Disruption of Lymphocyte Function and Signaling in CD45–associated Protein–null Mice

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Summary

CD45-AP specifically associates with CD45, a protein tyrosine phosphatase essential for lymphocyte differentiation and antigen receptor–mediated signal transduction. CD45 is thought to mediate antigen receptor signaling by dephosphorylating regulatory tyrosine residues on Src family protein tyrosine kinases such as Lck. However, the mechanism for regulating CD45 protein tyrosine phosphatase activity remains unclear. CD45-AP–null mice were created to examine the role of CD45-AP in CD45-mediated signal transduction. T and B lymphocytes showed reduced proliferation in response to antigen receptor stimulation. Both mixed leukocyte reaction and cytotoxic T lymphocyte functions of T cells were also markedly decreased in CD45-AP–null mice. Interestingly, the interaction between CD45 and Lck was significantly reduced in CD45-AP–null T cells, indicating that CD45-AP directly or indirectly mediates the interaction of CD45 with Lck. Our data indicate that CD45-AP is required for normal antigen receptor signaling and function in lymphocytes.

Key words: CD45-AP • CD45 • Lck • gene targeting • signal transduction

The earliest stages of lymphocyte antigen receptor signaling are mediated by protein tyrosine kinases (PTKs)[1] that are associated with the receptor complexes (1). The cytoplasmic segments of lymphocyte antigen receptors and their coreceptors recruit Src and Syk family PTKs by forming specific associations. There is increasing evidence that the activities of these PTKs are regulated by several different mechanisms, including alterations in the phosphorylation status of their key tyrosyl residues and interactions with other molecules. The intracellular domain of CD45 exhibits protein tyrosine phosphatase (PTP) activity and is essential for antigen receptor–mediated signal transduction in lymphocytes (2). It has been proposed that CD45 activates Src family PTKs such as Lck in T cells by dephosphorylating their downregulatory tyrosyl residues (3, 4). The essential role of CD45 in antigen receptor signaling was demonstrated when reconstitution of the cytoplasmic domain of CD45 in CD45-negative lymphocytes restored the ability of these cells to transduce antigen receptor–mediated stimulatory signals (5–7).

One of the central questions in signal transduction is how specificity is preserved among various interacting molecules in signaling cascades. In this respect, it remains unclear how CD45 PTP targets particular substrates and leads to amplification of specific stimulatory signals rather than indiscriminately dephosphorylating various tyrosine phosphorylated proteins, resulting in downregulation of the signal. Most of the CD45 in lymphocytes exists in a complex with a lymphocyte-specific protein, CD45-AP (associated protein; 8–10). The transmembrane segment of CD45-AP binds specifically to the transmembrane segment of CD45 (11–13). The role of CD45-AP has remained unknown, but it has been hypothesized that CD45-AP plays an adapter-like role for CD45, coordinating its interactions with molecules involved in signal transduction. We created CD45-AP–null mice by homologous recombination in order to examine the role of CD45-AP in CD45-mediated lymphocyte signal transduction. Unlike CD45-deficient (14) or -null mice (15), lymphocytes developed normally in CD45-AP–null mice. However, deletion of CD45-AP resulted in impaired T and B cell responses to antigen receptor stimulation. Defects in both proliferation and functional capacity were observed. In addition, T cells showed a reduced response to stimulation with a combination of PMA and ionomycin. The interaction between CD45 and Lck was significantly reduced in CD45-AP–null cells. These

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[1]*Abbreviations used in this paper:* AP, associated protein; ES, embryonic stem; PKC, protein kinase C; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase.

data indicate that CD45-AP is required for a normal interaction between CD45 and Lck and that this interaction in turn is required for normal signal transduction in T lymphocytes.

Materials and Methods

Creation of CD45-AP–null Mice. A targeting DNA construct was prepared by ligating two segments, of 3 kb and 1.6 kb, of the genomic clone (10) and an *HSV-tk* gene cassette (obtained from H. Westphal, National Institute of Child Health and Human Development, Bethesda, MD) into pMC1neo poly(A) vector (Stratagene, La Jolla, CA). The 625-bp KpnI-NsiI genomic fragment that contains all the coding region of the unspliced form of CD45-AP (9) and all but the first five amino acid sequences of the spliced form of CD45-AP (16) was replaced by the *neo* (neomycin resistance) gene cassette. RW4 embryonic stem (ES) cells (Genome Systems, St. Louis, MO) were transfected by electroporation with the targeting construct linearized by SalI, and colonies resistant to G418 and gancyclovir were screened for homologous recombination by genomic Southern hybridization with a probe that is immediately 3′ to the 1.6-kb genomic sequence. NsiI digestion of genomic DNA obtained from normal mice or from ES cells without homologous recombination results in a 6.1-kb fragment on Southern hybridization. 4 ES colonies out of 216 exhibited an altered Southern hybridization pattern as a result of homologous recombination. These clones were injected into blastocysts of C57BL/6 mice and chimeric mice were produced. Two out of five male chimeras had germline transmission of the mutant CD45-AP allele and mice heterozygous for the CD45-AP deletion were identified among their offspring. Homozygous mice were then bred by crossing the heterozygotes and identified by Southern hybridization of genomic DNA.

CD45 PTP Activity Assay. CD45 was isolated from equal numbers of cells obtained from thymus or spleen of 7–9-wk-old CD45-AP–null mice or age-matched wild-type counterparts by immunoprecipitation. CD45 was eluted by briefly subjecting the immunoprecipitates to high pH in 50 mM of diethanolamine (8). A portion of the CD45 preparation was used for PTP activity assay with Raytide (Oncogene Research Products, Cambridge, MA) as a substrate and another portion was analyzed by immunoblotting with anti-CD45 antibody (provided by J. Marth, University of California San Diego, La Jolla, CA). The substrate for PTP assay was prepared by labeling Raytide with γ-[^{32}P]ATP and Src PTK (Oncogene Research Products). PTP activity was determined by measuring the release of $^{32}PO_4$ from the labeled Raytide as previously described (8). The amount of CD45 in the samples was determined by densitometric analysis of the immunoblots.

Proliferation and Function Assays of T and B Lymphocytes. Splenic T and B cells of 7–9-wk-old CD45-AP–null mice or wild-type littermates were purified by a combination of one round of nylon-wool fiber column followed by negative selection with antibody-mediated affinity columns (Biotex Laboratories, Edmonton, Alberta, Canada). Typical T and B cell populations thus obtained were 90 and 95% pure, respectively, by flow cytometric analysis of surface markers. T cells were stimulated by plate-bound (at 5 μg/ml) anti–TCR-α/β Ab (PharMingen, San Diego, CA; reference 17), Con A (2 μg/ml), or a combination of PMA (10 ng/ml) and ionomycin (400 ng/ml). B cells were stimulated by F(ab′)$_2$ fragment of anti-IgM Ab (10 μg/ml; Jackson Immunoresearch Labs., West Grove, PA), anti-CD40 Ab (5 μg/ml; Serotec, Washington DC) (18), LPS (4 μg/ml), or a combination of PMA (10

ng/ml) and ionomycin (400 ng/ml). For some assays mitomycin C-treated spleen cells of wild-type mice were added to purified T cells as a source of accessory cells. Cell proliferation was determined after 72 h by measuring [^{3}H]thymidine incorporation (pulsed at 1 μCi/well overnight) into cells incubated at 10^5 cells per well in 200 μl DMEM with 10% FCS. For MLR, splenic T cells (MHC H-2^b haplotype) (4 $\times$ 10^6 cells/ml) were cultured with mitomycin C–treated spleen cells (4 $\times$ 10^6 cells/ml) derived from MHC-incompatible BALB/c (MHC H-2^d haplotype) or MHC-compatible C57BL/6 mice (MHC H-2^b haplotype) for 4 d before overnight [^{3}H]thymidine pulse. CTLs were generated from spleen cells (MHC H-2^b haplotype) (5 $\times$ 10^6 cells/ml) by coculturing with mitomycin C–treated spleen cells (3 $\times$ 10^6 cells/ml) derived from MHC-incompatible BALB/c mice (MHC H-2^d haplotype) in the presence of conditioned medium Rat T-STIM (Collaborative Biomedical Products, Bedford, MA) for 5 d. Nonadherent CTLs were then harvested and used as effector cells. Cells of H-2^d haplotype (T cell line S49.1) or H-2^b haplotype (spleen cells of C57BL/6 mice) were labeled with ^{51}Cr and used as targets for CTL in a radioactive chromium release assay.

Determination of Interaction between CD45 and Lck. Splenic T cells from 7–9-wk-old CD45-AP–null mice or age-matched wild-type counterparts were purified by two successive rounds of nylon-wool fiber columns. The typical T cell population thus obtained was 85% pure by flow cytometric analysis of surface markers. T cells, 4 $\times$ 10^7 cells/ml in IMDM, were incubated first with anti–TCR-α/β Ab (10 μg/ml; PharMingen; reference 17) on ice for 3 min followed by the addition of anti–hamster IgG (12.5 μg/ml) and further incubation at 37°C for 0, 5, 10, or 60 min for stimulation. After two washes in cold IMDM, the cells were lysed at 5 $\times$ 10^7 cells/ml in 0.8% polyoxyethylene 10 oleyl ether (BRIJ 96) containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM MgCl$_2$, 0.1 mM EGTA, 2.5 mM thioglycolic acid, 1 mM PMSF, 10 mM sodium fluoride, 1 mM sodium orthovanadate, and 10 mM pyrophosphate. Postnuclear supernatants of the lysates were first precleared with Protein G–Sepharose 4 beads (Pharmacia Biotech, Piscataway, NJ) and were then immunoprecipitated either with anti-CD45 Ab (19) cross-linked to Protein G–Sepharose 4 beads with dimethyl pimelimidate (20) or with unconjugated Protein G–Sepharose 4 beads. Material bound to the beads was analyzed by immunoblotting with Abs against CD45 (provided by J. Marth) and Lck (Zymed Labs., South San Francisco, CA) followed by horseradish peroxidase–conjugated Protein A or anti–mouse Ig and the Supersignal substrate system (Pierce, Rockford, IL).

Results and Discussion

Creation of CD45-AP–null Mice. There are two forms of CD45-AP mRNA with a minor difference at the NH$_2$ terminus as a result of splicing (9, 10, 16). The two resulting CD45-AP proteins are identical in their capacity for specific binding to CD45, but use different mechanisms for endoplasmic reticulum membrane translocation (10). The genomic DNA segment that encodes the entire coding region of the first form of CD45-AP and all but the first five amino acids of the second form was replaced with the *neo* gene cassette by homologous recombination in ES cells (Fig. 1 *A*). NsiI digestion of genomic DNA obtained from normal mice or from ES cells without homologous recombination results in a 6.1-kb fragment on Southern hybrid-

A

B

C
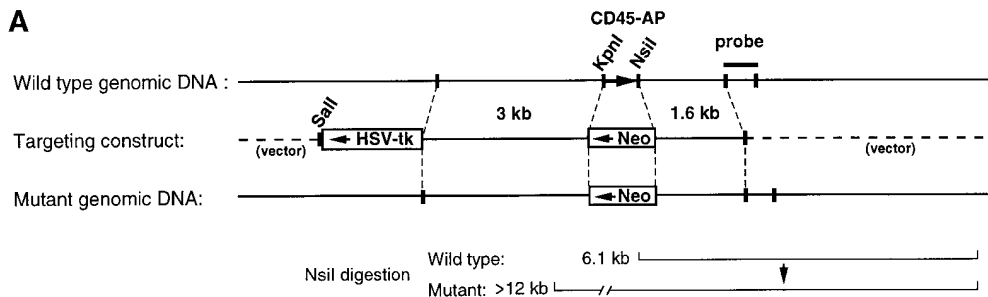
Figure 1. CD45-AP gene targeting. (*A*) A targeting DNA construct for homologous recombination in ES cells is shown in comparison to the wild-type and the mutant alleles. In the targeting construct, the *neo* gene that replaces the CD45-AP gene is flanked by a 3-kb and 1.6-kb genomic sequences, and the herpes simplex virus thymidine kinase (*HSV-tk*) gene cassette is ligated to the 3-kb genomic fragment. NsiI digestion of wild-type genomic DNA results in a 6.1-kb fragment by Southern hybridization with a probe that is immediately 3′ to the 1.6-kb genomic sequence. In the mutated allele, the fragment is larger than 12 kb. (*B*) Southern hybridization of genomic DNA obtained from tails of wild type (+/+), homozygous (−/−), and heterozygous (+/−) littermates for the mutated allele. The DNA was digested with NsiI and probed with the segment shown in *A*. (*C*) Cell lysates were prepared from equal numbers of cells obtained from thymus and spleen of wild-type (+/+) and homozygous (−/−) littermates for the mutated allele and were analyzed by immunoblotting with anti–CD45-AP antisera.
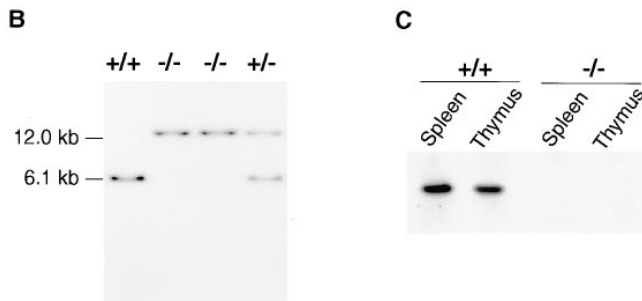
ization. ES colonies that exhibited an altered Southern hybridization pattern as a result of homologous recombination were injected into blastocysts of C57BL/6 mice. Mice heterozygous and homozygous for the CD45-AP deletion were identified by Southern blot analysis of genomic DNA (Fig. 1 *B*). No CD45-AP protein was detected in cell lysates of spleen cells and thymocytes obtained from the homozygous mutants by immunoblot analysis with antiserum against CD45-AP (11), confirming that these homozygous mice are indeed CD45-AP–null (Fig. 1 *C*).

Normal Lymphocyte Development but Reduced CD45 Expression in CD45-AP–null Mice. Gross morphological and histological analysis of all organ systems of CD45-AP–null mice did not show any abnormality, nor did flow cytometric analysis of cells obtained from thymus, spleen, lymph nodes, bone marrow, and peritoneal cavity. Therefore, leukocytes appeared to differentiate normally in these mice unlike in CD45-deficient (14) or -null mice (15). However, the surface expression of CD45 measured by flow cytometry was reduced in the mutant mice compared with their wild-type littermates (Fig. 2 *A*). The average fluorescence intensities of thymocytes and spleen cells obtained from CD45-AP–null mice were 86.2% (± 6.7% SD; 4 experiments) and 73.9% (± 2.4% SD; 6 experiments), respectively, of wild-type littermates. Expression of all isotypes of CD45 appeared reduced since the average fluorescence intensities of CD45-AP–null spleen cells stained with Abs against CD45RA (clone 14.8, PharMingen), CD45RB (clone 16A, PharMingen), and CD45RC (clone DNL-1.9, PharMingen) were 71.8% (± 5.3% SD; 4 experiments), 75.6% (± 2.5% SD; 4 experiments), and 79.7% (± 14.6% SD; 5 experiments), respectively, of wild-type cells. Interestingly, a subset of thymocytes that expresses high levels of CD45, seen as a second peak in the thymic CD45 profile of +/+ mice (Fig. 2 *A*), was absent in CD45-AP–null mice.

Thymocyte subpopulations known to express high levels of CD45 are mature CD4+ or CD8+ single-positive cells and large, cycling, positively selected CD4+CD8+ cells (21). Purified splenic T and B cells of CD45-AP–null mice exhibited equally reduced levels of CD45 expression, i.e., 73.5% (± 2.5% SD; 5 experiments) and 73.2% (± 1.8% SD; 4 experiments) average fluorescence intensities, respectively, of wild-type controls.

To determine whether the reduction in surface CD45 correlates with a reduction in total cellular CD45, the amount of total cellular CD45 in thymocytes and spleen cells was measured by immunoblotting of total cell lysates with anti-CD45 antisera (Fig. 2 *B*) followed by densitometric analysis. Total CD45 in thymus and spleen was reduced to 56.7% (± 12.5% SD; 5 experiments) and 69.8% (± 9.3% SD; 4 experiments), respectively, of wild-type counterparts. Thus, the amount of total CD45 in thymocytes is disproportionately small compared with the surface level detected by flow cytometry, whereas the total amount in spleen corresponds to the surface expression level. These results indicate that the thymocytes of CD45-AP–null mice contain a much smaller amount of intracellular CD45 compared with wild-type thymocytes and suggest the intriguing possibility that CD45-AP affects the subcellular localization of CD45 differentially in thymocytes and spleen cells.

The reduced expression of CD45 in CD45-AP–null cells suggests that CD45 is unstable in the absence of its binding partner, CD45-AP. Likewise, it has been reported that CD45-AP protein levels were much lower in three out of four CD45-negative T cell lines compared with their CD45-positive wild-type parental lymphocytes, suggesting that CD45-AP is unstable in the absence of CD45 (10).

Normal CD45 PTP Activity in CD45-AP–null Mice. Amino acid sequence analysis and protease susceptibility

A

B
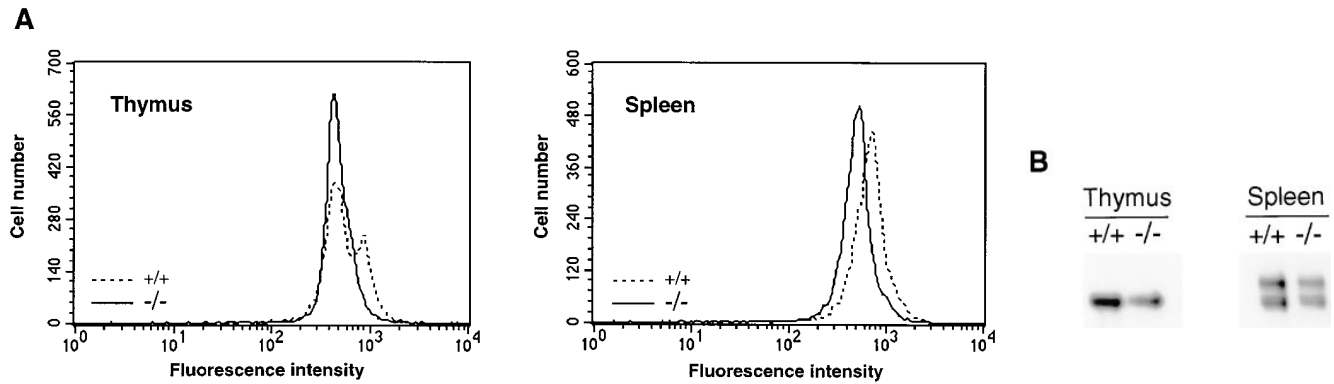
Figure 2. Expression of CD45 in CD45-AP–null mice. (*A*) Thymus and spleen cells obtained from CD45-AP–null (−/−) and wild-type (+/+) littermates were stained with FITC-conjugated anti-CD45 antibody (clone 30-F11; PharMingen). A representative flow cytometric analysis is shown. (*B*) Cell lysates were prepared from equal numbers of cells obtained from thymus and spleen of CD45-AP–null (−/−) and wild-type (+/+) littermates and were analyzed by immunoblotting with anti-CD45 antisera. A representative immunoblot is shown.

analysis of CD45-AP indicate that only a short segment at the NH_2 terminus of CD45-AP is located extracellularly, with the bulk of the protein being intracellular (11). CD45-AP and CD45 form a specific association through their respective transmembrane segments (11–13), whereas the two PTP domains of CD45 reside intracellularly (2). Therefore, it seems unlikely that binding of CD45-AP directly affects CD45 PTP activity since the binding does not involve the PTP domains. In fact, the PTP activity of CD45 isolated from a T cell line, YAC-1, was not altered by binding to recombinant CD45-AP in vitro (Takeda, A., unpublished observations). However, the possibility remains that CD45-AP coordinates the interaction between CD45 and other regulatory molecules and thus affects its PTP activity indirectly. Therefore, PTP activities of CD45 isolated from thymus and spleen of CD45-AP–null mice were compared with their wild-type counterparts by measuring the release of $^{32}PO_4$ from ^{32}P-labeled Raytide. The amount of CD45 was estimated by immunoblotting of the CD45 samples with anti-CD45 antisera followed by densitometric analysis of the immunoblots. Specific activities of CD45 PTP were obtained by dividing CD45 PTP activity by the amount of CD45. As shown in Table 1, the deletion of CD45-AP did not affect CD45 PTP activity in either thymus or spleen.

Reduced Proliferation and Function of T and B Lymphocytes of CD45-AP–null Mice in Response to Antigen Receptor Stimulation. In vitro analysis of proliferative responses of splenic T and B cells to various stimuli revealed interesting differences between CD45-AP–null mice and their wild-type littermates. Proliferative responses of CD45-AP–null T cells to TCR-mediated stimuli were significantly reduced regardless of the presence or absence of accessory cells (*AC*; Fig. 3 *A*). Strikingly, the response of CD45-AP–null T cells to a combination of PMA and ionomycin was reduced to <50% of wild-type whereas the response to Con A was comparable to that of the control. The proliferative response of mutant B cells to anti-IgM Ab was reduced to half of the wild-type, whereas their responses to anti-CD40

Ab, LPS, or a combination of PMA and ionomycin were comparable to control levels (Fig. 3 *B*).

No difference in fluorescence intensity was observed between CD45-AP–null and wild-type littermates when their spleen cells were stained with FITC-labeled anti–TCR-α/β (clone H57-597; PharMingen) or anti-IgM (clone R6-60.2; PharMingen) Ab and analyzed by flow cytometry (data not shown). Therefore, the reduced proliferative responses of CD45-AP–null lymphocytes to antigen receptor ligation are due to defects in signal transduction and not differences in receptor numbers. Furthermore, cells obtained by two successive rounds of nylon-wool fiber columns gave identical results in the proliferation assays to those obtained by negative selection with antibody-mediated affinity columns (data not shown). Thus, incubation of cells with antibodies during the cell purification procedures had no effect on the outcome of the cell proliferation and function assays.

B cells of both CD45-deficient and CD45-null mice differentiate normally but fail to proliferate in response to anti-IgM stimulation (14, 15). The mechanism by which

Table 1. *Relative Specific Activity of CD45 PTP*

	Specific activity (arbitrary units)	
Origin of cells	+/+	−/−
Thymus	28.1 ± 0.63	29.1 ± 1.58
Spleen	14.1 ± 0.26	14.4 ± 0.89

CD45 was isolated from equal numbers of cells obtained from thymus and spleen of CD45-AP–null (−/−) or wild-type (+/+) mice. A portion of the CD45 preparation was used for PTP activity assay with Raytide as a substrate and another portion was analyzed by immunoblotting with anti-CD45 Ab. Specific activity (arbitrary units) was obtained by dividing PTP activity by the amount of CD45. Data are expressed as an arithmetic mean ± SD.
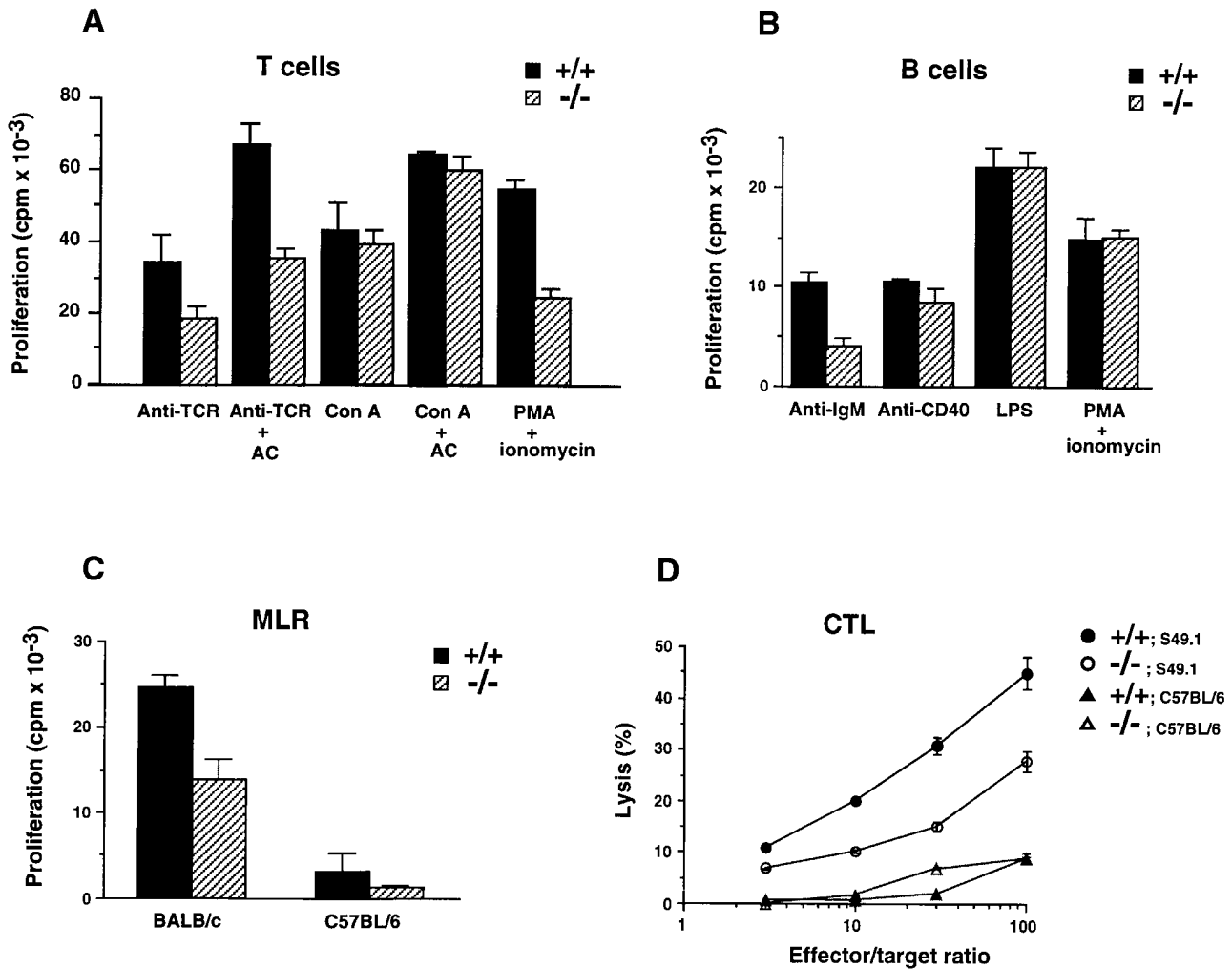
Figure 3. CD45-AP–null T and B cell responses to various stimuli. (*A*) Splenic T cells obtained from CD45-AP–null mice (−/−, *hatched bars*) or their wild-type littermates (+/+, *filled bars*) were stimulated by plate-bound anti–TCR-α/β Ab, Con A, or a combination of PMA and ionomycin. Some assays included accessory cells (*AC*). Cell proliferation was determined by measuring [³H]thymidine incorporation. Representative results of five experiments are shown. (*B*) Splenic B cells obtained from CD45-AP–null (−/−, *hatched bars*) or wild-type (+/+, *filled bars*) littermates were stimulated by F(ab')₂ fragment of anti-IgM Ab, anti-CD40 Ab, LPS, or a combination of PMA and ionomycin. Cell proliferation was determined by measuring [³H]thymidine incorporation. Representative results of five experiments are shown. (*C*) For MLR, splenic T cells obtained from CD45-AP–null (−/−, *hatched bars*) or wild-type (+/+, *filled bars*) littermates were cocultured with mitomycin C–treated spleen cells derived from either MHC-incompatible (H-2^d haplotype) BALB/c or MHC-compatible (H-2^b haplotype) C57BL/6 mice for 4 d before [³H]thymidine pulse. Representative results of three experiments are shown. (*D*) CTLs were generated from spleen cells of CD45-AP–null mice (−/−) or wild-type littermates (+/+) by coculturing with mitomycin C–treated spleen cells derived from MHC-incompatible (H-2^d haplotype) BALB/c mice for 5 d. ⁵¹Cr-labeled MHC-incompatible (H-2^d haplotype) S49.1 cells (*circles*) were used as targets and percentage of cell lysis was measured by ⁵¹Cr-release. ⁵¹Cr-labeled MHC-compatible (H-2^b haplotype) C57BL/6 spleen cells (*triangles*) were similarly used as a negative control. Representative results of three experiments are shown.

the absence of CD45 leads to failed proliferation is unknown. Thus, it is possible that the reduced B cell response to anti-IgM stimulation observed in CD45-AP–null mice (Fig. 3 *B*) is due to the decreased expression of CD45 in CD45-AP–null B cells. On the other hand, there is a considerable amount of evidence that CD45 plays a similar role in antigen receptor–mediated signal transduction of T and B cells, namely, by dephosphorylating regulatory tyrosine residues of their respective Src family PTKs (1, 2). As described below (Fig. 4 *B*), the interaction of CD45 with Lck in T cells is significantly decreased in CD45-AP–null mice.

Therefore, it is also possible that the reduced B cell response to anti-IgM stimulation observed in CD45-AP–null mice is a result of a reduced interaction of CD45 with B cell PTKs.

As expected from the impaired proliferative response of CD45-AP–null T cells to anti-TCR antibodies, spleen cells of CD45-AP–null mice exhibited reduced responses in MLR and CTL assays. CD45-AP–null T cells showed a markedly reduced proliferative response in MLR when they were cultured with mitomycin C–treated allogeneic lymphocytes obtained from BALB/c mice (Fig. 3 *C*). Syn-
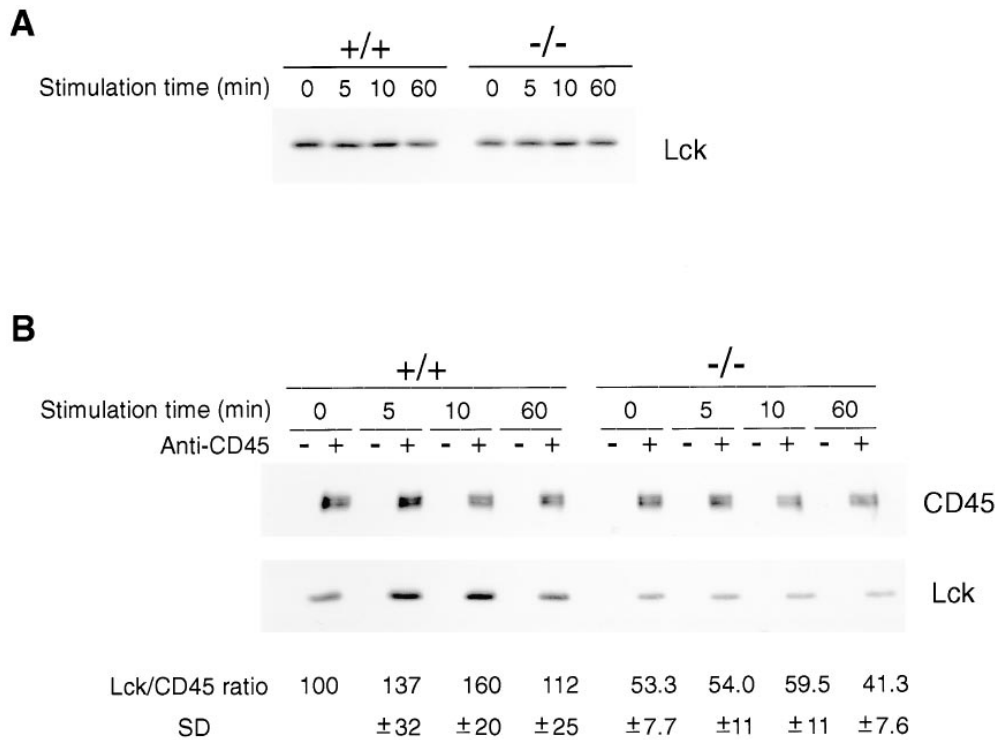
Figure 4. Interaction of CD45 with Lck in CD45-AP–null cells. (*A*) Splenic T cells obtained from CD45-AP–null (−/−) or wild-type (+/+) mice were stimulated by cross-linking of TCR-α/β receptors. Cell lysates prepared at various time points after stimulation were analyzed by immunoblotting with anti-Lck Ab. Representative results of five experiments are shown. (*B*) Cell lysates described in *A* were immunoprecipitated with either control Protein G beads (−) or Protein G beads conjugated with anti-CD45 Ab (+). The immunoprecipitates were analyzed by immunoblotting with anti-CD45 antisera or anti-Lck Ab. The ratios of Lck versus CD45 were obtained by densitometric analysis of the immunoblots. The Lck/CD45 ratios shown are the average of five experiments. The Lck/CD45 ratio obtained at 0 min stimulation of wild-type cells in each experiment was assigned a value of 100 and all other Lck/CD45 ratios of the same experiment were normalized relative to that value to obtain the arithmetic means ± SD.

geneic lymphocytes obtained from C57BL/6 mice did not trigger proliferation in either mutant or wild-type T cells. An ∼50% decrease in CTL activity against MHC-incompatible S49.1 cells was observed in spleen cells derived from CD45-AP–null mice compared with their wild-type littermates (Fig. 3 *D*). When MHC-compatible spleen cells obtained from C57BL/6 mice were used as targets no significant killing was observed with either mutant or wild-type CTLs.

These data show that T cells of CD45-AP–null mice failed to mount adequate responses not only with stimuli that trigger signaling events at the membrane surface but also with a combination of PMA and ionomycin. Stimulation with a combination of PMA and ionomycin provides a measure of cell response irrespective of upstream signaling events by directly stimulating protein kinase(s) C (PKC) and causing intracellular calcium influx. Since the response to a combination of PMA and ionomycin in CD45-AP–null B cells is unaffected, it is possible that CD45-AP has a role in signaling events that is T cell specific and downstream of PKC. PKC appears to have multifaceted and complex functions in TCR signal transduction pathways (22). PKC may function either upstream or downstream of Ras in the Ras pathway, or parallel to the Ras pathway for enhancing TCR signals (23, 24). Effectors of PKC in these pathways have not been established but candidates include the Ras GTPase-activating protein (p120-GAP), Lck, Raf-1, and Sos (guanine nucleotide exchange factor). Studies to iden-

tify the cause of PKC-mediated signaling blockage in CD45-AP–null mice are in progress.

Reduced Interaction between CD45 and Lck in CD45-AP–null T Cells. Most of the CD45 population in lymphocytes exists as a complex with CD45-AP with a stoichiometry of 1:1 (8). It has been hypothesized that CD45-AP coordinates the interaction between CD45 and other molecules involved in CD45-mediated signaling (9). Therefore, we examined the interaction of CD45 with Lck, a Src family PTK associated with the TCR complex and a likely CD45 substrate in vivo (3, 4). Splenic T cells of CD45-AP–null mice and wild-type mice were stimulated by TCR ligation with anti–TCR-α/β Ab and cell lysates were prepared at various time points after stimulation. The total amount of Lck present in the cell lysates was equal between the samples obtained from CD45-AP–null and wild-type cells and also remained unchanged during the stimulation as shown in Fig. 4 *A*. Material immunoprecipitated with anti-CD45 Ab or with control beads was analyzed by immunoblotting with anti-CD45 and anti-Lck Abs (Fig. 4 *B*). In wild-type mice, the amount of Lck associated with CD45 increased after 5 and 10 min of stimulation and returned to the baseline level after 60 min of stimulation. In contrast, in CD45-AP–null mice the amount of Lck coimmunoprecipitating with CD45 was significantly diminished compared with that seen in wild-type mice and was not affected by stimulation. The amounts of Lck and CD45 in immunoprecipitates were quantitated by densitometry of the immunoblots

and Lck/CD45 ratios were obtained. The Lck/CD45 ratio increased in wild-type cells after 5 and 10 min of stimulation but remained at constantly lower levels in CD45-AP–null cells. The Lck/CD45 ratios of wild-type T cells were 1.9-, 2.5-, 2.7-, and 2.7-fold more than those of CD45-AP–null T cells at stimulation times 0, 5, 10, and 60 min, respectively. The low, but detectable, levels of association between CD45 and Lck in CD45-AP–null mice indicate that CD45 is capable of associating with Lck at a lower level in the absence of CD45-AP. In fact, direct association has been observed between recombinant CD45 and Lck in vitro (25). However, when CD45 and Lck were expressed in fibroblasts that do not express CD45-AP, CD45 failed to stably dephosphorylate the kinase-active form of Lck (26), suggesting that an CD45-AP is required for optimally functioning association of CD45 with Lck.

Dephosphorylation of downregulatory tyrosine residues by CD45 is thought to be required for the activity of Src family PTKs that function early in lymphocyte signal transduction (1–4). However, the PTP activity of CD45 is potentially capable of counteracting the activity of PTKs by dephosphorylating their substrates (27, 28). Given the important role of CD45 in lymphocyte differentiation and signal transduction, there must be a mechanism in vivo that either regulates the CD45 PTP activity itself or restricts its access to substrates. Our results demonstrate that CD45-AP is essential for normal interactions of CD45 with a TCR-associated PTK, Lck, in vivo (Fig. 4). It remains to be determined whether CD45-AP directly mediates the interaction between CD45 and Lck. Combined with the functional impairments exhibited by T and B lymphocytes of CD45-AP–null mice (Fig. 3), our results strongly support the notion that CD45-AP plays an important role in coordinating the interaction between CD45 and the appropriate PTKs involved in CD45-mediated signal transduction. This function of CD45-AP may provide a means for regulation of CD45 PTP by targeting appropriate substrates.

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References

1. Weiss, A., and D.R. Littman. 1994. Signal transduction by lymphocyte antigen receptors. *Cell.* 76:263–274.
2. Trowbridge, I.S., and M.L. Thomas. 1994. CD45: an emerging role as a protein tyrosine phosphatase required for lymphocyte activation and development. *Annu. Rev. Immunol.* 12:85–116.
3. Mustelin, T., K.M. Coggeshall, and A. Altman. 1989. Rapid activation of the T-cell tyrosine protein kinase pp56[lck] by the CD45 phosphotyrosine phosphatase. *Proc. Natl. Acad. Sci. USA.* 86:6302–6306.
4. Ostergaard, H.L., D.A. Shackelford, T.R. Hurley, P. Johnson, R. Hyman, B.M. Sefton, and I.S. Trowbridge. 1989. Expression of CD45 alters phosphorylation of the *lck*-encoded tyrosine protein kinase in murine lymphoma T-cell lines. *Proc. Natl. Acad. Sci. USA.* 86:8959–8963.
5. Volarevic, S., B.B. Niklinska, C.M. Burns, C.H. June, A.M. Weissman, and J.D. Ashwell. 1993. Regulation of TCR signaling by CD45 lacking transmembrane and extracellular domains. *Science.* 260:541–544.
6. Hovis, R.R., J.A. Donovan, M.A. Musci, D.G. Motto, F.D. Goldman, S.E. Ross, and G.A. Koretzky. 1993. Rescue of signaling by a chimeric protein containing the cytoplasmic domain of CD45. *Science.* 260:544–546.
7. Desai, D.M., J. Sap, J. Schlessinger, and A. Weiss. 1993. Ligand-mediated negative regulation of a chimeric transmembrane receptor tyrosine phosphatase. *Cell.* 73:541–554.
8. Takeda, A., J.J. Wu, and A.L. Maizel. 1992. Evidence for monomeric and dimeric forms of CD45 associated with a 30-kDa phosphorylated protein. *J. Biol. Chem.* 267:16651–16659.
9. Takeda, A., A.L. Maizel, K. Kitamura, T. Ohta, and S. Kimura. 1994. Molecular cloning of the CD45-associated 30-kDa protein. *J. Biol. Chem.* 269:2357–2360.
10. Kitamura, K., A. Matsuda, S. Motoya, and A. Takeda. 1997. CD45-associated protein is a lymphocyte-specific membrane protein expressed in two distinct forms. *Eur. J. Immunol.* 27:383–388.
11. Kitamura, K., A. Maiti, D.H.W. Ng, P. Johnson, A.L. Maizel, and A. Takeda. 1995. Characterization of the interaction between CD45 and CD45-AP. *J. Biol. Chem.* 270:21151–21157.
12. Cahir McFarland, E.D., and M.L. Thomas. 1995. CD45 protein-tyrosine phosphatase associates with the WW domain-containing protein, CD45-AP, through the transmembrane region. *J. Biol. Chem.* 270:28103–28107.
13. Bruyns, E., L.R. Hendricks-Taylor, S. Meuer, G. Koretzky,

and B. Schraven. 1995. Identification of the sites of interaction between lymphocyte phosphatase–associated phosphoprotein (LPAP) and CD45. *J. Biol. Chem.* 270:31372–31376.

14. Kishihara, K., J. Penninger, V.A. Wallace, T.M. Kundig, K. Kawai, A. Wakeham, E. Timms, K. Pfeffer, P.S. Ohashi, M.L. Thomas, et al. 1993. Normal B lymphocyte development but impaired T cell maturation in CD45-exon6 protein tyrosine phosphatase-deficient mice. *Cell.* 74:143–156.

15. Byth, K.F., L.A. Conroy, S. Howlett, A.J.H. Smith, J. May, D.R. Alexander, and N. Holmes. 1996. CD45-null transgenic mice reveal a positive regulatory role for CD45 in early thymocyte development, in the selection of CD4$^+$CD8$^+$ thymocytes, and in B cell maturation. *J. Exp. Med.* 183: 1707–1718.

16. Shimizu, Y., H. Ogawa, Y. Oka, R. Mizuno, S. Sakoda, T. Kishimoto, and H. Sugiyama. 1994. Isolation of a cDNA clone encoding a novel membrane protein expressed in lymphocytes. *FEBS Lett.* 355:30–34.

17. Kubo, R.T., W. Born, J.W. Kappler, P. Marrack, and M. Pigeon. 1989. Characterization of a monoclonal antibody which detects all murine αβ T cell receptors. *J. Immunol.* 142:2736–2742.

18. Hasbold, J., C. Johnson-Leger, C.J. Atkins, E.A. Clark, and G.G.B. Klaus. 1994. Properties of mouse CD40: cellular distribution of CD40 and B cell activation by monoclonal anti-mouse CD40 antibodies. *Eur. J. Immunol.* 24:1835–1842.

19. Springer, T., G. Gafre, D.S. Secher, and C. Milstein. 1978. Monoclonal xenogeneic antibodies to murine cell surface antigens: identification of novel leukocyte differentiation antigens. *Eur. J. Immunol.* 8:539–551.

20. Schneider, C., R.A. Newman, D.R. Sutherland, U. Asser, and M.F. Greaves. 1982. A one-step purification of membrane proteins using a high efficiency immunomatrix. *J. Biol. Chem.* 257:10766–10769.

21. Ong, C.J., J.P. Dutz, D. Chui, H.-S. The, and J.D. Marth. 1997. CD45 enhances positive selection and is expressed at a high level in large, cycling, positively selected CD4$^+$CD8$^+$ thymocytes. *Immunology.* 91:95–103.

22. Szamel, M., and K. Resch. 1995. T-cell antigen receptor-induced signal-transduction pathways. Activation and function of protein kinases C in T lymphocytes. *Eur. J. Biochem.* 228:1–15.

23. Downward, J., J.D. Graves, P.H. Warne, S. Rayter, and D.A. Cantrell. 1990. Stimulation of p21^{ras} upon T-cell activation. *Nature.* 346:719–723.

24. Izquierdo, M., J. Downward, J.D. Graves, and D.A. Cantrell. 1992. Role of protein kinase C in T-cell antigen receptor regulation of p21^{ras}: evidence that two p21^{ras} regulatory pathways coexist in T cells. *Mol. Cell. Biol.* 12:3305–3312.

25. Ng, D.H.W., J.D. Watts, R. Aebersold, and P. Johnson. 1996. Demonstration of a direct interaction between p56^{lck} and the cytoplasmic domain of CD45 *in vitro. J. Biol. Chem.* 271:1295–1300.

26. Gervais, F.G., and A. Veillette. 1997. Reconstitution of interactions between protein-tyrosine phosphatase CD45 and tyrosine-protein kinase p56^{lck} in nonlymphoid cells. *J. Biol. Chem.* 272:12754–12761.

27. Furukawa, T., M. Itoh, N.X. Krueger, M. Streuli, and H. Saito. 1994. Specific interaction of the CD45 protein-tyrosine phosphatase with tyrosine-phosphorylated CD3 ζ chain. *Proc. Natl. Acad. Sci. USA.* 91:10928–10932.

28. Mustelin, T., S. Williams, P. Tailor, C. Couture, G. Zenner, P. Burn, J.D. Ashwell, and A. Altman. 1995. Regulation of the p70^{ZAP} tyrosine protein kinase in T cells by the CD45 phosphotyrosine phosphatase. *Eur. J. Immunol.* 25:942–946.