial substrates in vitro (23). More recently, work characterizing the phosphorylation of CD45 by CK2 has demonstrated that DII of CD45 contains characteristic CK2 phosphorylation sites within a 19-aa insert and that phosphorylation of those sites leads to an increase in the maximum velocity of CD45 in vitro (24). To further elucidate the nature of the physical interaction between CD45 and CK2 in lymphocytes, coimmunoprecipitation experiments were performed. Jurkat cells were incubated in medium alone or were stimulated with anti-CD3 mAb (OKT3) followed by immunoprecipitation of CD45 from detergent-soluble lysates. Western blot analysis of the CD45 immune complex with mAb specific for CK2α revealed that CK2α coprecipitates with CD45 from unstimulated cells and that upon stimulation, the amount of CK2α associated with CD45 increases compared with that in the unstimulated sample (Fig. 2A). Samples from unstimulated detergent-soluble Jurkat lysates incubated with protein G-Sepharose alone and probed with anti-CK2α did not contain CK2α. To determine whether a similar physical association occurs between CD45 and CK2α in B cells, coimmunoprecipitation experiments with the K46 B lymphoma cell line were performed. K46 cells were incubated in medium alone or were stimulated with anti-BCR mAb (B76), after which CD45 was immunoprecipitated from detergent-soluble lysates. Western blot analysis of the CD45 immunoprecipitates revealed, similar to T cells, that CK2α coprecipitates with CD45 from unstimulated B cells and that the amount of associated CK2 increases upon AgR stimulation (Fig. 2B). As before, samples from detergent-soluble K46 lysates incubated with protein G-Sepharose alone and probed with anti-CK2α mAb did not contain detectable amounts of CK2α. These findings suggest that CD45 and CK2 interact with one another in a constitutive manner in B and T cells and that upon stimulation through the AgR, the degree to which they interact increases. Western blotting could not be performed to monitor the recovery of CD45 due to a lack of anti-CD45 Abs that could be used for blotting. Nevertheless, control experiments to ensure that cellular activation does not affect recovery of CD45 were performed in which CD45 immune complexes were biotinylated before separation by SDS-PAGE. Loading of CD45 was detected by probing nitrocellulose membranes with streptavidin coupled to HRP, after which ECL was used to visualize CD45 on the membrane. Equivalent recovery of CD45 from both T and B cell lysates was consistently observed regardless of whether the cells had been stimulated (data not shown). In conclusion, these data provide the first evidence of a physical interaction between CD45 and the serine/threonine kinase CK2.

CK2 associates with CD45 in thymocytes and splenic B cells
To determine whether the physical association between CD45 and CK2 demonstrated in T and B cell lines also occurs in nontransformed cells, thymocytes were incubated in medium alone or were stimulated through the TCR complex with anti-CD3 mAb (145.2-C11). CD45 was immunoprecipitated from detergent-soluble lysates, and Western blotting with antiserum specific for CK2α was performed. These results demonstrated in T and B cell lines also occurs in nontransformed cells, thymocytes and splenic B cells.
performed to detect the presence of CK2. As shown in Fig. 3, CK2 associated with CD45 in thymocytes. Although the data clearly show the constitutive nature of the interaction, as seen in the unstimulated sample, CD45 and CK2 exhibited an enhanced interaction with one another in response to CD3 cross-linking. Splenic B cells were also incubated in medium alone or were stimulated through the BCR with anti-IgM mAb (B76). CD45 was immunoprecipitated from detergent-soluble lysates, separated by SDS-PAGE, and transferred to nitrocellulose membranes. The membranes were probed with antisera specific for the α subunit of CK2, and as shown in Fig. 3, a low level basal association between CK2 and CD45 could be seen that was enhanced upon cross-linking of the BCR complex. In both thymocyte and B cell experiments, protein G-Sepharose alone was incubated with cell lysates to demonstrate the specificity of the CD45/CK2 interaction. As described previously, recovery and loading of CD45 were monitored in selected experiments based on biotinylation of CD45 immune complex material and Western blotting using streptavidin coupled to HRP (data not shown). These studies clearly show that the serine/threonine kinase CK2 interacts with CD45 in nontransformed T and B cells.

Association between CD45 and CK2 requires the holoenzyme

The yeast two-hybrid assay was used to map the sites of physical interaction between the cytoplasmic domains of CD45 and CK2. The CK2 holoenzyme is a tetrameric protein consisting of two interchangeable catalytic subunits, α (45 kDa) and α′ (40 kDa), and two regulatory β subunits (26 kDa each) arranged in one of the following configurations: αββ′, αβ′β′, or αα′β′ (27, 28). Studies have demonstrated that CK2 can interact with its substrates as a holoenzyme or via either the catalytic α/α′ or regulatory β subunit. Previous studies have demonstrated that CK2α′ is the primary subunit responsible for phosphorylating CD45 in vitro, and that the CK2β subunit was not necessary for CD45 phosphorylation (24). Based on these observations we used the AD-CK2α, AD-CK2α′, and AD-CK2β GAL4 activation domain-CK2 fusion constructs to determine whether individual CK2 subunits exhibit the ability to interact with the cytoplasmic domain of CD45 in the yeast two-hybrid assay. The BD-CD45 fusion construct was generated by amplifying the entire 702-aa cytoplasmic domain of CD45 (Tyr564-Thr1268) and subcloning it in-frame into the GAL4 binding domain fusion vector pGBT9. Yeast were cotransformed with BD-CD45, and each of the GAL4 activation domain-CK2 fusion constructs. None of the CK2 subunits was observed to interact with the cytoplasmic domain of CD45 in the yeast two-hybrid assay as assessed by growth on uracil-deficient selection medium (Table I).

Two distinct possibilities could explain the disparate results obtained from the coimmunoprecipitation experiments and the yeast two-hybrid analysis. First, it is formally possible that the lack of detectable protein–protein interaction in the yeast two-hybrid assay could be explained by the fact that the physical association between CD45 and CK2 is indirect and requires an intermediate protein. Alternatively, it is possible that CK2 and CD45 do indeed interact directly with one another in vivo; however, the ability to interact might be dependent on the physical presence of both the α/α′ and β subunits, the intact CK2 holoenzyme, or both. To test the latter possibility, a second CK2β construct was generated (p14MET25-CK2β) that could be used in conjunction with the BD-CD45, AD-CK2α, and AD-CK2α′ constructs in a yeast three-hybrid assay. Yeast were cotransformed with constructs that encoded either the AD-CK2α + BD-CD45 + CK2β or the AD-CK2α′ + BD-CD45 + CK2β polypeptides. They were then selected on Ura−, Trp−, and His+ medium before initiating the yeast three-hybrid screen on uracil-deficient selection medium. Yeast that expressed any of the three-protein combinations exhibited comparable growth in the absence of uracil, thereby demonstrating that both the catalytic and regulatory subunits must be present before CK2 can physically associate with the cytoplasmic domain of CD45 (Table I). These results suggest that the CK2 holoenzyme may be required for direct physical interaction with the cytoplasmic domain of CD45.

Association between CK2 and CD45 is mediated by the unique 19-aa acidic insert in PTP DII

Because CD45 contains tandem repeat PTP domains, and each of these domains contains consensus CK2 phosphorylation sites, it was of interest to determine whether CK2 interacts with both D1 and DII or selectively with only one domain. To address this question, the yeast three-hybrid assay was used once again in conjunction with GAL4 binding domain fusion protein constructs that encoded either D1 or DII of CD45. The ability of the full-length CD45 and CK2: Analysis of Physical/Functional Relationships

Yeast Transformed With Growth on Selective Mediuma

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a Transformed yeast were plated on dropout medium to select for those cells that had taken up the BD and AD vectors (yeast two-hybrid) as well as the p14MET25 vector (yeast three-hybrid) before plating them on uracil-deficient medium to assay for the interaction between CD45 and CK2. Growth on uracil-deficient plates was assessed 48 h after plating the yeast.

b The yeast two-hybrid assay was used to determine whether the individual subunits of CK2 interact with the cytoplasmic domain of CD45. For these experiments, yeast were cotransformed with the BD-CD45 construct encoding the cytoplasmic domain of CD45 (aa 564–1268) with each of the AD constructs containing individual subunits of CK2.

c The yeast three-hybrid assay was used to determine whether the interaction between CD45 and CK2 requires both α/α′ and β subunits of CK2. The BD-CD45 vector was cotransformed into yeast with the CK2β subunit that had been subcloned into the p14MET25 vector. Yeast were then transformed with either the AD-CK2α or AD-CK2α′ vectors.
CD45 cytoplasmic domain (BD-CD45), CD45 PTP DII alone (BD-CD45 PTPDII), or CD45 PTP DII alone (BD-CD45 PTPDII) to interact with AD-CK2α + CK2β or AD-CK2α’ + CK2β was assessed based on the growth of cotransformed yeast on uracil-deficient selection medium (Fig. 4). Yeast containing BD-CD45 + AD-CK2α + CK2β, or BD-CD45 + AD-CK2α’ + CK2β grew in the absence of uracil as previously described. Yeast containing BD-CD45 PTPDII + AD-CK2α + CK2β or BD-CD45 PTPDII + AD-CK2α’ + CK2β also grew in the absence of uracil. It should be noted that the growth characteristics of yeast were identical regardless of whether the BD-CD45 full-length construct or the BD-CD45 PTPDII mutant was used for transformation. In contrast, yeast cotransformed with BD-CD45 PTPDII + AD-CK2α or α’ + CK2β did not grow in the absence of uracil (Fig. 4). These results clearly demonstrate that PTP DII, but not DI, mediates the interaction between CK2 and CD45. These results further suggest that the ability of DII to interact with CK2 may be due to the presence of one or more unique motifs in DII that are not contained within DI.

PTP DII of CD45 contains four CK2 consensus phosphorylation sites at positions 965, 968, 969, and 973 within an acidic 19-aa insert. The amino acid sequence within this insert is 100% homologous in human, mouse, rat, chicken, and shark, with the exception of aa position 968 in shark (2). To determine whether the 19-aa insert in DII is required for the binding of CK2 to DII of CD45, BD-CD45, and BD-CD45 PTPDII, deletion constructs were generated in which the 19-aa acidic insert was deleted. Each of these constructs was then tested for the ability to interact with the CK2 α/α’ and β subunits in the yeast three-hybrid assay. As determined by monitoring the growth of yeast on uracil-deficient medium, there was no interaction between the CK2 subunits and either the full-length CD45 cytoplasmic mutant (BD-CD45Δ958–973) or the DII mutant (BD-CD45 PTPDIIΔ958–973; Table II). These data demonstrate that the physical interaction between CK2 and DII of CD45 is dependent on the 19-aa acidic insert.

**CD45 function is altered by deletion of the acidic insert in DII**

Structure/function experiments were conducted using the CD45-negative Jurkat cell line J45.01 (29, 30). Compared with CD45-positive parental Jurkat cells (clone E6-1), these cells exhibit a complete lack of responsiveness to stimulation through CD3 due to the loss of CD45 expression (Fig. 5). However, when the J45.01 cell line is transfected with minigene expression constructs encoding either wild-type mouse CD45RA or CD45RO, it is possible to reconstitute full responsiveness to ligands that cross-link the TCR complex. As shown in Fig. 5, stimulation of the J45.CD45RA and J45.CD45RO transfectant cell lines with anti-CD3 mAb resulted in a Ca2+ mobilization response comparable to that observed for parental Jurkat cells. Thus, expression of either mouse CD45RA or CD45RO is sufficient to restore the cell’s responsiveness, presumably through the maintenance of a pool of active Src family PTKs that transduce a signal in response to TCR complex ligation. To assess the importance of the DII acidic motif in the regulation of CD45 function, the CD45RA and CD45RO minigene constructs were mutagenized, resulting in deletion of the 19-aa acidic insert in DII. After transfection with these constructs, J45.01 cells that

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**FIGURE 4.** CK2 selectively interacts with PTP DII of CD45. The yeast three-hybrid assay was used to determine whether CK2 selectively interacts with either DI or DII of CD45, or with both domains. A, The MAV203 strain of yeast was cotransformed with vectors encoding BD-CD45, BD-CD45 PTPDI, or BD-CD45 PTPDII in conjunction with CK2β and AD-CK2αα’. Transformed yeast were plated on dropout medium (Trp-, Leu-, His-) to select for those cells that had taken up all three vectors. These yeast were then plated on uracil-deficient medium and incubated at 30°C for 48 h to assay for the interaction between CD45 and CK2β and AD-CK2αα’. Transformed yeast were plated on dropout medium (Trp-, Leu-, His-) to select for those cells that had taken up all three vectors. These yeast were then plated on uracil-deficient medium and incubated at 30°C for 48 h to assay for the interaction between CD45 and CK2β and AD-CK2αα’. Transformed yeast were plated on dropout medium (Trp-, Leu-, His-) to select for those cells that had taken up all three vectors. These yeast were then plated on uracil-deficient medium and incubated at 30°C for 48 h to assay for the interaction between CD45 and CK2β and AD-CK2αα’. Transformed yeast were plated on dropout medium (Trp-, Leu-, His-) to select for those cells that had taken up all three vectors. These yeast were then plated on uracil-deficient medium and incubated at 30°C for 48 h to assay for the interaction between CD45 and CK2β and AD-CK2αα’. Transformed yeast were plated on dropout medium (Trp-, Leu-, His-) to select for those cells that had taken up all three vectors. These yeast were then plated on uracil-deficient medium and incubated at 30°C for 48 h to assay for the interaction between CD45 and CK2β and AD-CK2αα’.
expressed the mutant forms of CD45RA and CD45RO were selected based on immunofluorescence staining and cell sorting to isolate nonclonal transfectant cell lines. J45.CD45RA:958–973 and J45.CD45RO:808–826 cell lines that express comparable levels of CD45 compared with J45.CD45RA and J45.CD45RO transfectants (data not shown) were analyzed to assess CD3-dependent signal transduction. As shown in Fig. 6, deletion of the 19-aa insert affects the function of both CD45RA and CD45RO based on changes in the Ca\(^{2+}\) mobilization response detected in cells treated with anti-CD3 mAb. The results depicted in Fig. 6 are representative of nine independent experiments with the same mutation results in a significant decrease in the magnitude of the Ca\(^{2+}\) flux in cells that express CD45RO. This finding suggests that the function of specific CD45 isoforms may be differentially controlled by CDK2-dependent post-translational modification.

Additional experiments were performed to determine whether MAPK activation is affected in cells that express wild-type vs mutant CD45 molecules. For these experiments, parental Jurkat cells as well as J45.CD45RA and J45.CD45RA:958–973 transfectants were incubated in the presence or the absence of OKT3 for 1–10 min. The cells were lysed in buffer containing 1% Nonidet P-40, and equivalent amounts of lysate were separated by SDS-PAGE. Activation of Erk1/2 and Jnk was analyzed using phospho-specific Abs directed against key threonine and tyrosine residues that are phosphorylated upon activation of these kinases. The data depicted in Fig. 7A demonstrate that cross-linking of CD3 on parental Jurkat cells leads to increased phosphorylation of Erk1, as detected by Western blotting with phospho-specific Ab. Similarly, reconstitution of wild-type CD45RA expression in the J45.01 cell line promotes activation of predominantly Erk1 in response to CD3 cross-linking. In contrast, Erk1/2 activation in the J45.CD45RA:958–973 transfectants was significantly inhibited, indicating that the 19-aa insert in PTPDII is important for promoting CD3-dependent signaling leading to MAPK activation. As can be seen, stimulation of all three cell lines with PMA promotes comparable activation of Erk1/2. Differences in Erk1/2 phosphorylation were not due to unequal loading of Erk1/2, as determined by stripping the membranes and reprobing with an Ab that recognizes Erk1/2. Similar results were obtained when phosphorylation associated with activation of Jnk was examined (Fig. 7B). Mutation of the 19-aa insert in PTPDII was observed to result in decreased phosphorylation of Jnk compared with that in Jurkat cells reconstituted with wild-type CD45RA. Again, differences in Jnk phosphorylation were not due to unequal loading, as determined by immunoblotting with anti-Jnk Ab. Comparable results were observed when MAPK activation was analyzed in J45.CD45RO vs J45.CD45RO:958–826 transfectants (data not shown).

### Table II. Association between CD45 and CK2 is mediated by the unique 19-aa acidic insert in PTP DII

<table>
<thead>
<tr>
<th>Yeast Transformed With ( ^{a} )</th>
<th>Growth on Selective Medium ( ^{b} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD-CCK2α + CK2β+</td>
<td>Trp, Leu, His, Ura</td>
</tr>
<tr>
<td>BD-CD45 cyto</td>
<td>+ + +</td>
</tr>
<tr>
<td>BD-CD45 cyto:958–973</td>
<td>+ + +</td>
</tr>
<tr>
<td>BD-D11P14MET25</td>
<td>+ + +</td>
</tr>
<tr>
<td>BD-CD45 PTPDII:958–973</td>
<td>+ + +</td>
</tr>
</tbody>
</table>

\(^{a}\) Yeast were cotransformed with the AD-CCK2α vector and p14MET25 vector containing CK2β. Subsequently, the yeast were transformed with the BD vectors containing either the cytoplasmic domain of CD45 or PTP DII from which the unique 19-aa insert had been deleted.

\(^{b}\) Transformed yeast were plated on dropout medium to select for those cells that had taken up the BD, AD, and p14MET25 vectors. The yeast were then plated on uracil-deficient medium to assay for the interaction between CD45 and CK2. The growth of yeast on uracil-deficient medium was assessed 48 h after plating.
CD45 function is altered by mutagenesis of serine residues within the DII acidic insert

Results obtained from yeast three-hybrid analyses demonstrated that the 19-aa insert in DII is important for the physical association between CD45 and CK2. Thus, it was logical to conclude that the suboptimal reconstitution of CD3-mediated signaling observed in the J45.CD45RA:D958–973 and J45.CD45RO:D808–826 cell lines is due to the inability of CK2 to associate with CD45. However, because deletion of the 19-aa insert inherently removes the serine residues that are phosphorylated by CK2 (24), it was not possible to elucidate the relative importance of the association between CD45 and CK2 vs phosphorylation of CD45 by CK2. Therefore, additional mutations were introduced into the CD45RA minigene construct, resulting in the conversion of four serine residues within the acidic insert (S965, 968, 969, and 973) to alanine. J45.CD45RA:S965/968/969/973 transfectants were isolated that expressed comparable levels of CD45 compared with J45.CD45RA and J45.CD45RA:D958–973 based on immunofluorescence staining and cell sorting (data not shown). Analysis of Ca\textsuperscript{2+} mobilization in response to CD3 cross-linking revealed that mutation of the four serine residues to alanine resulted in a shift of the Ca\textsuperscript{2+} mobilization response comparable to that observed in the J45.CD45RA:D958–973 transfectant cell line (Fig. 8).

To confirm that mutation of the serine residues within the acidic insert does not prevent the association with CK2, a solid phase immunoprecipitation technique was used to assess the ability of CK2 to interact with the various mutants of CD45 expressed in the J45.01 cell line. As depicted in Fig. 9, CK2 coprecipitated with both CD45RA and CD45RO, although there were slight differences in the amount of CK2 detected. In contrast, deletion of the acidic 19-aa insert was observed to decrease detectable levels of CK2 to background. Whereas deletion of the insert abrogated the specific interaction between CD45 and CK2, mutation of the four serine residues to alanine caused only a slight decrease in the amount of CK2 that coprecipitated with CD45. These results support the conclusion that inhibition of CK2-dependent phosphorylation of CD45 is responsible for the observed decrease in CD45 function.
Discussion

CK2 is a multifunctional serine/threonine kinase that is ubiquitously expressed in the cytoplasm and nucleus of all eukaryotic cells (27, 28). CK2 expression is elevated in rapidly proliferating and transformed cells, and overexpression in transgenic mice results in the development of lymphomas (31–34). Additionally, it has been shown that overexpression of CK2α in MRL-lpr/lpr mice dramatically potentiates the lymphoproliferative and autoimmune syndrome associated with this strain (35). Studies have demonstrated that CK2 phosphorylates multiple substrates, including proteins involved in gene transcription, the synthesis of nucleic acids and polypeptides, and signal transduction, thereby providing a potential explanation for its ability to regulate cellular proliferation and transformation (27, 28). In this study, immunoprecipitation experiments demonstrated that CK2 physically interacts with CD45 in T and B lymphocytes. The observations that CD45 and CK2 are constitutively associated with one another in unstimulated lymphocytes and that CD45 is constitutively phosphorylated by CK2 (24) suggest that phosphorylation of CD45 could play an important role in regulating its basal activity. Additionally, activation-dependent recruitment of CK2 to CD45 was observed in both T and B cells. Based on studies in vitro demonstrating enhancement of CD45 activity in conjunction with phosphorylation by CK2 (24), it is possible that activation-dependent recruitment of CK2 leads to potentiation of CD45 function.

In this study, the nature of the interaction between CD45 and CK2 was further investigated using yeast two-hybrid analysis, demonstrating that the individual α, α′, or β subunits of CK2 do not associate with CD45. This finding is in contrast to previous work demonstrating that individual subunits of CK2 exhibit the ability to interact with a large number of substrates and/or regulatory proteins. For example, the CK2α subunit alone interacts with PP2A, e-Abl, nucleolin, and insulin receptor substrate 1 (36–38), whereas CK2β has been shown to interact with the serine/threonine kinase Mos (39) and the cell surface receptor CD5 (40).

Although it was formally possible that the lack of a detectable interaction between the individual subunits of CK2 and CD45 in the yeast two-hybrid assay could be due to the requirement for an intermediate protein that physically couples CD45 and CK2, this hypothesis was not supported by the results from yeast three-hybrid analysis. The yeast three-hybrid assay revealed that the physical interaction between CD45 and CK2 requires the presence of both the α or α′ and β subunits of CK2. It is interesting to note that previous studies have shown that either the CK2α or α′ subunit is sufficient to mediate phosphorylation of CD45 in vitro in the absence of the β subunit. Although the β subunit may not be required for phosphorylation of CD45 in vitro, the results obtained in this study demonstrate that it is important for the physical interaction between CD45 and CK2. Thus, the intact CK2 holoenzyme may be required for recruitment and binding to CD45, which presumably lead to phosphorylation of the acidic 19-aa insert in DII.

CD45 is unique among the transmembrane tandem repeat PTPs in that DII contains an acidic 19-aa insert. This insert is highly conserved among all species and contains within it four CK2 consensus phosphorylation sites (Ser967, Ser968, Ser969, and Ser973) in CD45RA (41). Indeed, studies have demonstrated that these residues are phosphorylated by CK2 leading to a 3-fold increase in the maximum velocity of CD45 and that the increase in CD45 catalytic activity can be reversed by treatment with the phosphatase PP2A (24). Mapping studies performed using the yeast three-hybrid assay revealed that the binding site for CK2 is also located within the 19-aa insert (residues 958–973 in CD45RA). Although a detailed analysis of the specific residues involved in binding of CK2 was not performed, it is likely that the interaction involves residues surrounding the four serines in the insert because both the α/α′ and β subunits are required. Presumably, the α/β subunits interact with one or more residues that flank the conserved serines, leaving these critical residues available for phosphorylation by the α or α′ subunits. Further evaluation of the specific residues important for CK2 binding will require scanning alanine mutagenesis of the 19-aa insert in conjunction with the yeast three-hybrid system. Nevertheless, it is apparent that the unique insert in DII of CD45 is required for binding of CK2, which presumably facilitates phosphorylation of CD45 by this serine/threonine kinase.

The functional importance of the 19-aa insert in CD45 was demonstrated by mutational studies in which CD45RA and CD45RO isoforms lacking this insert were expressed in CD45-deficient Jurkat cells. The results demonstrate that deletion of the acidic insert alters the kinetics of the CD3-mediated Ca2+ mobilization response in Jurkat transfectants that express the CD45RA:Δ958–973 isoform. The equivalent mutation in CD45RO (CD45RO:Δ808–826) has a much more significant effect on the ability of this isoform to reconstitute signaling via CD3 compared with that of CD45RA:Δ958–973. Deletion of the acidic insert in CD45RO decreased the overall magnitude of the Ca2+ response, suggesting that the function of this isoform may be differentially regulated by CK2-dependent phosphorylation. In contrast, CD3-mediated activation of Erk1/2 and Jnk was affected to a similar extent in cells expressing CD45RA:Δ958–973 and CD45RO:Δ808–826 mutant molecules. Thus, it remains to be determined whether distinct CD45 isoforms are differentially regulated by CK2-dependent post-translational modification. Additional studies with the CD45RA isoform revealed that mutation of the CK2 phosphocarrier sites within the acidic insert results in a similar shift in the kinetics of the Ca2+ response compared with the CD45RA mutant lacking the entire insert. This finding supports the conclusion that phosphorylation of specific serine residues within the insert may be directly involved in regulation of CD45 function as opposed to the association with CK2 per se. In support of this conclusion, the interaction between CK2 and the serine to alanine mutant of CD45 was only slightly decreased compared with that of wild-type CD45RA.

Previous studies have demonstrated that stable transfection of CD45-deficient H45.01 T cells with CD45RO in which the four serines within the DII acidic insert were mutated to alanine (Ser815, Ser818, Ser819, and Ser823 to Ala) results in a sustained Ca2+ flux after TCR cross-linking, without affecting the magnitude of the response (42). In contrast, experiments performed in this study did not reveal a sustained elevation in the free intracellular concentration of Ca2+ in cells expressing CD45RO:Δ808–826 compared with that in cells that express wild-type CD45RO (data not shown), whereas a significant decrease in the magnitude of the overall Ca2+ mobilization response was observed in response to CD3 cross-linking. Additionally, the CD3-mediated Ca2+ response was not sustained in Jurkat cells transfected with either CD45RA:Δ958–973 or CD45RA:Δ965/968/969/973A compared with wild-type CD45RA (data not shown). Thus, both studies support the conclusion that the 19-aa insert is important for regulating CD45 function, although it is not clear whether CK2-dependent phosphorylation affects the ability of CD45 to regulate the initiation of the signaling response after TCR cross-linking and/or the resolution of the response. It is formally possible that experimental differences in the two studies, related to the cell lines and/or the activation stimuli used, could be responsible in part (42).

The mechanism by which CK2-dependent post-translational modification of CD45 regulates its function is unknown at present. Nevertheless, it is possible that phosphorylation of the insert alters...
the conformation of DII, which, in turn, regulates the formation of intramolecular bonds between DI and DII. Studies have shown that the catalytic activity of CD45 is negatively regulated by intramolecular dimerization (43, 44). This is thought to be due to the reciprocal insertion of a wedge located in the membrane-proximal region of one CD45 molecule into the substrate binding pocket in PTP DI of another. It has further been hypothesized that intramolecular dimerization and wedge insertion are regulated by the intramolecular association between DI and DII in a given CD45 molecule (20). Studies suggest that the formation of an intramolecular interaction between DI and DII prevents intramolecular dimerization, thus enhancing CD45 catalytic function (20, 45, 46). This prediction is supported by numerous studies demonstrating that the catalytic activity and/or substrate specificity of PTP DI are regulated by DII (18–20, 47). In this regard it is possible that phosphorylation of the acidic insert in DII may promote the formation of an intramolecular bond between DI and DII, resulting in increased CD45 activity. Another possible mechanism by which CK2-dependent phosphorylation of CD45 could regulate its function relates to the potential role that DII plays in substrate recruitment. Previous studies have shown that DII of CD45 appears to be located adjacent to the substrate binding pocket of DII might alter its affinity and/or specificity for substrates. In summary, the results from this study support the conclusion that there is a direct physical interaction between CD45 and the CK2 holoenzyme, and that this interaction is important for post-translational modification of CD45 resulting in alteration of its catalytic activity and/or substrate specificity.

Acknowledgments
We thank Dr. Matt Thomas for supplying the CD45 minigene constructs, Dr. Kerry Campbell for providing the p14 MET25 yeast vector, and Dr. David W. Litchfield for providing rabbit antisera specific for CK2α.

References