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REGULATION OF CD45 TYROSINE PHOSPHATASE ACTIVITY BY CASEIN KINASE 2

By

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ABSTRACT

REGULATION OF CD45 TYROSINE
PHOSPHATASE ACTIVITY BY CASEIN KINASE 2

By

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CD45, a transmembrane protein tyrosine phosphatase (PTP), is critical in lymphocyte activation and proliferation. Previous work in our lab established that casein kinase2 (CK2) is a major kinase that phosphorylates CD45 at the 19 amino acid acidic insert of D2 domain. In this study, recombinant CD45 and its CK2 site mutants were expressed and purified from E. *coli* and these proteins were used to examine the effects of phosphorylation on its PTP activities. PTP activities of phosphorylated and non-phosphorylated CD45 were compared using pNPP, Raytide, Myelin Basic Protein (MBP) and recombinant Lck as substrates. It was found that phosphorylation of CD45 increased its PTP activity toward myelin basic protein three fold and the increased activity was reversed through dephosphorylation by PP2A, a serine phosphatase. Kinetic analysis indicated that the increase of CD45 activity by phosphorylation was mainly due to an increase of V_{max}. However, no significant differences of PTP activity between unphosphorylated and CK2-phosphorylated CD45 were observed using pNPP, Raytide and recombinant Lck. In addition, our data showed that CD45 was able to differentially dephosphorylate two Lck phosphorylation sites. Taken together, these data suggest that CD45 has strong substrate specificity and phosphorylation of CD45 by CK2 may be one of the mechanisms to regulate the PTP activity of CD45.

To my wife, Ying Wang

&

to my parents and sisters

Without their love and sustained support,

this could not be achieved.

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PTP, protein tyrosine phosphatase; PTK, protein tyrosine kinase; TCR, T cell antigen receptor; RPTPs, receptor-like protein tyrosine phosphatases; DSPs, dual-specificity phosphatases; LMPs, low molecular weight (acid) phosphatases; MHC, major histo-compatibility complex; ITAMs, immunoreceptor tyrosine-based activation motifs; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; Csk, c-Src kinase; CK2, casein kinase 2; MBP, myelin basic protein; His_6-cytCD45, 6-His tagged form of cytoplasmic domain of CD45; pNPP, p-nitrophenylphosphate.

Chapter 1.

Literature Review

Introduction

Immune system is an exquisite and complicated system functioning to protect vertebrates from invasion of pathogens. This protection is carried out by two steps: immune recognition and immune response. Recognition of self- and foreign antigens by the immune system will trigger appropriate responses by a variety of molecules and cells. Lymphocytes are one of the most important players in this process. Lymphocytes include T and B lymphocytes, both of which originate from hematopoietic stem cells. Although T and B cells have distinct functions in immune recognition and response, the molecular signaling mechanisms employed by these two cells are similar in many respects. Due to the crucial role of lymphocytes in the immune system, understanding lymphocyte function at the molecular level has always been an important subject in immunology.

The response of lymphocytes to antigen stimulation requires the activation of different signaling pathways. As noted in many other cell types, protein phosphorylation and dephosphorylation is a general mechanism used in lymphocyte activation. The importance of protein phosphorylation in control of cellular process was recognized as early as the 1950's. Tyrosine phosphorylation was noticed after a protein tyrosine kinase (PTK), Src kinase, had been purified. Since their catalytic domains are relatively conserved, many protein tyrosine kinases have been identified by screening of cDNA libraries at low stringency with kinase probes. To date, it has been well established that protein tyrosine phosphorylation plays important roles in regulation of cellular growth, differentiation, morphology and movement. In contrast, protein tyrosine dephosphorylation was poorly understood until the first protein tyrosine phosphatase (PTP) — PTP1B was characterized about ten years ago (Tonks et al., 1988a). In recent

years, advances in PTP studies have enriched our knowledge about how cells use delicate phosphorylation/dephosphorylation mechanism to maintain their normal functions. Lymphocyte signaling is a good example to promote the understanding of the dual regulation by both PTKs and PTPs.

This review summarizes the functions of PTPs in the context of antigen stimulated lymphocyte signaling in three parts. The first part provides general information about the PTP family including the structure, catalytic action and activity regulation. T cell antigen receptor (TCR) signaling will be discussed in the second part. The third part focuses on how CD45, one of most important PTPs, acts as a key regulator of lymphocyte activation and development. Evidence presented here strongly supports the notion that PTPs are crucial for cellular response to extracellular stimuli as well as having housekeeping functions.

Protein Tyrosine Phosphatases

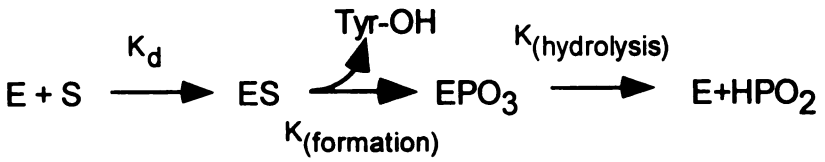
PTP Family. It is estimated that there are as many as 500 human PTPs based on genomic data (Neel and Tonks, 1997). To date, at least 75 PTPs have been identified. These PTPs can be divided into four families: receptor-like PTPs (RPTPs), nonreceptor PTPs, dual-specificity phosphatases (DSPs) and low molecular weight (acid) phosphatases (LMPs). The RPTPs and nonreceptor PTPs are also referred to as "classical PTPs". Every PTP has at least one catalytic domain, which is around 240 amino acids with a unique signature motif ([I/V]HCxAGxxR[S/T]). The invariant cysteine is important for its enzymatic activity. Mutation of this residue eliminates all enzymatic activity in PTPs (Streuli et al., 1989). Most RPTPs have two tandem catalytic domains

— a proximal domain (D1 domain) and a distal domain (D2 domain) based on their relative position to the cytoplasmic membrane. It is generally believed that only the D1 domain has catalytic activity and the D2 domain exists in an inactive form. RPTPs have a variety of extracellular domains and a short transmembrane region. Several structural motifs such as fibronectin type-III, MAM, and "carbonic anhydrase" have been identified in the extracellular domain of different RPTPs, which may relate to different physiological functions. In contrast, all nonreceptor PTPs have only one catalytic domain and no extracellular or transmembrane region. Some domains, such as SH2, PEST and Band 4.1, have been found in nonreceptor PTPs fused to either the amino- or carboxyl-terminal of the PTP domain. These additional domains are believed to regulate localization, enzymatic activity or protein-protein-protein interaction, and therefore serve as regulatory domains. DSPs dephosphorylate phosphotyrosine and phosphothreonine in a sequence specific way. MKP-1, PAC-1, cdc25A,B,C and VH-1 are all members of this family. LMPs have the lowest homology with other three families but they retain the basic structure of the PTP catalytic domain. Little is known about this family.

Structural Features. Crystal structures of PTPs resolved in recent years provide insights to the function of PTPs in signal transduction pathways. These structures suggest that all classic PTPs and DSPs have similar three dimension architecture, although overall sequence identity between them is only less than 5% (Barford et al., 1994; Yuvaniyama et al., 1996). Basically, the catalytic domain has a $\alpha+\beta$ sandwich structure. Four paralleled β-strands form a twisted sheet in the center with several surrounding α helices. The residues of PTP signature motif are within a single loop between β strand and α helix, and they form a pocket for incoming phosphotyrosine on

the substrate. At the base of the pocket is the catalytic cysteine (Cys215, numbered according to PTP1B). Negatively charged Asp181 interacts directly with substrate phosphate and undergoes a dramatic conformation change. The guanidinium group of Arg221 facing the active site assists the phosphate binding. Tyr46 on a loop between $\alpha 1$ and $\beta 1$ set the depth of the pocket and this depth determines the specificity of PTPs (Jia et al., 1995). Compared with DSPs, tyrosine specific phosphatases have a deeper cleft in which only phosphotyrosine moiety is long enough to reach the catalytic cysteine. The domain structure of RPTPs is similar to the structure of PTP1B. A resolved structure showed that RPTP exists as a symmetrical dimer. The helix-turn-helix structure from one monomer acts as a wedge and inserts into the catalytic cleft of the other monomer. The symmetrical insertion of the wedges from each monomer blocks the access of substrates into catalytic clefts and thus results in a inactive enzyme. Two highly conserved acidic residues within the wedge are thought to be critical to inhibit the enzymatic activity. Therefore, the unique structure of RPTP implies that dimerization is a important regulatory mechanism used by the RPTP family (Bilwes et al., 1996). Although the overall structure of LMPs is totally different from the other three families, the structures in the active site among these families are all similar suggesting that PTPs utilize a common catalytic mechanism (Su et al., 1994; Zhang et al., 1994).

Enzymatic Reaction and Kinetics. The enzymatic mechanism for PTPs has been extensively studied using a number of specific PTP inhibitors. The two-step reaction can be represented by the equation shown below (Denu et al., 1996):



First, the unprotonated thiolate group of Cys215 attacks the phosphorus atom of the substrate and releases dephosphorylated substrate. As a consequence, a thiol-phosphate intermediate is formed. Then, this thiol-phosphate is hydrolyzed due to the nucleophilic attack of activated water toward the phosphorus atom again. The second reaction is generally considered as a rate-limiting step. Evidence from X-ray structures suggests that both Arg221 and Asp181 are important for the catalysis. Protonated Asp181 as a general acid binds with the phosphotyrosine substrate through hydrogen bond while the nitrogens from Arg221 side chain and the amide groups from the active-site loop stabilize the dianions of the phosphotyrosine. Once the phosphoenzyme immediate is formed, Asp181 as a general base activates the water to facilitate hydrolysis. Dramatic movement of Asp181 (about 8-12 Å) has been noticed during the formation of phosphoenzyme immediate. Asp181 to Ala mutant abolishes the catalytic activity and thus this mutant is useful for substrate trapping.

Kinetics studies have showed that PTPs have high specific activities for artificial substrates. However, free pTyr is a poor substrate (K_m = 5 mM) demonstrating additional interaction is important for the reaction. The specific activities of PTP1B using RCML and MBP as substrates are up to 1000 fold higher than those of receptor-linked PTKs (Tonks et al., 1988b). This implied that maintenance of low level of dephosphorylation by PTPs might be necessary in control of normal cellular function. Although each PTP has a preference for particular substrates, the K_m of PTPs ranges within three orders of magnitude.

Regulation of PTP Activity. The mechanism on the regulation of PTP activity is not fully understood. Since some PTPs can be phosphorylated by serine/threonine and tyrosine kinases, it is possible that PTP activities are regulated through phosphorylation. Indeed, some studies indicated that phosphorylation of PTPs influence their activity. However, these studies are inconsistent on the issue of whether phosphorylation up- or down- regulates PTP activity. For example, the overall PTP activity increased when CV-1 kidney cells were treated with PKA/PKC activators or phosphatase inhibitors (Brautigan and Pinault, 1991). Similarly, decreased CD45 activity induced by ionomycin treatment correlated with decreased phosphorylation (Ostergaard and Trowbridge, 1991). However, TPA treatment of human peripheral lymphocytes increased the phosphorylation of CD45 but decreased its activity (Yamada et al., 1990). In addition, several other reports showed that the *in vitro* phosphorylation of PTPs had no effect on their activities (Pot et al., 1991; Stover et al., 1991; Tonks et al., 1990).

Based on the similarity of RPTPs with RPTKs, a regulation mechanism via ligand binding has been speculated. To date, in spite of tremendous effort, few ligands have been identified, and some ligands of RPTPs are not necessary to regulate enzymatic activities of RPTPs. For example, no changes of the phosphatase activity and downstream signaling have been detected after PTPβ/ζ bound with its ligand—contactin (PELES et al., 1995). However, evidence derived from the crystal structure of PTPα supports the hypothesis. EGF also can inhibit the T cell signaling mediated by EGF-receptor/CD45 chimeras of ligand-mediated regulation through dimerization (Desai et al., 1993; Majeti et al., 1998). The current model is that transducing of extracellular signals involves a synergistic control of tyrosine phosphorylation, the activation of RPTKs and

inhibition of RPTPs by surface bound ligands (Neel and Tonks, 1997). Additional evidence will be required to support this hypothesis.

T Cell Antigen Receptor Signaling

T cell activation is one of the important events during immune response against pathogen and foreign substances. Activation also plays an important role during the development of T cells in the thymus. Great progress has been made in the understanding of the details of how this process occurs since the T cell antigen receptor was identified in early 1980's. The study of T cell activation set a paradigm for signal transduction, and yet many questions still remain to be answered. Overall, T cell signaling includes four steps: 1) TCR binding to antigen-bound major histocompatibility complex (MHC) -- this binding initiates the T cell signaling; 2) TCR-regulated protein tyrosine kinases (PTKs) activation -- multiple tyrosine phosphorylation events result in the activation of downstream signal pathways. 3) Cellular responses and related genes are expressed. 4) TCR activation is terminated by negative regulators.

T Cell Antigen Receptors. The TCR complex consists of eight subunits derived from six different polypeptide chains. These eight subunits are grouped into four hetero- or homo- dimers: α/β, CD3γ/CD3ε, CD3ε/CD3δ and TCRζ/ζ. While α/β dimers function as a ligand-binding site, the other three dimers function in receptor assembly, cell surface expression and signal transduction. Less than 5% of peripheral blood T cells express γ/δ chains, instead of α/β, as an alternative ligand-binding site. An important feature of the TCR is the presence of ten immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic domains of CD3γ/CD3ε and CD3ε/CD3δ. In

contrast, each TCRζ/ζ has three pairs of ITAMs in a tandem arrangement. The consensus sequence on each ITAM has been identified as $YxxL(x)_{6-8}YxxL$ (Qian and Weiss, 1997). These ITAMs are phosphorylated upon TCR activation, and phosphorylated tyrosines recruit SH2-containing proteins to TCR complex. Since one TCR contains as many as ten ITAMs, these ITAMs are thought to function in amplifying signals received by TCR (Weiss et al., 1994). Studies also suggest that the binding of SH2-containing proteins with ITAMs is sequence specific, the specificity decides which downstream pathways will be turned on under certain condition (Exley et al., 1994). Thus, it is possible that T cells may be differentially activated via TCR-controlled signaling. In addition, an intact ITAM is required for the association of TCRζ and CD3ε with cytoskeleton (Caplan and Baniyash, 1995; Rozdzial et al., 1995).

TCR-regulated Protein Tyrosine Kinases (PTKs). Tyrosine phosphorylation is an important step for the initiation of downstream signaling pathways. Upon engagement of TCR by antigen or anti-TCR antibodies, PTKs were activated and many proteins are immediately phosphorylated by PTKs.. At least four families of PTKs including Src, Csk, Tec and Syk families have been found in T cells (Qian and Weiss, 1997). Many of these are positive regulators while some kinases such as Csk have negative effects on TCR signaling. A few domain structures have been identified in these families. All four families have SH2 domains, and three families have SH3 domain. The Tec family has additional pleckstrin homology (PH) and Tec homology (TH) domains. These domains are modules responsible for protein-protein and protein-lipid interaction (Pawson, 1995). Extensive studies of these PTKs in recent years provide a lot of details about T cell activation. Several important PTKs are briefly discussed as follows.

1) *Lck and Fyn.* Both of these PTKs belong to Src kinase family and they have similar structures: a myristylated glycine at the N-terminal, a kinase domain at its C-terminal, a tandem SH3 and SH2 domain in the middle. The myristylated site facilitates association with the plasma membrane. For Lck, this binding is related to its function in TCR signaling (Veillette et al., 1989). There are two phosphorylated sites on Lck (Y505 and Y394). When Y505 is phosphorylated, the intramolecular interaction between Y505 and SH2 domain folds Lck into a loop and this conformation is inactive. Once this conformation is formed, the autophosphorylation site Y394 is exposed and can be accessed by other molecules. Studies showed that Lck is associated with CD4 or CD8 and this association is required for TCR mediated activation. In $CD4^-CD8^-$ T cells, TCR function was restored only by expression of CD4 with Lck binding site. CD4 mutants without this binding site failed to restore normal TCR signaling (Glaichenhaus et al., 1991).

Evidence suggests that Lck is critical in T cell activation. Lck negative cell lines were unable to mobilize Ca^{2+} and to express activated antigens (Straus and Weiss, 1992). Overexpression of Lck in murine T cells increased the level of tyrosine phosphorylation and secretion of IL-2 (Abraham et al., 1991). Lck is also important for thymocyte development. In Lck deficient mice, thymocyte numbers were dramatically decreased (Molina et al., 1992). While Lck was found to be associated with CD4 or CD8, co-immunoprecipitation and immunofluorescence demonstrated that Fyn was associated with TCR ζ chain. Generally, Fyn has similar functions with Lck in TCR signaling, but Fyn was not required for thymocyte development and proliferation in peripheral cells

(Appleby et al., 1992; Stein et al., 1992) . In addition, Fyn is not able to compensate for Lck deficiency in Lck negative cells (Karnitz et al., 1992).

2) *Syk and ZAP-70.* Syk and ZAP-70 play different roles in lymphocyte activation and development although they have similar structures. Syk is important for B cell antigen receptor signaling and B cell development. However, the T cell development and TCR signaling were normal in Syk deficient mice (Cheng et al., 1995; Turner et al., 1995) . In contrast, ZAP-70 was essential for TCR mediated pathways. Abnormally expressed ZAP-70 in human cells resulted in severe combined immunodeficiency (Arpaia et al., 1994; Chan et al., 1994; Elder et al., 1994). At least three tyrosine phosphorylation sites, Y292, Y492 and Y493, have been identified within ZAP-70 (Watts et al., 1994). While Y492 and Y493 locate inside the kinase domain, Y292 sits on the loop between SH2 and kinase domain. Studies showed that Y493 is phosphorylated by a Src family kinase and the phosphorylation further activates the kinase activity of ZAP-70. Also phosphorylation of Y493 is required of the subsequent phosphorylation of Y492, Y292 and other sites, but how the sequential phosphorylation occurs is unclear. Y492F mutants increase the kinase activity of ZAP-70, phosphorylation on Y492 probably turns down the kinase activity. Unlike Y492 and Y493 mutants, Y292F had no effects on the kinase activity and may only serve as a binding site for recruitment of other signal molecules (Wange et al., 1995).

As its name indicated, ZAP-70 is a ζ chain associated protein. The association of ZAP-70 with ζ chain upon TCR activation is important for initiating T cell signaling. Two tandem SH2 domains on ZAP-70 bind two tyrosine phosphates on the ITAM of ζ chain. This high affinity binding facilitates the phosphorylation and activation of ZAP-

70 by Src family kinases. Crystal structure of ITAM bound ZAP-70 showed that they interact with each other in an anti-parallel way. In other words, the N-terminal SH2 of ZAP-70 binds with C-terminal tyrosine phosphate on ITAM while the C-terminal SH2 of ZAP-70 binds with N-terminal tyrosine phosphate on ITAM (Hatada et al., 1995). A constitutive association between ZAP-70 and TCR ζ chain was observed in murine thymocytes and lymph node T cells. However, ZAP-70 in resting T cells was unphosphorylated and inactive until TCR was activated (vanOers et al., 1994). This provides evidence that phosphorylation and activation of ZAP-70 is critical to TCR signaling.

3) *Csk and Itk.* As mentioned above, Csk has negative effects on Src family kinase. Phosphorylation on the C-terminal end of Lck and Fyn by Csk inactivated their kinase activities. Itk is a member of Tec family kinases. Itk is a potential positive regulator of TCR signaling since the number of thymocytes and $CD4^+$ T cells was abnormally low in Itk deficient mice (Liao and Littman, 1995). Further studies are needed to elucidate the details.

Downstream Signaling Pathways. It is believed that antigen presentation to T cells activates multiple pathways although some links to these pathways are still missing. Activation of PTKs transduces signals in at least three parallel pathways: 1) ***PLCγ mediated lipid hydrolysis, Ca^{2+} mobilization and PKC activation.*** Ca^{2+} mobilization results in the activation of Calcineurin, a serine/threonine phosphatase. Then NFAT is dephosphorylated and activated by Calcineurin. 2) *Ras/MAPK pathway.* Activated MAPK phosphorylates Elk, which then activates Fos transcription. 3) ***Rac/JNK pathway.*** Activated JNK phosphorylates Jun, which combines with Fos to form AP-1. Once

transcription factors, such as NFAT, AP-1, NF-κB and Oct-1, are activated, transcription of cytokine genes like IL-2 will be stimulated. Thus, these activated pathways finally result in T cell functional responses including cytokine secretion, proliferation and cytolytic activity (Osman et al., 1996). Accumulated evidences suggest that ZAP-70 was required for Ca^{2+} mobilization and activation of MAP kinase. ZAP-70 has been shown to interact with many signaling molecules such as Lck, Abl, GAP, Vav, SHP-1, Cbl, LAT and SLP-76 (Qian and Weiss, 1997; Peterson et al., 1998). Some of these signaling proteins are substrates of ZAP-70. The interactions of ZAP-70 with downstream pathways were partly, if not all, mediated by LAT. LAT is one of the adaptor proteins specifically expressed in hematopoietic cells excluding B cells (Zhang et al., 1998). A transmembrane domain was identified within LAT, and several tyrosines were found to mediate the interactions of LAT with other SH2 containing proteins. Co-immunoprecipitation showed those LAT associates with several signaling molecules including Grb2, PI3K, PLCγ1, SLP-76, Cbl and Sos. This provided evidence that LAT may couple Ca^{2+} mobilization by interacting with PLCγ and couple Ras/MAPK pathway by interacting with Grb2/Sos in T cells. In B cells, Shc instead of LAT recruits Grb2/Sos to membrane and activates Ras/MAPK pathway. Besides LAT, other adaptor proteins such as SLP-76, SLAP/Fyb and SKAP-55 also participate in the activation of Ras/MAPK pathway. In addition, it has been known that SLP-76 can associate with Vav upon stimulation (Michel et al., 1998; Wu et al., 1996). However, this pathway has not been completely defined.

Negative Regulation of T Cell Activation. In order to maintain their normal functions, T cells must shut down the activation by antigen presentation. To date, little is

known about how TCR signaling is terminated and how T cells keep a dynamic balance between activation and inhibition. Studies suggest that the termination of T cell signaling is controlled by multiple signal proteins including receptors, PTKs, PTPs and adaptor proteins. Csk is the only PTK which has negative effects on TCR activation. Evidence indicated that several receptors such as CD5, CTLA-4 and KIRs were involved in negative regulation of T cell signaling. It has been known that the negative regulation by CTLA-4 and KIRs is mediated by SHP-1 or SHP-2 (Burshtyn and Long, 1997; Marengere et al., 1996). However, the mediators of CD5 have not been found. In addition, some adaptor proteins are suggested to be negative regulators. One of these adaptors is Cbl which is able to associate with a variety of SH2 containing proteins such as Vav, Crk, PI3K, Fyn and Syk family kinases (Peterson et al., 1998). There are two hypotheses about the inhibitory mechanisms by Cbl. One is that the association of Cbl with Syk family kinases blocks the recruitment of these kinases to activated receptors (Ota and Samelson, 1997). The other is that Cbl can interact with CrkL/C3G complex and result in the activation of Rap1, the activated Rap1 competes with Ras for binding Raf kinase, which results in the blockage of the Ras/MAPK pathway (Boussiotis et al., 1997; Ichiba et al., 1997).

Extracellular Interactions and T cell Activation. Extensive investigation of the signaling pathways in T cells have advanced our understanding of TCR activation. In contrast, studies on how surface molecules of T cells initiate the signaling cascade are just under way. Several questions have been raised about the interactions of TCR and MHC complex such as steric hindrance, ligand concentration and affinity. 1) *Steric hindrance*. 30% of T cell surface is occupied by negatively charged glycoproteins.

These proteins have as long as 45 nm of extracellular domains. Thus TCRs are deeply buried by these proteins (Cyster et al., 1991). To reach these TCRs, peptide-MHC complexes must overcome not only electrical repulsion but also the steric hindrance. 2) *Low ligand concentration.* It is estimated that the density of TCRs is about 200 molecules/μm^2 while the density of the peptide-MHC is only 0.1-1 molecules/μm^2 (Shaw and Dustin, 1997). How can a single peptide-MHC activate up to 100 TCRs? 3) *Low affinity*. The dissociation constants between soluble TCR and peptide bound MHC range from 10^{-4} to 10^{-7} M while the dissociation constants between antigens and immunoglobulins are less than 10^{-9} M. How can this weak interaction trigger a long lasting activation event?

To answer these questions, a topological model for T cell activation has been proposed (Shaw and Dustin, 1997). This model suggests that T cells must pass through three checkpoints to reach maximal activation. The first checkpoint is integrin mediated cell adhesion. Since the interaction between integrins is not affected by charge repulsion, integrin mediated cell adhesion shortens the distance between two cells and thus peptide-bound MHC can contact the buried TCRs. The second checkpoint is the formation of a zone excluding PTPs, especially CD45. The exclusion of PTPs provides time for TCRs initial tyrosine phosphorylation and Ca^{2+} mobilization. The third checkpoint is the formation of contact cap. If the amount of TCR and peptide-MHC complex is sufficient, a stable contact will be formed. In this model, time is thought to be a decisive factor. It takes seconds for first step, minutes for second step, hours for third step. If a contact cap can maintain several hours, a fully activated T cells is achieved. This model argues that the rearrangement of proteins within a contact cap, rather than the engagement of TCR by

antigens, is critical to TCR activation. However, further evidence is needed to substantiate this hypothesis.

CD45 and Its Biological Functions in Lymphocyte Activation and Development.

CD45 is one of the most abundant surface glycoproteins expressed exclusively in all leukocytes. Due to its abundance and unique distribution, CD45 has been extensively studied since mid 70's (Trowbridge and Thomas, 1994). After the identification of the first PTP (PTP1B) in late 80's, CD45 was predicted to be a tyrosine phosphatase based on sequence comparison (Charbonneau et al., 1988). CD45s PTP activity was quickly confirmed (Tonks et al., 1988a). Further studies determined that CD45 is required for T cell signaling since CD45 negative cell lines fail to respond to antigen-induced activation (Pingel and Thomas, 1989). Although great advance has been made to understand the biological functions of CD45 in hematopoietic cells, there are still a lot of questions remained. For example, a ligand has not been found, the regulation of activity has not been determined, and the function of the D2 domain has not been defined. Thus, the further study of CD45 is important to fully understand lymphocyte development and activation.

Structural Features. There are eight isoforms of CD45 (α, β, γ, δ, ε, ξ, η, θ and ι.) differentially expressed on the surface of leukocytes (Chang et al., 1989; Chang et al., 1991). The multi-isoforms result from the alternate splicing of the exons from a single gene. For example, mouse CD45 gene has 34 exons spanning 110 kb on the chromosome 1. Through alternate splicing, it produces a cDNA encoding 1291 amino acids for the human protein (Streuli et al., 1987).

Like other RPTPs, CD45 contains three structural domains: An extracellular domain (1-542 amino acids in human), a single transmembrane region (543-563 amino acids) and cytoplasmic domain (564-1268 amino acids). There are two glycosylation regions, O-linked glycosylation and N-linked glycosylation regions exist on the extracellular domains. At the most N-terminal (1-177 amino acids), alternate exon use generates eight isoforms. In addition, there are three cysteine rich fibronectin III like repeats within the N-linked glycosylation region.

Role of CD45 in Lymphocyte Activation and Development. Accumulated evidence has demonstrated the importance of CD45 in TCR-mediated signaling. However, the function of CD45 in lymphocytes is not fully understood. Clearly, CD45 can act as a positive regulator of T cell signaling. The regulation of CD45 may vary depending on different developmental stages of lymphocytes.

1) Regulation of PTK Activities. It has been established that CD45 functions to regulate activities of PTKs such as Lck and Fyn. This view is support by several studies. First, Lck was hyperphosphorylated in CD45-deficient cell lines (Mustelin and Altman, 1990; Ostergaard et al., 1989). Second, CD45 has been suggested to be associated with Lck through either direct or indirect interaction. The SH2 domain was critical for Lck/CD45 interaction. One explanation of this interaction is that CD45 can be phosphorylated by Csk, thus creating a binding site for SH2 domain on Lck (Autero et al., 1994). However, in vitro studies showed that unphosphorylated recombinant CD45 bound to Lck suggesting a unconventional SH2 domain interaction (Ng et al., 1996). An indirect interaction mediated by CD45-AP was also suggested (McFarland and Thomas, 1995). Third, CD45 can dephosphorylate the negative regulatory tyrosine residues of

Lck and Fyn in vitro (Mustelin et al., 1989; Mustelin et al., 1992). Usually CD45 is regarded as a positive regulator for Lck and Fyn. However, in CD45-deficient YAC-1 cells Lck and Fyn were hyperphosphorylated and their activities, instead of being decreased, were also elevated (Burns et al., 1994). The observation implies that activities of Lck and Fyn are not controlled by a single site, the phosphorylation of other site within kinase domain could also contribute to the regulation. Although Lck and Fyn are similar, their modulation by CD45 is structurally specific. While hyperphosphorylation of Lck in CD45 deficient T cell lines increased 8-10 fold as compared to its wild type counterpart, there was only 2-3 fold increase of hyperphosphorylation for Fyn in same cell line (Hurley et al., 1993). Either deletion of unique domain of Lck or replacement of this domain with corresponding sequence from Fyn dramatically decreased the hyperphosphorylation of Lck suggesting differential regulation between these two kinases by CD45 (Gervais and Veillette, 1995). Since CD45 has different isoforms and these isoforms are differentially expressed in a variety of lymphocytes, Lck and Fyn may also be differentially regulated by CD45 isoforms depending on developmental stage (see below).

2) Differential Regulation of CD45 on Lymphocyte Development. The importance of CD45 in lymphocyte development has been proven by studies from CD45 deficient mice. Two kinds of CD45 deficient mice have been generated and their developmental deficiencies on T cells and B cells have been investigated (Byth et al., 1996; Kishihara et al., 1993). Since exon 6 is alternatively used in splicing process, CD45 deficient mice at exon 6 were not complete nulls. In other words, CD45 isoforms without exon 6 were normally expressed in these mice. In contrast, expression of CD45 in exon 9 targeted

mice was abnormal. Studies on these two deficient mice confirm that CD45 is required for TCR, BCR signaling and lymphocyte development. Development of T cells can be divided into two steps: 1) positive selection from $CD4^-CD8^-$ double negative (DN) to $CD4^+CD8^+$ double positive (DP); and 2) negative selection from $CD4^+CD8^+$ double positive to $CD4^+$ and $CD8^+$ single positive (SP). Generally, deficiencies exhibited in exon 6 and exon 9 targeted mice were similar. Both of them showed a lower number of mature T cells. The DN to DP conversion decreased fivefold while the DP to SP conversion decreased twofold. Thus CD45 was critical for each step of T cell development, and it was more important in second step than in first step. It seems that the function of CD45 was closely related to TCR signaling rather than other pathways since only apoptosis through TCR was severely affected in these deficient mice. TCR signaling of CD45 targeted mice was defective even in mature T cells from those deficient mice. Similarly, T cells expressing certain CD45 isoforms in exon6 targeted mice were unable to respond to antigen stimulation (Byth et al., 1996; Kishihara et al., 1993). Either these T cells were intrinsically abnormal or different isoforms may have distinct function. Compared with CD45 deficient mice, the impairment from DN to DP in Lck deficient mice was more severe (Molina et al., 1992). It is possible that Lck is regulated by CD45 in different stages or by different CD45 isoforms. Based on these data, a threshold-regulating model was proposed (Neel, 1997). In this model, CD45 sets the TCR signaling threshold by regulating Lck activity. Only strong signals beyond this threshold can activate T cells in CD45 deficient mice and as a result those stimulated cells are positively selected.

Although the number of B cells was normal, the development of B cells and BCR signaling in CD45 deficient mice was affected. Unlike wild type mice in which most of B cells are $IgM^{lo}IgD^{hi}$, CD45 deficient mice had increased number of $IgM^{hi}IgD^{hi}$ and $IgM^{hi}IgD^{lo}$ (Benatar et al., 1996). Like in T cells, CD45 may act as a positive regulator for B cell selection, and the selection process is abnormal in CD45 deficient mice due to the change of threshold set by CD45 (Cyster et al., 1996). BCR signaling stimulated by IgM or IgD was also impaired in CD45 deficient mice while response to lipopolysaccharrides and stimulation through CD40 in the same B cells was normal. Upon antigen stimulation, deficient B cells lost their ability to proliferate. Although intracellular Ca^{2+} mobilization was normal in these cells, extracellular Ca^{2+} influx is abolished (Benatar et al., 1996; Byth et al., 1996). These data indicate that CD45 is required for both lymphocyte development and proliferation in response to antigen stimulation.

3) Function of CD45 Extracellular Domain in Cell Adhesion and TCR Signaling. CD45 is a surface molecule with an extended and highly glycosylated extracellular domain. However, the function of the extracellular domain in lymphocyte development and proliferation are unknown. Accumulated evidence suggests that CD45 may interact with other surface molecules and these interactions have impact on antigen mediated signaling. The interaction of extracellular domains between CD45 and CD2 modulated the signaling through CD2 stimulation (Verhagen et al., 1996). Also CD45 can formed complex with CD4 and this association influenced antigen recognition. Interestingly, only the CD45RO isoform, rather than CD45RABC, participated in this interaction. This interaction may have also been related to the regulation of Lck activities (Leitenberg et

al., 1996). CD45 may participate in homotypic cell adhesion of lymphocytes. Apparently, TCR signaling is required for T cell homotypic adhesion because only activated T cells can aggregate. Furthermore, CD45 mediated homotypic adhesion probably depends on its isoforms. Antibodies to some CD45 isoforms induced adhesion while antibodies to other isoforms inhibited adhesion (Zapata et al., 1995). Cell adhesion mediated by integrin was an important step for the initiation of TCR signaling. Some evidence suggests that CD45 was also involved in this process. With the formation of focal adhesions, multiple tyrosine phosphorylation events occur. Phosphorylation of focal adhesion kinase (FAK) and paxillin was regulated by CD45 because these proteins failed to be phosphorylated in CD45 deficient cells (Pao et al., 1997). Although these data predict that CD45 is correlated with cell adhesion, details regarding this process are still in question.

Regulation of CD45. Sufficient evidence supports the proposal that CD45 is a regulator for lymphocyte activation, but how CD45 is regulated has not been defined. Since CD45 is a receptor-like PTP, ligand binding is considered as a potential mechanism for CD45 regulation. Nonetheless, the fact that a ligand for CD45 has not been found up to date forces us search for other possible mechanisms. As mentioned before, it is possible that CD45 is regulated by phosphorylation and dimerization (see regulation of PTP activities). CD45 can be phosphorylated *in vitro* or *in vivo* by multiple serine/threonine kinases such as PKC and casein kinase 2 and by tyrosine kinases like Csk (Autero et al., 1994; Charbonneau and Tonks, 1992). However, the phosphorylation of CD45 either increased or decreased its activity. One explanation for this inconsistency is that CD45 is differentially regulated by multiple kinases. Although dimerization is a

tantalizing mechanism for CD45 regulation, EGF-CD45 chimera is not equivalent to native CD45 molecules. It is difficult to confirm this hypothesis since the ligand of CD45 is unknown. Another possibility for CD45 regulation is through protein-protein interaction. CD45 can interact with a variety of molecules through either extra- or intra-cellular domain interaction. Beside proteins mentioned earlier such as Lck, CD2 and CD4, CD45 can also specifically associated with a glycoprotein (gp116) and a cytoskeleton protein—fodrin (Arendt and Ostergaard, 1995; Okumura and Thomas, 1995). In addition, CD45 interacted with CD45AP through transmembrane domain (Bruyns et al., 1995; Kitamura et al., 1995). It has been known that CD45AP co-expressed with CD45 in T and B cells and a complex of CD45/CD45AP protected CD45AP from degradation (Schraven et al., 1994). There is a putative WW domain within the cytoplasmic domain of CD45AP. This WW domain can bind with proline-rich sequences suggesting CD45AP may mediate the interaction of CD45 with a proline-rich containing protein. Whether these proteins can regulate CD45 remains unknown due to lack of details about their interactions with CD45.

Conclusions

Lymphocyte activation is an important event for immune recognition and immune response. Numerous signaling molecules and multiple signaling cascades are involved in this process. Integration of these signal pathways results in expression of specific genes and ultimately cellular responses occur. Protein phosphorylation and dephosphorylation are rapid chemical reactions and their fast nature predicts that these reactions are extensively used in control of intracellular signals. Another advantage of

phosphorylation/dephosphorylation reactions is that they have capacity to amplify transduced signals. Phosphorylation/dephosphorylation are not two absolutely opposite actions in the process of signal transduction. Positive regulation of Src family kinase by PTPs is a good example to indicate the importance of the cooperation between PTKs and PTPs. As the first identified RPTP, CD45 is critical to the lymphocyte activation and development. Great progress has been achieved to dissect the biological functions of CD45 within the past few years. Many questions such as ligands of CD45, regulation of PTP activities and the functions of D2 domain still remain. In addition, the differential effects of CD45 isoforms in lymphocyte signaling are unclear. Without doubt, resolution of these problems is a key step to deepen our understanding of how cells link and equilibrate their intracellular signaling networks.

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Chapter 2.

Regulation of CD45 Tyrosine Phosphatase Activity by Casein Kinase 2

Introduction

CD45 is a transmembrane protein tyrosine phosphatase (PTP) expressed on the surface of nucleated hematopoietic cells. Because of its importance in lymphocyte signaling, CD45 has been extensively investigated in recent years (Justement et al., 1994; Kung and Thomas, 1997; Neel, 1997). There are at least eight different isoforms of CD45, which distribute differentially on various lymphocyte types and in certain lymphocyte developmental stages (Frearson and Alexander, 1996). CD45 isoforms result from alternate usage of exons at the O-linked glycosylated region of its extracellular domain (Chang et al., 1989; Chang et al., 1991). The function of extracellular domain of CD45 in lymphocyte signaling remains unclear because chimeric proteins containing only the cytoplasmic domain of CD45 were sufficient to restore T cell receptor activation (Desai et al., 1993; Hovis et al., 1993; Volarevic et al., 1993). The cytoplasmic domain of CD45 contains two tandem repeated PTP domains designated D1 and D2. The D1 domain is constitutively active and its catalytic activity is required for complementing T cell receptor (TCR) signaling in CD45-deficient cell lines (Desai et al., 1993). The D2 domain is inactive. CD45 activates Src family kinase by dephosphorylating the inhibitory pTyr near the C terminus *in vitro* and possibly *in vivo*. CD45 is a positive regulator of TCR signal transduction. CD45 can also dephosphorylate several artificial substrates in *vitro*. But it is unknown how the substrate specificity of CD45 is achieved and how its PTP activity is regulated.

It has been shown that CD45 can be phosphorylated *in vitro* by a variety of protein kinases, including protein kinase C, glycogen synthase kinase, casein kinase 2 (CK2), and Csk (Kung and Thomas, 1997). The relationship of CD45 phosphorylation to

its function in T cell signal transduction, however, remains unclear. Several studies on the correlation of CD45 phosphorylation with its PTP activity have been reported but the results are inconsistent (Autero et al., 1994; Ostergaard and Trowbridge, 1991; Stover and Walsh, 1994). Previous work in our lab has shown that CK2 is a major kinase responsible for phosphorylation of CD45 *in vitro* and *in vivo*. The phosphorylation sites of CK2 has been identified in the 19 amino acid acidic region of the D2 domain of CD45. In this study, the effects of CD45 phosphorylation on its PTP activity were examined using recombinant CD45 and its CK2 site mutants. The data presented herein demonstrate that phosphorylation of CD45 by CK2 increased CD45 activity three fold using myelin basic protein as a substrate. In contrast, phosphorylation of CD45 by CK2 did not change the PTP activity toward other substrates such as pNPP, Raytide and recombinant Lck. However, we found that CD45 could differentially dephosphorylate two phosphorylation sites of Lck. The C-terminal inhibitory site of Lck was much more easily dephosphorylated by CD45 than the autophosphorylation site.

Materials and Methods

Cell Lines, Antibodies and Regents. Sf21 insect cells were provided by Dr. D. Dewitt (Michigan State University) and the cells were cultured in either TC-100 (Sigma) or HyQ medium (HyClone) with 1x Antibiotic-Antimyotic (Life Technologies). Polyclonal rabbit anti-Lck serum was kindly provided by Dr. B. M. Sefton (Salk Institute). Myelin basic protein (MBP) was purchased from Sigma and enhanced Raytide was obtained from Calbiochem. Recombinant human Csk was a gift from Dr. P. A. Cole (Rockefeller University).

Expression Vectors. pET3d-CD45, which expresses the cytoplasmic domain of wild type murine CD45 with an amino terminal His_6-tag, was provided by Dr. Pauline Johnson (University of British Columbia). pET3d-CD45(S/A) and pET3d-CD45(S/E) were generated by mutating serine at residues 965, 968, 969 and 973 in the acidic insert of the D2 domain of CD45 into alanine or glutamate respectively (Wang et al., 1999). pGEX-Src was obtained from Dr. J. E. Dixon (University of Michigan). Wild type murine His_6-tagged pFastBac-Lck was generously provided by Dr. B. M. Sefton (Salk Institute) and pBluescript-Lck by Dr. R. Schwartz (Michigan State University). The catalytically inactive form of Lck containing a Lys273Arg mutation, designated pGEX-Lck(K/R) and pFastBac-Lck(K/R), were prepared by mutagenesis as described below.

Expression and Purification of Recombinant CD45, Lck and Src Kinase from *E. Coli*. Recombinant His_6-tagged wild type and mutant CD45 were purified from *E. coli* BL21(DE3) as described (Ng et al., 1995). Cultures were grown in LB containing 100 μg/ml ampicillin at 37°C until the optical density at 600 nm reached 0.6-0.8. IPTG (Boehringer Mannheim) was added to a final concentration of 0.1 mM and cultures were

incubated at 30°C overnight. Then cells were collected by centrifugation and resuspended in Buffer I [0.1% Triton X-100, 20 mM Tris-HCl (pH7.5), 150 mM NaCl, 20 mM imidazole 0.025% β-mercaptoethanol]. Cells were lysed by fast freezing in a dry ice/ethanol bath followed by slow thawing at room temperature. DNase I (10 μg/ml) (Boehringer Mannheim) was added to digest nuclear DNA. The cell debris was removed by centrifugation at 10,000 x g for 10 min and the supernatant was filtered with a 0.22 μm low binding filter (Millipore). The protein sample was loaded onto a pre-equilibrated Ni^{2+}-NTA column (Qiagen) with Buffer I. After washing with Buffer I, proteins were eluted from the column using Buffer II (1 M imidazole, pH7.2). A PD10 column (Amersham Pharmacia Biotech) was used to remove the high concentration of imidazole and proteins were eluted in Buffer III (0.1% Triton X-100, 20 mM Tris-HCl, pH8.0, 150 mM NaCl]. Finally, a Mono Q anion exchange column (Amersham Pharmacia Biotech) was employed for the further purification of recombinant proteins.

Recombinant GST-Src was expressed in E. *coli* BL21(DE3) at 1mM IPTG. E. *coli* cells were lysed by fast freezing and slow thawing as described above and the proteins were purified using GST-column according to the manufacturer's directions (Amersham Pharmacia Biotech). GST-Lck(K/R) was expressed using a previously described method with modification (Hlavac and Rouer, 1997). Cultures were induced with 0.1 mM IPTG at room temperature for 24 h. After induction, cells were collected and resuspended in Buffer IV (50 mM Tris-HCl, pH7.4, 1mM EDTA, 100 mM NaCl, 10% glycerol and 1% NP-40) followed by sonication (Branson Sonofier 450, two 30-s bursts at 50% duty cycle). The insoluble material was solubilized with 1% Sarcosyl and diluted with Buffer III (1:10) and then centrifuged again to remove insoluble material.

The supernatant was loaded onto a glutathione-agarose affinity column (Amersham Pharmacia Biotech) and eluted with 20 mM reduced glutathione (Sigma) in 50 mM Tris-HCl (pH 8.0). Purified proteins were aliquoted and stored at -80°C.

Site-directed Mutagenesis and Construction of pFastBac-Lck(K/R). Point mutation was carried out using 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 mutagenesis of lysine to arginine at residue273 were as following: 5'-GGTGGCGGTGAGGAGTCTGAAACAAGGG-3' and 5'-CCCTTGTTT-CAGACTCCTCACCGCCACC-3'. The primers were extended by *pfu* DNA polymerase during a short temperature cycling (95°C for 30 sec, 55°C for 1 min, 65°C for 13.5 min, 18 cycles) and the parental DNA template was then digested by *Dpn* I endonuclease. Mutants were selected after the synthesized DNA was transformed into *E. Coli* XL1-Blue and later verified by sequencing.

Transposition and Isolation of Recombinant Bacmid DNA. The mutated DNA of pFastBac-Lck(K/R) from mini-preparation was transformed into DH10BAC (Life Technologies) for transposition into the bacmid. Transposition and isolation of bacmid DNA were carried out according to the manufacturer's instruction (Life Technologies). Briefly, transformants were plated on LB plate containing 50 μg/ml kanamycin, 7μg/ml gentamicin, 10 μg/ml tetracycline 100 μg/ml X-gal and 40 μg/ml IPTG. The plate was incubated at 37°C for at least 24 h and then white colonies containing the recombinant bacmid were picked. Selected colonies were further confirmed for the presence of Lck DNA by PCR analysis. The bacmid DNA was then isolated using a method specific for

large plasmids as follows: a bacterial colony was inoculated into 2ml of LB supplemented with 50 μg/ml kanamycin, 7μg/ml gentamicin, 10 μg/ml tetracycline and grown at 37°C to stationary phase. The culture was spun and resuspended in 0.3 ml of Solution I [15 mM Tris-HCl (pH 8.0) 10 mM EDTA, 100 μg/ml RNase A]. 0.3 ml of Solution II (0.2N NaOH, 1% SDS) was added and the lysate was incubated at room temperature for 5 min followed by slowly adding 0.3 ml of 3 M potassium acetate (pH 5.5). After standing on ice for 5 to 10 min, the sample was centrifuged at 14,000 x g for 10 min. Transferred supernatant was precipitated by isopropanol and resuspended into 40 μl TE. A 0.5% agarose gel was used to analyze for the presence of high molecular weight DNA.

Transfection of Sf21 Cells with Bacmid DNA and Screening of Recombinant Baculovirus. $9X10^5$ Sf21 cells with a viability of more than 97% were seeded into 2ml of TC-100 medium with antibiotics and incubated at 27°C for at least 1 h followed by washing with TC-100 without antibiotics. A mixed suspension of bacmid DNA and CellFECTIN (Life Technologies) in TC-100 without antibiotics was prepared and overlaid onto cells. After incubation at 27°C for 5h, the medium was replaced with TC-100 medium containing antibiotics and incubated for 72 h. Recombinant baculovirus was screened by either Western blot or Csk kinase assay.

Infection and Purification of Recombinant Baculovirus Lck from Sf21 Insect Cells. Sf21 insect cells were grown in 15 ml of TC-100 with a 75 cm^2 flask at 27°C for several days and confluent cells were collected with 50 ml of mixed TC-100 and HyQ (1:1). The cell suspension was then transferred into a spinner and grown at 27°C with gentle stirring. These cells were split with HyQ medium once a day until the desired

number of cells was achieved. Sf21 cells were infected with virus stock at a multiplicity of infection (MOI) of 0.1. Infected cells were then harvested by centrifugation for 10 min at 1000 x g, and the cell pellet was resuspended in lysis buffer containing 50 mM Tris-HCl (pH 8.5), 5 mM 2-mercaptoethanol, 100 mM KCl, 1 mM PMSF and 1% NP-40. After a brief sonication, the cell debris was removed by centrifugation at 10,000 x g for 10 min and the supernatant was loaded onto pre-equilibrated Ni^{2+}-NTA column as described above. A PD-10 column was also used to remove high concentration of imidazole. Purified Lck was aliquoted and stored at -80°C.

Radioactive Labeling of Protein Substrates. The ^{32}P-labelling of MBP and Raytide substrates was performed as described previously (Melkerson-Watson et al., 1994). Briefly, 50 μg of enhanced Raytide (Calbiochem, San Diego, CA) or 250 μg of MBP (Sigma) was labeled with 50 μCi of [γ–^{32}P]ATP (3000 Ci/mmol, Dupont NEN) by incubation with 500 ng of recombinant Src tyrosine kinase for 1 h at 30°C, in 160 μl of reaction mixture containing 500 μM ATP, 10 mM $MgCl_2$, 16 mM HEPES (pH 7.5), 0.03 mM EDTA, 0.07% β-mercaptoethanol. Labeled ^{32}P-Raytide or MBP was added to 100 μl of 5 mg/ml BSA and 70 μl of 50% cold TCA followed by centrifugation at 12,000 rpm for 15 min. The pellet was then washed twice with 10% cold TCA and resuspended in 200 μl of 200 mM Tris-HCl (pH 8.0).

Recombinant Lck (200 μg) was labeled in 200 μl of reaction mixture containing 10 mM HEPES (pH7.4), 10 mM $MnCl_2$, 10 mM $MgCl_2$ 5 μM ATP and 50 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) in presence or absence of 100 μg of Csk at 30°C for 30 min. Labeled Lck then was precipitated by adding 100 μl of 50% Ni^{2+}-NTA bead slurry. The slurry was incubated for 10 min at room temperature and the beads were washed twice

with Buffer I [0.1% Triton X-100, 20 mM Tris-HCl (pH7.5), 150 mM NaCl, 20 mM imidazole 0.025% β-mercaptoethanol] and twice with 25 mM HEPES (pH7.5) 0.1% NP-40. Finally, the beads were resuspended in 200 µl of 100 mM imidazole.

PTP Assays. The PTP assay using ^{32}P-labelled MBP, Raytide and recombinant Lck 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 H_2O, 5 µl of ^{32}P-labeled substrate 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 acidic charcoal suspension [0.9 M HCl, 90 mM $Na_4P_2O_7$, 2 mM NaH_2PO_4, and 4% (w/v) active charcoal (Sigma)] to stop the reaction. After centrifugation, the amount of released ^{32}P in the supernatant was measured in a scintillation counter.

PTP assay using pNPP as substrate was performed in a 96-well plate. Each well contained 200 µl of solution containing 40 mM MES (pH 5.0), 1.6 mM DTT and 25 mM pNPP. Reaction was initiated by adding 0.05-5 µg of purified protein and incubated at 30°C for 30 min. The process of reaction was monitored with a microtiter plate reader (Molecular Devices) at 410 nm and results were analyzed using SoftMaxPro (Version 2.1.0) software.

CK2 Phosphorylation of His_6-cytCD45. Wild type and mutant His_6-cytCD45 protein (1 µg) was either mock treated or treated with 0.04 mU recombinant CK2 (Boehringer Mannheim) at 30°C for 30 min in the presence of 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT and 5 µM ATP. Treatment with PP2A treatment was performed as follows. His_6-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 treated with PP2A (Promega) for an additional 30 min at 30°C in 40 µl of reaction mixture containing 20 mM $MgCl_2$, 50 mM Tris-HCl (pH 8.5), 1 mM DTT, 1 µl of PP2A (0.5 units/µl)for. The mixture was then subjected to PTP assay as described above.

FPLC Analysis. FPLC analysis was performed using a Mono Q anion exchange column (Amersham Pharmacia Biotech) using Buffer A [0.1% Triton X-100, 20mM Tris-HCl (pH8.0)] and Buffer B [0.1% Triton X-100, 20mM Tris-HCl (pH8.0), 2M NaCl] and a NaCl gradient from 150 mM to 600 mM as described previously (Ng et al., 1995). Eluted proteins were assayed by release of phosphate using pNPP as substrate.

SDS PAGE and Western Blot. 10% or 12% SDS polyacrylamide gel was ran at 130V for 1.5 h. Gel was stained with GELCODE blue stain reagent (Pierce). Immunoblotting was performed on PVDF membrane with 1:1000 dilution of first antibody followed by incubation with HRP-conjugated goat anti-rabbit secondary antibody (BioRad) and visualization with chemiluminescence (Amersham Pharmacia Biotech).

Kinetic Analysis. For the His_6-cytCD45 kinetics, specific activity was expressed as nmol/min/mg protein and was plotted against substrate concentration. Kinetic parameters were calculated by nonlinear curve fitting of the data to the Michaelis-Menten equation using Origin software (Microcal Software). The kinetic parameters of CK2 (Boehringer Mannheim) with His_6-cytCD45 as substrate were determined with a CK2 assay using Whatman P81 phosphocellulose paper squares as described previously (Tonks et al., 1990).

Protein Quantitative Analysis. Protein concentration was determined by the Bio-Rad protein assay (Bio-Rad) using BSA (Sigma) as standard with a range of 1μg to 5μg.

Results

Expression and Purification of CD45 and Its CK2 Site Mutants. Previous work in our lab showed that CK2 is a major kinase responsible for the phosphorylation of CD45 *in vivo* and *in vitro* (Wang et al., 1999). Sequence analysis of CD45 indicated that there are four consensus sequences, Ser-X-X-acidic group, residing in the 19-amino acids acidic insert of the D2 domain and the consensus sequence is recognized by CK2 (Pearson And Kemp, 1991). Homology comparison showed that four serine residues (Ser965, 968, 969 and 973) within the consensus sequences were highly conserved among different species, and these four serines are potential CK2 phosphorylation sites (Figure 1). To investigate the effect of phosphorylation by CK2 on the activity of CD45, all these serines were mutated to either alanine, or all four to glutamate. Wild type and mutant cytoplasmic domain CD45 (designated as His_6-cytCD45 wt, S/A or S/E respectively) were expressed in *E. coli* BL21 (DE3). Expressed proteins were purified using Ni^{2+}-NTA column followed by Mono-Q anion exchange column chromatography (Figure 2). All three proteins appeared as single peaks. The S/A mutant had an elution profile identical to wild type CD45, both eluting at 240 mM NaCl (Figures 2A and 2B). In contrast, the S/E mutant bound to Mono-Q more tightly and eluted at 340 mM (Figure 2C). The four additional negative charges introduced in the acidic insert enhanced the interaction of CD45 with the matrix in the column and increased the binding affinity of CD45. This observation suggested that the acidic insert was exposed on the surface of native CD45 where it interacted with the matrix. Approximately 1 mg of recombinant protein was obtained from 500 ml of bacterial culture. All three proteins appeared as one

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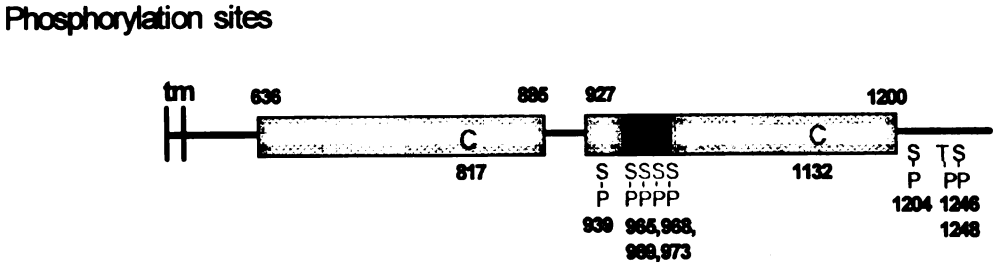

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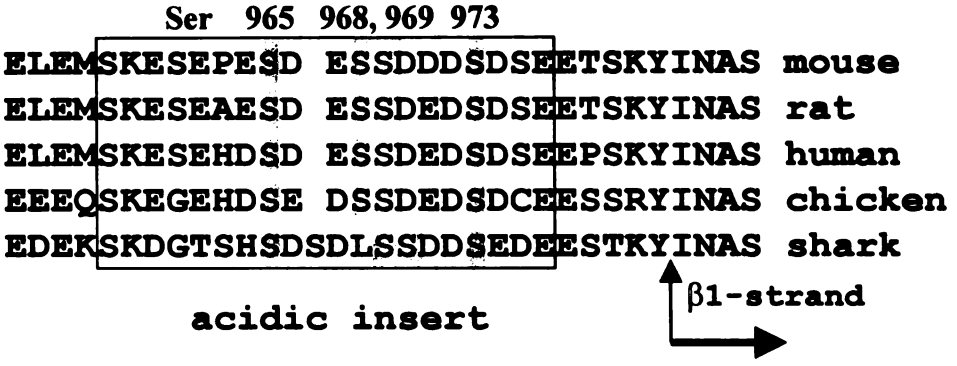

Figure 1 Schematic representation of CD45 cytoplasmic domain and its phosphorylation sites. **(A)** Two tandem catalytic domains are shown as gray solid boxes and the 19 amino acid acidic insert is shown as the black box. Serine and threonine phosphorylation sites are indicated underneath the structure. tm: transmembrane region; C: invariant cysteine residue. **(B)** Comparison of homologous sequences of CD45 acidic insert among different species. The region of acidic insert is framed with an open box and four potential serine phosphorylation sites are shaded. The starting position of β1-strand is indicated by an arrow.

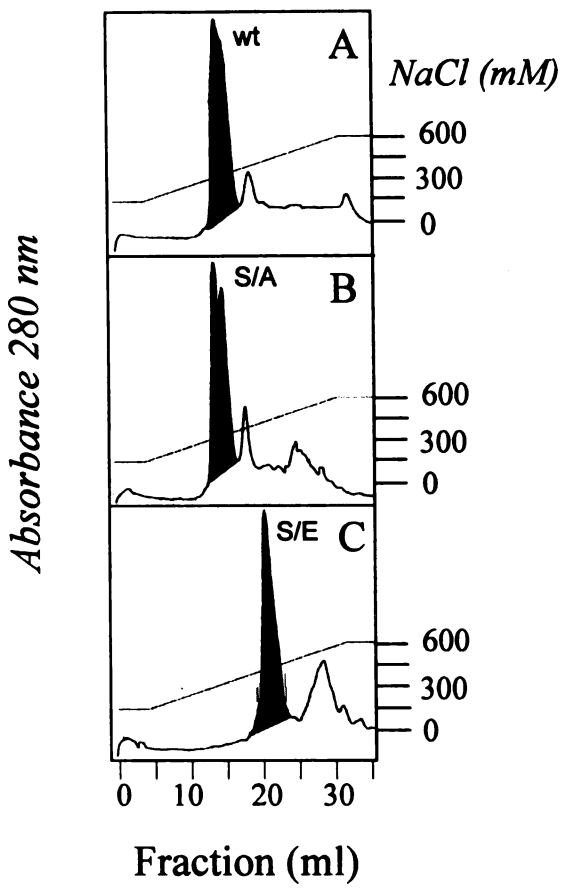

Figure 2. FPLC chromatography of three types of recombinant CD45. **(A)** Wild type CD45, **(B)** S/A and **(C)** S/E mutants were purified by Ni^{2+}-NTA and PD-10 column column chromatography and were loaded onto a Mono-Q ion exchange column and eluted using a NaCl gradient from 150 to 600 mM. PTP active fractions were determined by pNPP hydrolysis and are shaded black. The full scale absorbance was 0.5.

major band of 95 kDa on 12% SDS-PAGE stained with Coomassie brillant blue (Figure 3).

CD45 Is Multiply Phosphorylated by CK2. To study the kinetics of phosphorylation of CD45 by CK2, a time course analysis was performed using purified wild type CD45 as a substrate (Figure 4). The results indicated that CD45 could be easily phosphorylated by CK2. Phosphorylation of CD45 reached a plateau within less than 30 min. Based on the amount of incorporation of ^{32}P into CD45, a stoichiometry of 2.5 mol of phosphate/mol of protein was estimated suggesting that at least three serines on CD45 were phosphorylated by CK2 under the experimental conditions.

To confirm that CD45 was phosphorylated by CK2 at multiple sites, CK2 treated CD45 wild type was subject to FPLC analysis using Mono-Q column (Figure 5). Free ATP in the reaction mixture had a high absorbance at 280 nm but did not interfere with the elution of CD45 since the mocked-treated CD45 eluted at same salt concentration as purified wild type CD45 (compare Figures 5A and 2A). In contrast, CK2 phosphorylated CD45 was retained by the column and eluted at a NaCl concentration of 340 mM to 360 mM. The phosphorylated CD45 eluted as two peaks probably resulting from differently phosphorylated forms (Figure 5B). The fact that CK2 phosphorylated CD45 eluted near the position of the S/E mutant supports the notion that the S/E mutant can mimic the charge effect of phosphorylation. Taken together, the results indicate that there are at least three sites (possibly four) on CD45 which can be phosphorylated by CK2 *in vitro*.

Kinetic Analysis of CD45 Phosphorylation by CK2. A kinetic analysis was performed to further characterize the nature of CK2 phosphorylation of CD45 (Figure 6). The K_m was 0.51 μM and the V_{max} was 35.5 nmol/min/mg with CD45 as a substrate of

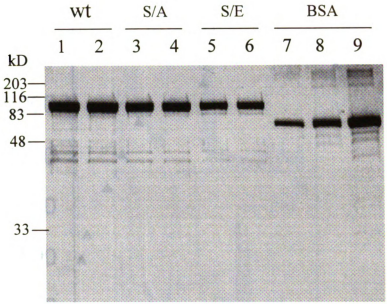

Figure 3. SDS-PAGE analysis of purified recombinant CD45 proteins. A 5μl sample of wild type (wt, lane 1-2), S/A (lane 3-4) and S/E (lane 5-6) were loaded onto 12% SDS-PAGE gel followed by electrophoresis and Commassie Blue staining. CD45 protein amounts were estimated by comparison to 1 μg (lane7), 2 μg (lane 8) and 5 μg (lane 9) of BSA.

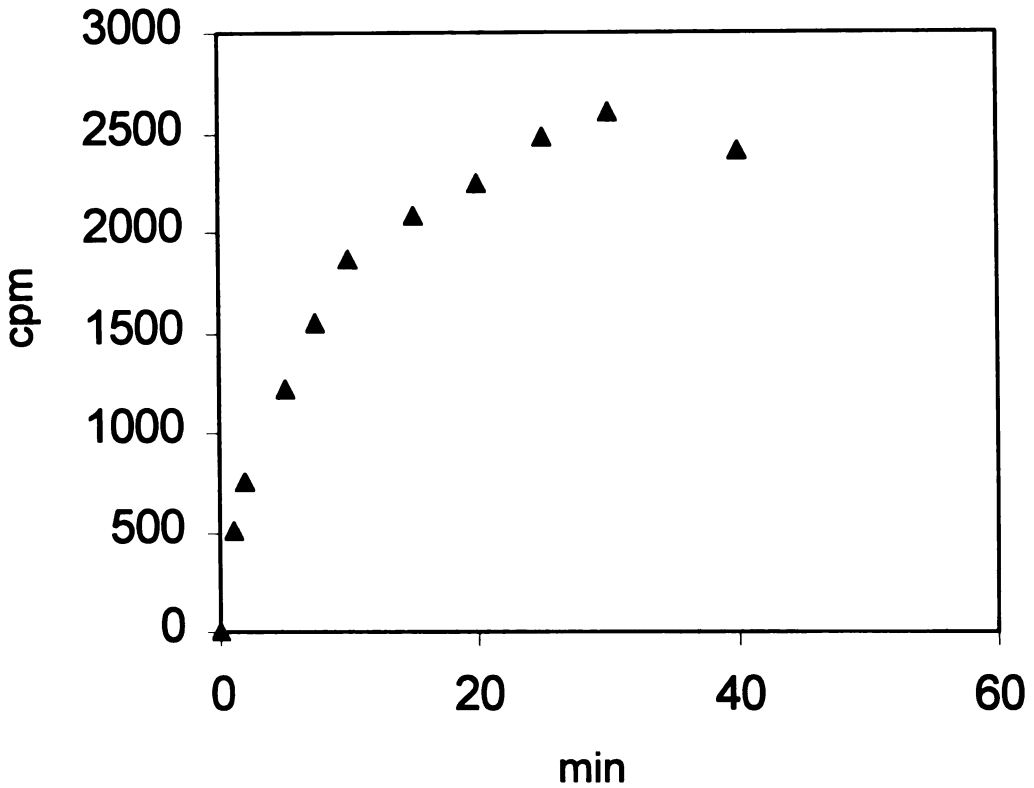

Figure 4. Time course analysis of phosphorylation of CD45 by CK2. Recombinant wild type CD45 protein (1 μg) was mixed with CK2 (0.04 mU) in a total volume of 50 μl under the conditions described in materials and methods. An aliquot (5 μl) of the reaction was taken out at the indicated times and used for measurement of ^{32}P incorporation into CD45.

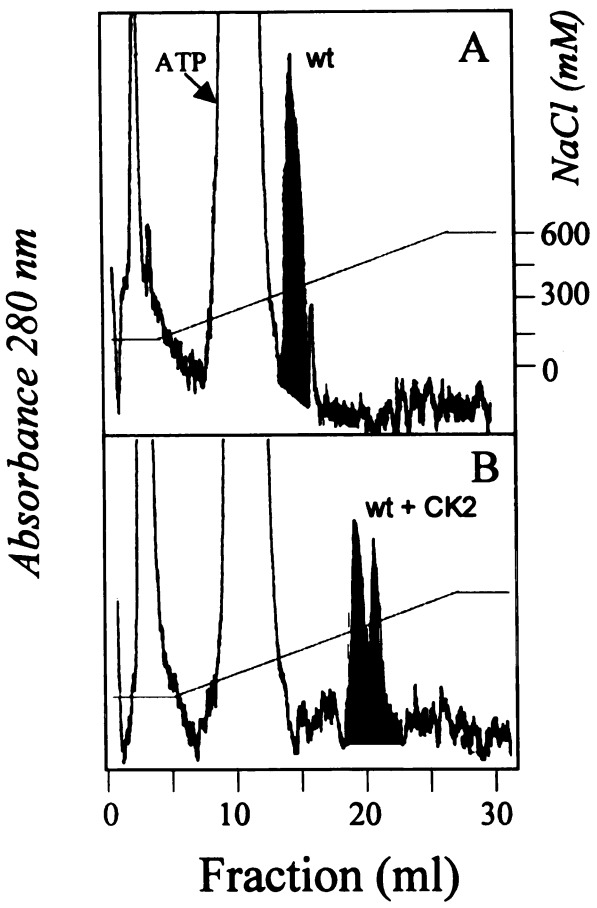

Figure 5. Analytical FPLC separation of CK2-phosphorylated CD45. (A) Mock-treated and **(B)** CK2 treated recombinant wild type CD45 were loaded onto Mono-Q ion exchange column and eluted using NaCl gradient from 150 mM to 600 mM as shown. PTP active fractions were determined by pNPP hydrolysis are shaded black. An ATP peak before PTP active fractions is shown by the arrow. The full scale absorbance was 0.02.

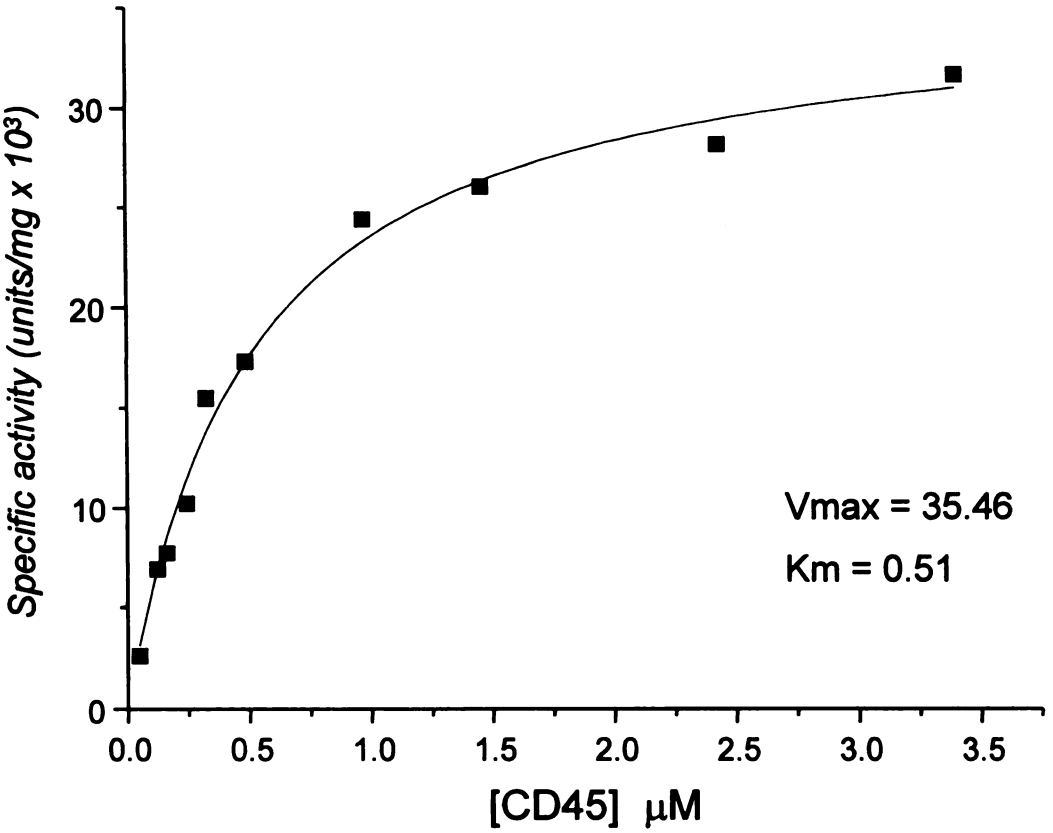

Figure 6. Kinetic analysis of phosphorylation of CD45 by CK2. Specific activity was determined by measuring ^{32}P incorporation of recombinant wild type CD45 from 0 to 3.5 mM. Kinetic parameters were generated by nonlinear curve fitting of the data to the Michaelis-Menten equation using Microcal Origin software.

CK2. These parameters were comparable to reports of CK2 kinetics with other protein substrates. For example, the K_m and V_{max} of CK2 was 1.1 μM and 82.5 nmol/min/mg using eIF-2 as substrate (Gonzatti-Haces and Traugh, 1982). With a K_m in the sub-micromolar range, CD45 is an excellent substrate for CK2 (Tuazon and Traugh, 1991).

Serine Phosphorylation of The Acidic Insert in The D2 Domain Increases CD45 Activity Using MBP As Substrate. 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 the hypothesis, We evaluated the effect of CK2 phosphorylation on CD45 activity using several artificial substrates--pNPP, Raytide and MBP. No significant effects of phosphorylation on CD45 PTP activities were observed using pNPP as a substrate at different concentrations (2.25 mM, 5.62 mM and 11.25 mM) (Figure 7). Specific activities of CD45 toward ^{32}P-raytide increased from 1.74 x 10^4 to 3.38 x 10^4 ng/min/mg upon comparison of unphosphorylated and CK2 phosphorylated CD45 wt. Both phosphorylated and non-phosphorylated CD45 S/A mutant had similar specific activities compared to unphosphorylated CD45wt (1.57 x 10^4 ng/min/mg). In contrast, both forms of S/E mutants (phosphorylated and non-phosphorylated) demonstrated a slight activity increase when compared with non-phosphorylated CD45 (from 1.74 x 10^4 to 2.33 x 10^4 ng/min/mg) (Figure 8). We concluded that phosphorylation of CD45 by CK2 has little effect on PTP activity toward pNPP or ^{32}P-Raytide substrates.

Since pNPP is an analog of pTyr, and Raytide is a short peptide (2476 Da), we attributed the unchanged activity after phosphorylation of CD45 to the small size of these two substrates. In other words, the substrates may not be big enough to interact with the

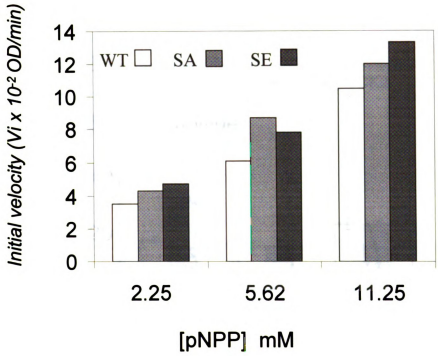

Figure 7. Comparison of PTP activities of recombinant wild type CD45 and CK2 site mutants using pNPP as substrate. The assays were performed with three different concentrations of pNPP under the experimental conditions described in materials and methods. 2.5 μg of enzyme was used in each assay.

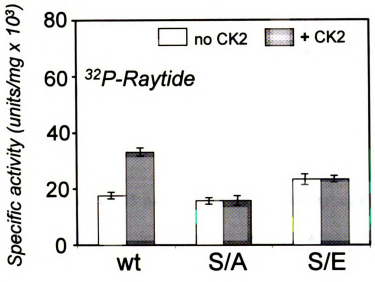

Figure 8. Comparison of PTP activity of recombinant wild type CD45 and CK2 site mutant CD45 using ^{32}P-Raytide as substrate. CK2 phosphorylated (gray bar) and untreated (white bar) recombinant wild type CD45 (wt), S/A and S/E mutants were incubated with ^{32}P-Raytide as substrate at 30°C for 10 minutes. PTP activities were determined by measurement of the release of ^{32}P after incubation.

acidic insert in the D2 domain of CD45. Therefore, potential regulatory effects of phosphorylation on CD45 activity could not be observed using these substrates. To examine this possibility, we compared the phosphorylated and non-phosphorylated forms of CD45 wild type and the CK2 site mutants using a 18 kD protein – MBP (Figure 9). Wild type CD45 activity was enhanced after CK2 phosphorylation by about 3-fold. Neither the S/A mutant nor phosphorylated S/A increased the activity of CD45. However, S/E mutant exhibited a three-fold activity increase compared to unphosphorylated CD45 and there was no change in the activity of the S/E mutant after phosphorylation with CK2. This result suggested that the contact between the acidic insert in the D2 domain of CD45 and its substrates enhances the apparent regulation of PTP activity and of CD45.

Effect of PP2A on CD45 Activity. In many cases the regulation of proteins by phosphorylation acts like a molecular switch with a two-directional function. If phosphorylation of CD45 up-regulates its PTP activity, then dephosphorylation of CD45 could possibly down-regulate its PTP activities. To determine the effect of de-phosphorylation on the CD45 activity, PP2A, a serine phosphatase, was used to remove the phosphate on CD45 after CK2 phosphorylation. As shown in Figure 10, CK2 increased the activity of CD45 and subsequent sequential dephosphorylation with PP2A reversed the activation. Treatment of non-phosphorylated CD45 with PP2A did not alter CD45 activity. In this experiment, a 10 μg/ml of heparin was added to inhibit CK2 activity before incubating with PP2A. The amount of heparin to be added was determined by titration to achieve a balance in which the heparin inhibited the CK2 without inhibiting CD45 or PP2A (data not shown). Complete reversal had not been

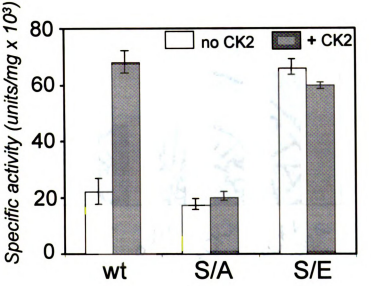

Figure 9. Effect of phosphorylation of CD45 on PTP activity using ^{32}P-MBP as substrate. CK2 phosphorylated (gray bar) and untreated (white bar) recombinant wild type CD45 (wt) and the S/A and S/E mutants were incubated at 30°C for 10 minutes with ^{32}P-MBP. PTP activities were determined by measurement of the release of ^{32}P after incubation.

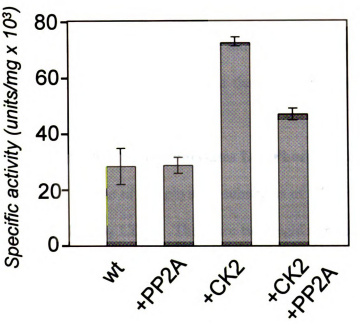

Figure 10. Dephosphorylation of CD45 by PP2A: Effect on PTP activity. Wild type CD45 was treated with CK2 and then incubated with PP2A (+CK2, +PP2A) or without PP2A (+CK2) at 30°C. PTP activity was measured using MBP as substrate. Wild type alone (wt) and wild type treated with PP2A only (+PP2A) under same condition were used as controls.

achieved during the short incubation possibly due to residual CK2 activity and the presence of excessive ATP.

Kinetic Comparison of CD45 and Its CK2 Mutants. To further understand the enzymatic mechanism of regulation of CD45 by phosphorylation, a kinetic analysis of CD45 wild type and its CK2 mutants was carried out using MBP as a substrate. As shown in Figure 11 and Table I, the S to A mutation (S/A) only slightly altered the basic kinetic parameters of His_6-cytCD45 while the introduction of acidic residues, glutamate, into the serine CK2 sites (S/E) resulted in a three fold increase in V_{max} and a small increase in K_m.

CD45 Differentially Dephosphorylates Lck Phosphorylation Sites. Since Lck has been suggested to be one of the physical substrates of CD45 *in vivo*, we studied the dephosphorylation of Lck by CD45. There are two regulatory phosphorylation sites on Lck (Y505 and Y394 in human Lck). Y505 is phosphorylated by c-Src kinase (Csk) and Y394 is the site of autophosphorylation. Both phosphorylation sites are believed to be subject to dephosphorylation by CD45. To examine whether CD45 has distinct ability to remove phosphate from these two sites, it was necessary to prepare the kinase-inactive form of Lck (designated as I-Lck(K/R)) which abolished the autophosphorylation of Lck. For this purpose, wild type murine His_6-tagged pFastBac-Lck was mutated by replacing lysine at position 273 with arginine. Mutated pFastBac-Lck(K/R) was then transposed into bacmid. The bacmid DNA containing I-Lck(K/R) was transfected into Sf21 insect cells and transfectants were screened by precipitating the cell lysate with Ni^{2+}-NTA beads followed by an evaluation of the ability of Csk to phosphorylate bead bound I-Lck(K/R) (*in vitro* Csk kinase assay). Two positive transfectants (out of six) were selected as

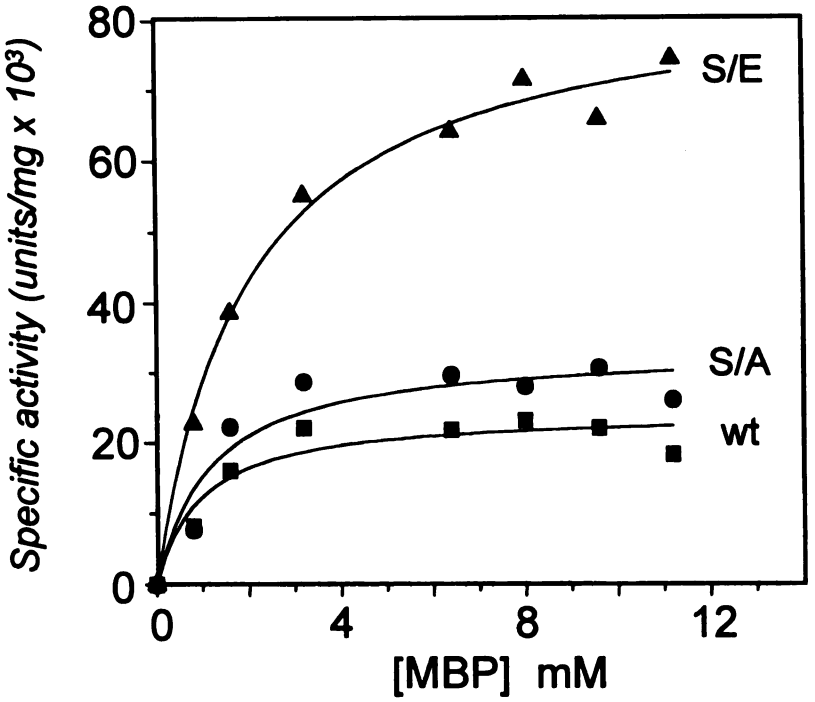

Figure 11. Kinetic comparison of wild type CD45, S/A and S/E mutants using ^{32}P-MBP as substrate. The specific activity of each form was determined by measuring ^{32}P release from labeled substrate over the range of 0 to 12 mM. Kinetic parameters were generated by nonlinear curve fitting the data to Michaelis-Menten equation using Microcal Origin software.

Table I. Kinetic parameters of the CK2 site-mutated His_6-cytCD45 with ^{32}P MBP as substrate.

CD45 form	K_m [a] (μM)	V_{max} (μmol/min/mg)
wt	0.94 ± 0.47[b]	24.2 ± 2.4
S/A[c]	1.18 ± 0.42	33.3 ± 2.7
S/E	1.95 ± 0.29	85.1 ± 3.5

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

b. Average and standard deviation of three determinations.

c. S/A and S/E refer to the corresponding Ala and Glu mutations at Ser residues 965, 968, 969 and 973.

shown in Figure 12. To confirm that the positive transfectants were inactive, one was chosen to compare the effectiveness of autophosphorylation to that of phosphorylation by Csk (Figure 13). When lowest amount of I-Lck(K/R) was present there was strong phosphorylation by Csk with no detectable autophosphorylation (Figure 13, lane 1 and 2). At this level the ratio of I-Lck(K/R) to Csk was 1/7 (w/w). When higher amount of I-Lck(K/R) was present, a basal level of autophosphorylation was detected while the I-Lck(K/R) was strongly phosphorylated by Csk. This result showed that mutated I-Lck(K/R) had extremely low kinase activity.

The decreased kinase activity of I-Lck(K/R) also led to a higher expression in insect cells. Usually, 400 μg of active Lck was obtained from 500 ml of insect cell culture. In contrast, as high as 15 mg of I-Lck(K/R) was purified from 250 ml of culture. The difference of yield between active and inactive form of Lck reflects the possible toxicity in insect cells resulting from overexpression of active Lck.

To test whether phosphorylation of CD45 at CK2 sites had an impact on the dephosphorylation of Lck, we treated the ^{32}P labeled active and inactive forms of Lck with the CK2 mutant forms of CD45 (S/A and S/E) (Figure 14). In this experiment no significant difference in dephosphorylation of either form of Lck was detected between wild type CD45 and the S/A or S/E mutants. That is, all the forms of CD45 had similar PTP activity toward either the active or inactive form of Lck. This result implied that phosphorylation status of CD45 in the acidic insert had no direct effect on functional dephosphorylation of Lck. However, the enzymatic activity of CD45 toward the Csk-phosphorylated, ^{32}P I-Lck(K/R) was 400-fold greater than that for autophosphorylated ^{32}P Lck as substrate. This suggests a high substrate specificity of CD45 for the tail

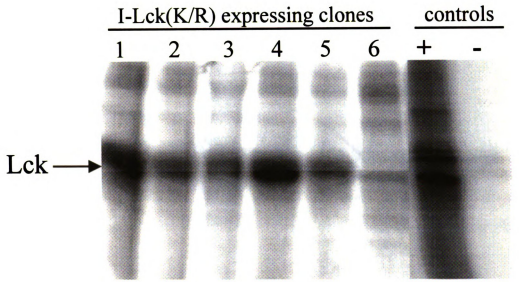

Figure 12. Selection of Sf21 clones expressing inactive Lck (I-Lck(K/R). Clones of infected Sf21 cells (6 x10^5) were lysed with 1% NP-40 followed by precipitation with Ni^{2+}-NTA beads. *In vitro* kinase was performed by incubation of the beads with 2.5 µg of Csk and [^{32}P]γATP. The reaction was analyzed using 10% SDS-PAGE followed by phosphorimage analysis. Equal amount of Lck transfected cells (+) and uninfected Sf21cells (-) were used as positive and negative controls.

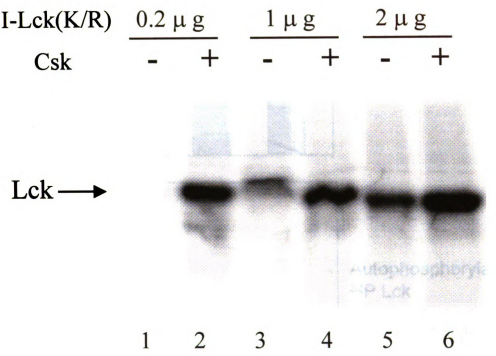

Figure 13. Comparison of autophosphorylation and Csk phosphorylation of inactive Lck [I-Lck(K/R)]. Different amounts of inactive Lck (0.2, 1 or 2 μg) were incubated [^{32}P]γATP in presence or absence of Csk (1.5 μg) at 30°C for 15 min. The reaction was terminated, analyzed by SDS-PAGE and radioactivity was visualized by phosphorimage analysis.

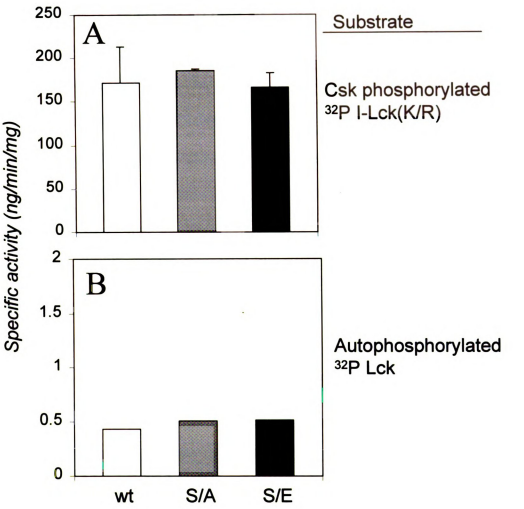

Figure 14. Comparison of PTP activity of mutant forms of CD45 with Csk or autophosphorylated Lck as substrates. (A) Recombinant wild type CD45 (1 ng) (wt) and the S/A and S/E mutants were incubated at 30°C for 10 minutes with Csk-phosphorylated ^{32}P I-Lck(K/R) (5 μg). The data represent the average and SD of three determinations. **(B)** Recombinant wild type CD45 (100 ng) (wt) and the S/A and S/E mutants were incubated with autophosphorylated ^{32}P Lck (1 μg). One representative determination is shown. PTP activities were determined by measurement of the release of ^{32}P after incubation.

phosphorylated form of Lck and raises the possibility that CD45 differentially regulates the two phosphorylation sites. Because Y505 is an inhibitory site for Lck kinase activity, the preference of CD45 for this site supports the notion that CD45 is a positive regulator for Lck.

Discussion

This study was designed to characterize the nature of phosphorylation of CD45 by CK2 and to establish the relationship of phosphorylation of CD45 with its phosphatase activity. The PTP activity of three forms of CD45 (wild type, S/A and S/E mutants) with and without CK2 phosphorylation were compared using several different substrates. The similarity in activities between S/E mutant and phosphorylated wild type CD45 showed that S/E mutant was indeed a good analog to a phosphorylated form of protein. Both of them have higher PTP activities toward MBP substrate than either unphosphorylated wild type or S/A mutant suggesting that phosphorylation of CD45 by CK2 at the acidic insert increases the phosphatase activity.

Protein phosphorylation is a universal regulatory mechanism used by cells to respond to extracellular stimuli. Phosphorylation of CD45 by protein kinases had been noticed for a long time, but little was known about how the phosphorylation of CD45 might regulate its biological function. The experiments of others suggested that CD45 activity either increased or decreased after cells were treated with PKC activators or phosphatase inhibitors (Brautigan and Pinault, 1991; Ostergaard and Trowbridge, 1991; Yamada et al., 1990). The discrepancy between these studies implicates that CD45 may have various phosphorylation regulatory mechanisms depending on cell types, status or conditions of stimuli. In other words, multiple phosphorylation sites may exist on CD45 that are differentially regulated by different protein kinases. Previous studies suggested that the PTP activity of CD45 purified from lymphocytes was unchanged after phosphorylation by CK2 (Tonks et al., 1990). A possible explanation is that the CD45 used in this study was purified from lymphocyte cells and was already highly

phosphorylated. This explanation can be supported by the results from our lab, which showed that immunoprecipitated CD45 from Jurkat T cells could not be labeled easily by exogenous CK2 (Wang et al., 1999).

Kinetic analysis shows that the increase of CD45 activity after phosphorylation by CK2 is due to a large increase in its $V_{max.}$ instead of a decrease in its K_m value. As mentioned before, CD45 consists of two tandem PTP domains that are structurally similar. The 19 amino acid acidic insert exists only in the D2 domain of CD45 but not in D1 domain and not in other PTPs. Also, the acidic insert is highly conserved among different species. The unique nature of this acidic insert suggests that it may be functionally important for CD45.

Solution of the crystal structure of another receptor type PTP, RPTPα showed that RPTPα may exist as an inhibited dimer in which the helix-turn-helix wedge from the N terminal of each monomer blocks the mouth of catalytic cleft of the other monomer (Bilwes et al., 1996). If CD45 has a similar overall structure with RPTPα, the 19 amino acid acidic insert in D2 domain is potentially located in a loop at the N-terminal of β1 and this loop is close to the D2 catalytic cleft. Since the loop sticks out of the molecular surface, phosphorylation of this loop may disrupt the inhibitory dimer structure and make substrate interaction more accessible. As a consequence, the V_{max} of CD45 might increase. However, after CD45 was phosphorylated by CK2, the large increase of PTP activity was only observed using MBP as a substrate while there were slight or no increases of PTP activity using Raytide and pNPP. This result may suggest that CD45 has some substrate specificity.

In this study, we also developed the PTP assay using Lck as a substrate. Because of the role of Lck in lymphocyte signaling, understanding the interaction of CD45 and Lck is crucial to the dissection of the TCR signal pathways. For this purpose, we attempted to express Lck using both E. coli and insect cell expression systems. It was very difficult to purify active or inactive Lck from E. coli since the expressed Lck protein formed inclusion bodies. Although detergents such as N-lauroylsarcosine dissolved the inclusion bodies, the Lck was unable to bind to GST-column (data not shown). In contrast, Lck protein expressed in insect cells was soluble. This observation suggested that post-translational modifications unique to eukaryotic cells might be important to maintain the correct protein conformation. In addition, the dramatic difference in yield observed for expression of active and inactive Lck indicated the potential functional effect of expression of this proto-oncogene in normal cells. Our data showed that phosphorylation of CD45 at CK2 sites did not change the PTP activities toward Lck substrate as it did for MBP. This result indicates that the regulation of CD45 PTP activity might involve other mechanisms besides phosphorylation. Regulation of Lck by CD45 might need the participation of other molecules. The fact that coimmunoprecipitation of CD45 with Lck has a low stoichiometry (Ng et al., 1996) implies that the interaction between Lck and CD45 is weak and not sufficient to hold them in a correct position to allow detectable phosphorylation regulation of CD45.

In this study, CD45 was also shown to have a high substrate specificity toward two different phosphorylation sites (Y505 and Y394) on the same Lck molecule. According to resolved crystal structure of the inactive, Y505 phosphorylated Src kinase, the phosphate on Y505 is buried inside of the SH2 domain of Lck, while phosphorylated

Y394 is on an exposed loop on the outside of Lck. However, our data show that CD45 dephosphorylated Lck preferentially at position of Y505 instead of Y394. Comparing the enzyme activity of CD45 toward different ^{32}P labeled substrates, Raytide, MBP and I-Lck(K/R) seemed to serve as good substrates of CD45, while pNPP and autophosphorylated Lck served as poor substrates of CD45. The substrate specificity of CD45 strongly suggests that the environment in which the tyrosylphosphate resides, or the steric conformation of the protein is a decisive factor in the PTP activity of CD45.

It has been known that mutation of these four serine sites to alanine abolished greater than 95% of the ability of CK2 to phosphorylate CD45 (Wang et al., 1999). Here, we demonstrate that there are multiple CK2 phosphorylation sites on CD45 based on the stoichiometry of phosphorylation and on the fact that phosphorylated CD45 has similar elution profile with S/E mutant. In addition, the FPLC elution profile indicated that CK2 treated CD45 had two elution peaks suggesting multiple forms of CD45 existed after CK2 phosphorylation. However, the data presented here is unable to prove whether these four phosphorylated serines contribute equally or preferentially to the increase of CD45 PTP activity. Mutation of each single site plus different site combination is needed to answer this question. Analytic FPLC showed that the introduction of four negative charges have significant effects on the interactions of CD45 with the Mono-Q column. It may be that keeping the D2 insert acidic correlates with the function of CD45. However, it is hard to understand why CK2 makes this region more acidic and more negatively charged and why these four introduced phosphates significantly increases PTP activity.

CK2 is ubiquitously expressed in all cell types and it can phosphorylate a variety of important signaling molecules, such as Jun, Myc, Myb, Rb and p53 (Litchfield and

Luscher, 1993; Litchfield et al., 1996; Tuazon and Traugh 1991). However, the function of CK2 in signal transduction is still a puzzle. Recent studies showed that CK2 is actively expressed in transformed and proliferating cells, and it can also act as an oncogene in cooperation with Myc in transgenic mice (Seldin and Leder, 1994; Seldin and Leder, 1995). Thus, CK2 is proposed to be a critical and essential serine/threonine protein kinase in control of cell growth and differentiation.

Evidence from CD45 deficient mice suggested that regulation of Src family kinase activity by CD45 varies in a development and cell type-dependent way. For example, regulation of Lck activity by CD45 is less important in the early stages of thymic development than in the late stages. These observations led to the suggestion that CD45 may control lymphocyte signaling by setting different thresholds according to the cellular environment and to the developmental stage of cells. If this is true, then overexpression of CK2 should have an impact on the threshold set by CD45 since it can up-regulate the PTP activity. Considering the high abundance and constitutively high expressed activity of both CK2 and CD45, it is tantalizing to speculate that both CK2 and CD45 act as "buffer molecules", which are important in the maintenance of the basal level of phosphorylation and in the adaptability of cells.

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