CD45-associated protein promotes the response of primary CD4 T cells to low-potency T-cell receptor (TCR) stimulation and facilitates CD45 association with CD3/TCR and Lck

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Summary

Although it is clear that the CD45 tyrosine phosphatase is required for efficient T-cell activation and T-cell development, the factors that regulate CD45 function remain uncertain. Previous data have indicated that there is an association of CD45 with CD4 and the T-cell receptor (TCR) complex controlled by the variable ectodomain of CD45 and, following activation, by high- and low-potency peptides. This suggests that controlling substrate access to CD45 may be an important regulatory mechanism during T-cell activation. In the present study we have examined the role of the transmembrane adapter-like molecule CD45-associated protein (CD45-AP) in regulating the association of CD45 with CD3/TCR and lck, and in regulating primary $CD4^+$ T-lymphocyte activation. In $CD4^+$ T cells from CD45-AP-deficient mice, coimmunoprecipitation of CD45 with the CD3/TCR complex, in addition to lck, is significantly reduced compared with wild-type T cells. Functionally, this correlates with a decreased proliferative response, a decrease in interleukin (IL)-2 production, and a decrease in calcium flux upon stimulation with a low-potency altered peptide ligand. However, the response of CD45-AP-deficient T cells to stimulation with a high-avidity agonist peptide was largely intact, except for a modest decrease in IL-2 production. These data suggest that CD45-AP promotes or stabilizes the association of CD45 with substrates and regulates the threshold of T-cell activation.

Keywords: T lymphocyte; phosphatase; kinase; CD45

Introduction

CD45 is the predominant transmembrane protein tyrosine phosphatase in T lymphocytes and plays a critical role in regulating T-lymphocyte activation.[1,2] Studies in CD45-deficient T-cell lines as well as in genetically deficient mice and humans have demonstrated that CD45 expression is required for efficient T-cell receptor (TCR) signal transduction and subsequent growth and development. The positive role of CD45 in T-cell activation has largely been attributed to the role of CD45 in promoting src family kinase activity by dephosphorylating the negative regulatory carboxy-terminal tyrosine, thus maintaining an 'open' active conformation. However, despite the importance of CD45 in T-cell activation, the factors that regulate CD45–substrate interactions before and after T-cell activation are not well understood

We have hypothesized that CD45 function is modulated by altering substrate access to the tyrosine phosphatase domains of CD45 via specific interactions with other integral membrane proteins and/or membrane-associated signalling intermediates. We, and others, have previously shown by fluorescent colocalization and co-immunoprecipitation experiments that CD45 associates with CD4 and the CD3/TCR complex.[3–7] In addition, the basal association of CD45 with CD4/Lck and the CD3/TCR complex is regulated in part by the size of the heavily glycosylated variable CD45 ectodomain[4,6,7] and is dynamically regulated following activation by high- and low-potency peptides.[6] Importantly, changes in the association of CD45 with these signalling complexes correlate with changes in the sensitivity of the TCR to low-potency stimulation,[4,7–10] supporting the idea that the regulation of the association of CD45 with substrates is an important factor in regulating T-cell activation. However, the precise nature of this interaction and whether it is mediated by other molecular intermediates remain uncertain.

CD45-associated protein [CD45-AP, or lymphocyte-associated phosphoprotein (LPAP)] is a 32-kDa transmembrane protein expressed primarily in lymphoid lineage cells.[11,12] CD45-AP has a short extracellular domain with a relatively long cytoplasmic tail that contains a region with homology to WW domains and an acidic region that has been shown to mediate association with Lck.[13] CD45-AP directly associates with CD45 via their respective transmembrane domains[14,15] and is thought to serve as an adaptor molecule that regulates the association of CD45 with appropriate substrates. Indeed, previous reports have found that CD45-AP can interact directly with Lck and stabilizes the association of CD45 with Lck.[13,15–17]

The physiologic role of CD45-AP in regulating T-cell activation is controversial. An initial report describing the function of T cells from CD45-AP-deficient mice indicated a defect in T-cell proliferation following anti-TCR stimulation, leading to the conclusion that CD45-AP was required for optimal activation of mature T cells.[17] Subsequently, two additional independent reports failed to detect defects in CD45-AP-deficient T-cell proliferation following stimulation with anti-CD3 or anti-TCR, suggesting that CD45-AP was not essential for T-cell activation.[18,19] The reason(s) for the differences in these data is not clear, but possibilities include differences in the technical factors used in gene targeting, strain differences, and/or differences in the assays used to assess T-cell activation. Specific analysis of CD4 T-cell responses to physiological peptide stimulation has not been described.

In order to specifically assess the role of CD45-AP in regulating CD4 T-cell activation, we utilized CD45-AP-deficient mice crossed with and TCR transgenic mice specific for moth cytochrome c (MCC) peptide. Using this system, we observed a defect in T-cell proliferation, interleukin (IL)-2 production and calcium mobilization upon stimulation with a low-potency partial agonist peptide. In contrast, stimulation with a high-potency agonist peptide for this TCR was largely unaffected by the absence of CD45-AP, except for a modest decrease in IL-2 production. In total, these data suggest that CD45-AP enhances the sensitivity of the TCR to low-potency stimulation. These defects in $CD4^+$ T-cell activation correlate with decreased association of CD45 with the CD3/TCR complex and Lck. Our findings suggest that CD45-AP plays an important role in regulating CD45–substrate interactions and promotes efficient T-cell activation.

Materials and methods

Mice

The AND TCR transgenic mice in which $CD4^+$ T cells express a TCR specific for the carboxy terminus of moth cytochrome c have been previously described[20] and are maintained in our animal facility as heterozygotes on a C57Bl/6 background. The CD45-AP-deficient mice have been described previously[17] and were backcrossed onto a CD57Bl/6 background and intercrossed with and transgenic mice. B10.A(5R) mice were obtained from The Jackson Laboratory (Bar Harbor, ME).

Peptides

Moth cytochrome c (81–103) pMCC = VFAGLKKANER ADLIAYLKQATK and K99R = VFAGLKKANERADLIAY LRQATK peptides were synthesized by the W. M. Keck Foundation Biotechnology Resource Laboratory (New Haven, CT). All peptides were purified by high-pressure liquid chromatography prior to use.

Preparation of antigen-presenting cells (APCs) and $CD4^+$ T cells

T-cell-depleted APCs were prepared by antibody-mediated complement lysis of B10.A(5R) splenocytes treated with mitomycin C as previously described.[21] $CD4^+$ $CD8^-$ T cells from lymph nodes and spleens were isolated by immunomagnetic negative selection using antibodies against CD8, CD32/CD16, B220, NK1·1 and major histocompatibility complex (MHC) class II followed by incubation with anti-mouse and anti-rat immunoglobulin-coated magnetic beads (Perspective Diagnostics, Cambridge, MA) as previously described.[21] The purity of the recovered $V\alpha11^+$ $CD4^+$ T cells was usually 85–95% as determined by staining with anti-CD4 and anti-Vα11 monoclonal antibody (mAb).

T-cell proliferation and activation

Briefly, 10^5 purified T cells were cultured in triplicate in 96-well flat-bottom microtitre plates and stimulated with the indicated concentrations of plate-bound anti-CD3 or with different doses of peptide in the presence of 10^5 mitomycin C-treated APCs. After 3 days the cells were pulsed with 1 μCi/well of [^{3}H]thymidine and harvested 18–24 hr later, and the level of incorporation was determined by scintillation counting. Anti-CD3 coated microtitre plates were prepared by incubating with the indicated concentrations of anti-CD3 (2C11), diluted in phosphate-buffered saline (PBS), for 4 hr at 37° and then washed three times before purified $CD4^+$ T cells were added.

For analysis of biochemical signalling following peptide stimulation, T-cell-depleted splenocytes from B10.A(5R) mice were pulsed with varying doses of the indicated peptide for 2–4 hr at 37° in Hank's balanced salt solution supplemented with 5% fetal calf serum (FCS). The pulsed APCs were washed once and mixed 1 : 1 with purified MCC-specific T cells in microcentrifuge tubes, quickly

pelleted, incubated at 37° for the indicated times, and then lysed in ice-cold 1% NP-40 lysis buffer [20 mM Tris-Cl, pH 7·5, 150 mM NaCl and 5 mM ethylenediaminetetraacetic acid (EDTA)] supplemented with protease and phosphatase inhibitors [10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulphonyl fluoride (PMSF) and 1 mM Na_3VO_4]. Stimulation of CD4 T cells following anti-CD3 and/or anti-CD28 stimulation and analysis of phosphotyrosine indcution were performed as previously described.[22] Phosphorylated and total extracellular signal-regulated kinase (ERK), cellular homologue of viral oncogene v-akt (Akt) and phospholipase C gamma-1 (PLCγ-1) were detected by western blot using specific antibodies (Cell Signaling Technology, Beverly, MA).

Intracellular cytokine staining

Intracellular analysis for IL-2 production was performed following 36 hr of stimulation with peptide and the addition of monensin (Golgistop; BD Biosciences, San Diego, CA) for the final 6 hr of culture. Cells were harvested and surface-labelled with anti-CD4 prior to fixation and permeabilization (Fix and Perm; Caltag, Burlingame, CA), and labelling with antibody to IL-2 (BD Biosciences).

Calcium mobilization

Calcium signalling following antigen-specific stimulation was monitored as described previously.[22] Briefly, $CD4^+$ T cells loaded with 5 μM fluo-3/AM ester (Molecular Probes, Eugene, OR) were plated by centrifugation in 96-well plates at a concentration of 5×10^5 cells/50 μl. The cells were then scanned using the ACAS 570 video laser cytometer (Meridian Instruments, Okemos, IL). After initiation of scanning, 4×10^6 T-depleted splenocytes (APCs) pulsed with 20 μM of peptide were added to the $CD4^+$ T cells. The initial average fluorescence of each cell was digitized and normalized to 1, and the results were expressed as changes in normalized fluorescence intensity of individual cells over time. The percentage of responding cells was determined by dividing the number of cells demonstrating an increase in intracellular calcium of more than 50% by the total number of scanned cells.

Immunoprecipitation and western blotting

Co-immunoprecipitation of CD45 with CD3/TCR was performed using a 1% Brij 97 (Sigma, St Louis, MO) lysis buffer as previously described.[6] Western blot analysis was carried out following sodium dodecyl sulphate (SDS) polyacrylamide electrophoresis and transfer onto nitrocellulose paper (Schleicher and Schuell, Keene, NH). CD45 was detected using a biotinylated anti-CD45 antibody (30-F11), which is pan-specific for all isoforms, followed by avidin-coupled horseradish peroxidase. In some experiments, cells were surface-biotinylated prior to lysis and immunoprecipitation. In this case, surface-biotinylated CD45 was detected by western blot using avidin-coupled horseradish peroxidase. The zeta chain of the TCR, lck, CD45-AP and Coronin 1B were detected using polyclonal antisera[17,23] (Coronin 1B antibody was purchased from Santa Cruz Biotechnology, Santa Cruz, CA) followed by protein A coupled horseradish peroxidase (Sigma-Aldrich, St Louis, MO) or goat anti-immunoglobulin coupled horseradish peroxidase. All of the immunoblots were developed with the ECL chemiluminescent detection system (Amersham, Piscataway, NJ).

Results

CD45-AP expression is required for optimal proliferation in response to low doses of anti-CD3 in conjunction with anti-CD28

The generation and initial characterization of the CD45-AP-deficient mice used in this study have been previously described.[17] In the present study, we utilized CD45-AP-deficient mice backcrossed 7–9 times onto the C57/Bl6 background. Western blot analysis confirms the absence of CD45-AP, while the expression levels of TCR-ζ and Coronin 1B (previously known as Coronin 2), encoded by an adjacent gene to that encoding CD45-AP, are equivalent (Fig. 1a) in wild-type and CD45-AP-deficient cells.[24] Matsuda *et al*. have previously described a defect in splenic T-cell proliferation following stimulation *in vitro* with anti-TCR antibodies,[17] while another, independent group reported an increase in lymphocyte cellularity in CD45-AP-deficient older animals.[19] These data suggest a role for CD45-AP in regulating lymphocyte responsiveness that may also influence lymphoid homeostasis. However, the role of CD45-AP in regulating T-cell activation remains controversial. In addition to the mice developed by Matsuda *et al.*, two other studies describing independently derived CD45-AP-deficient mice did not report any alterations in T-lymphocyte activation *in vitro*.[18,19]

It is unclear why there is a disparity in the literature regarding the role of CD45-AP in regulating lymphocyte responsiveness, but it may be a result of differences in the assay systems used and/or in the cell populations analysed. In particular, alterations in the efficiency of TCR signalling may not be evident unless the strength of signals used to stimulate the TCRs are altered experimentally. In addition, many of the previous reports on CD45-AP-deficient T cells have not specifically examined the response of $CD4^+$ T cells. As CD4 has been reported to more closely associate with CD45 and with Lck, it is likely that CD45-AP plays a more important role in regulating CD4 T-cell responses than CD8 T cells. Thus, analysis of total T cells may underestimate the role of CD45-AP in regulating CD4 T-cell activation.

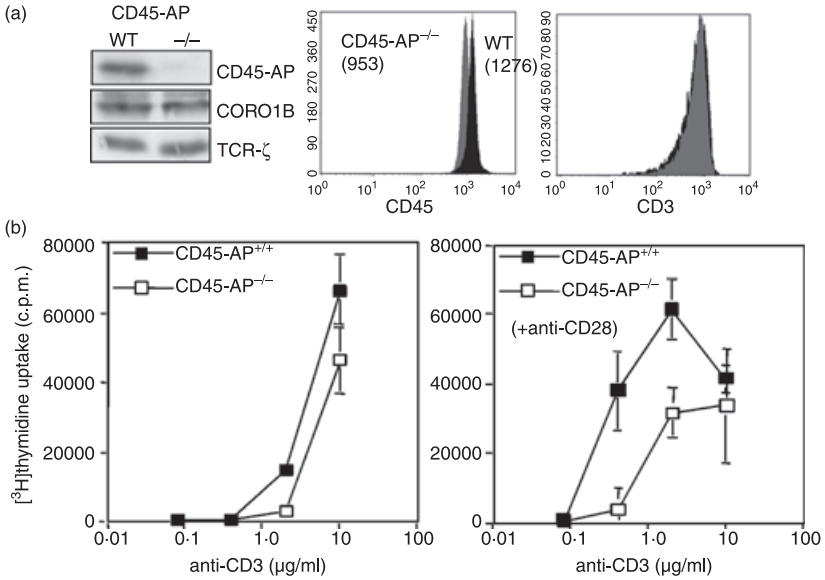

Figure 1. CD45-associated protein (CD45-AP) promotes T-cell proliferation at suboptimal levels of anti-CD3 stimulation. (a) Characterization of CD45-AP-deficient cells. Western blot analysis of CD45-AP, coronin 1B (CORO1B) and T-cell receptor (TCR)-ζ protein expression in cell lysates and flow cytometric analysis of CD45 and CD3 expression from wild-type (filled histograms) and CD45-AP-deficient (shaded histograms) animals is presented. The mean fluorescence intensity of CD45 expression is indicated for wild-type and CD45-AP-deficient cells adjacent to the corresponding histogram. (b) Purified $CD4^+$ T cells from wild-type or CD45-AP-deficient mice were stimulated with different concentrations of immobilized anti-CD3 alone (left panel), or in conjunction with anti-CD28 (2·5 μg/ml) (right panel). Proliferation was assessed by pulsing with 1 μCi [^{3}H]thymidine for 16 hr after 3 days in culture. The data are representative of three independent experiments. c.p.m., counts per minute; WT, wild type.

In order to specifically investigate the role of CD45-AP in regulating $CD4^+$ T-cell activation, purified $CD4^+$ T cells were isolated from wild-type and CD45-AP-deficient mice and stimulated with different concentrations of plate-bound anti-CD3 antibodies. As shown in Fig. 1(b), the absence of CD45-AP caused a small reduction of $CD4^+$ T-cell proliferation when cells were stimulated with anti-CD3 antibody alone. However, when CD45-AP-deficient T cells were stimulated with lower doses of anti-CD3 in the presence of a fixed dose of anti-CD28 (required for optimal stimulation of naïve T cells), there was a more prominent defect in stimulation compared with control T cells (Fig. 1b). Importantly, CD3/TCR and CD28 expression levels were identical in wild-type and CD45-AP-deficient cells, consistent with previous reports[17–19] (Fig. 1a; data not shown). However, as previously reported, CD45 expression was reduced by approximately 25% in the CD45-AP-deficient mice (Fig. 1a).[17,19] As the level of CD45 expression may affect T-cell responsiveness, we compared the response of CD4 T cells from wild-type ($CD45^{+/+}$) mice with that of cells from $CD45^{+/-}$ littermates, which have a greater decrease in surface CD45 expression than the CD45-AP-deficient cells. Although the $CD45^+$ cells expressed approximately 40% less CD45 than wild-type controls, the proliferative response to low doses of anti-CD3 and anti-CD28 was not significantly affected (data not shown). Thus it is unlikely that the small decrease in cell surface expression of CD45 can fully explain the proliferative defect observed in the absence of CD45-AP.

The decrease in proliferation of the CD45-AP-deficient CD4 T cells observed in Fig. 1(b) could be a result of a specific defect in CD28 signalling or of diminished sensitivity to stimulation with low levels of anti-CD3. In order to detect any differences in early biochemical TCR-dependent and/or CD28-mediated signalling events, wild-type and CD45-AP-deficient cells were stimulated with anti-CD3 and/or anti-CD28 and analysed for induction of total tyrosine phosphorylation. The cells were also stimulated with a combination of anti-CD3 and anti-CD4 to induce a maximal response. As shown in Fig. 2(a), no significant differences in the overall pattern of tyrosine phosphorylation were detected in the CD45-AP-deficient cells following stimulation with anti-CD3 alone or anti-CD3 in combination with anti-CD28 or anti-CD4. In specific analyses of Akt phosphorylation, which is enhanced upon anti-CD28 stimulation, and ERK phosphorylation, no differences were found between wild-type and CD45-AP-deficient cells following anti-CD3 and anti-CD28 stimulation (Fig. 2b).

Role of CD45-AP in regulating antigen-specific CD4 T-cell stimulation

In order to further investigate the role of CD45-AP in regulating CD4 T-cell activation in response to more

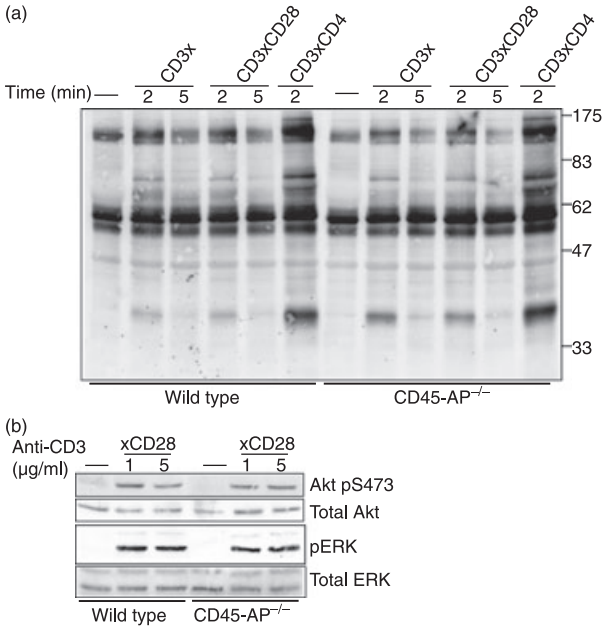

Figure 2. Analysis of tyrosine phosphorylation and cellular homologue of viral oncogene v-akt (Akt) activation following stimulation with anti-CD3 and/or anti-CD28. (a) NP-40 soluble cell lysates of purified CD4 T cells (1×10^6) from CD45-associated protein (CD45-AP)-deficient or wild-type control mice were analysed for total tyrosine phosphorylation (pY) by western blot following stimulation with either anti-CD3 (5 μg/ml) with or without anti-CD28 (2 μg/ml) or CD4 (1 μg/ml) for the indicated period of time. Cells were preincubated on ice for 10 min with the indicated primary antibodies and then goat anti-hamster antibody was added for cross-linking for the indicated times. (b) Cells were stimulated for 2 min as described in (a) (except that anti-CD3 was used at 1 or 5 μg/ml, as indicated) and analysed for Akt and extracellular signal-regulated kinase (ERK) phosphorylation using phosphospecific antibodies. The blot was then stripped and probed for total Akt and ERK. The data shown are representative of two independent experiments.

physiological ligands, the CD45-AP-deficient mice were backcrossed with the AND TCR transgenic mouse specific for peptides of moth cytochrome c presented by MHC class II IE. We previously identified and characterized an altered peptide ligand specific for this TCR that functions as a partial agonist.[23] Thus, we used this system to stimulate purified $CD4^+$ T cells from CD45-AP-deficient mice with different doses of agonist peptide (pMCC), or with an altered peptide ligand (K99R) with a single amino acid substitution that affects TCR recognition and poorly stimulates these T cells. Somewhat surprisingly, we found that there was no reproducible difference in stimulation of CD45-AP-deficient cells compared to control wild type cells at any of the tested doses of the agonist peptide (Fig. 3a). In contrast, there was a marked decrease in the stimulation of CD45-AP-deficient cells upon stimulation with the low-avidity peptide K99R (Fig. 3b). The addition of exogenous IL-2 to the K99R-stimulated cells partially restored the proliferative defect seen with the CD45-AP-deficient T cells (Fig. 3c). These data suggest that there may be a decrease in IL-2 production upon low-potency peptide stimulation that may contribute to the poor proliferative response.

In order to directly examine IL-2 production by CD45-AP-deficient T cells after agonist and partial agonist peptide stimulation, the cells were stimulated and assessed for the presence of intracellular IL-2 (Fig. 4a). Similar to the proliferation data, there was a significant decrease in IL-2 production upon stimulation with the partial agonist peptide K99R in the CD45-AP-deficient cells compared with wild-type controls. In addition, there was a more modest, but consistent decrease in IL-2 production upon stimulation with the agonist peptide in the absence of CD45-AP. In addition to IL-2 production, we also assessed induction of the IL-2 receptor as defined by CD25 expression. Again, following peptide stimulation there was a modest decrease in the induction of CD25 expression in the CD45-AP-deficient cells (Fig. 4b). For both IL-2 production and CD25 expression, the defects were more pronounced upon stimulation with lower doses of peptide and/or stimulation with the partial agonist peptide. These data suggest that CD45-AP is required

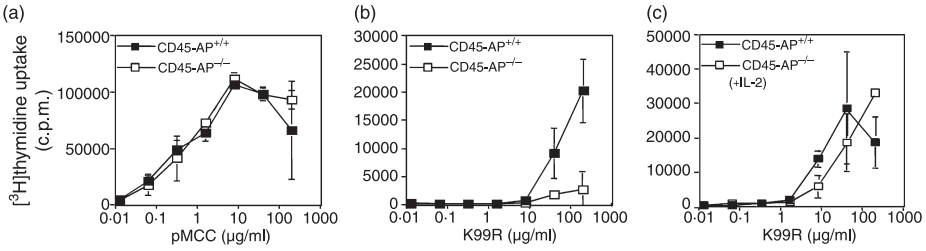

Figure 3. CD45-associated protein (CD45-AP) is required for proliferation in response to a low-avidity peptide ligand but not to an agonist peptide. Purified $CD4^+$ T cells from wild-type or CD45-AP-deficient AND T-cell receptor transgenic mice were stimulated with different doses of agonist peptide (pMCC) (a), or partial agonist peptide (K99R) (b, c). In (c), 10 U/m of exogenous interleukin (IL)-2 was added at all antigen doses. Antigen stimulation was performed in the presence of B10.A(5R) mitomycin c-treated, T-cell-depleted splenocytes as antigen-presenting cells. Proliferation was assessed by pulsing with [^{3}H]thymidine for 16 hr after 3 days in culture. The data are representative of three independent experiments. c.p.m., counts per minute.

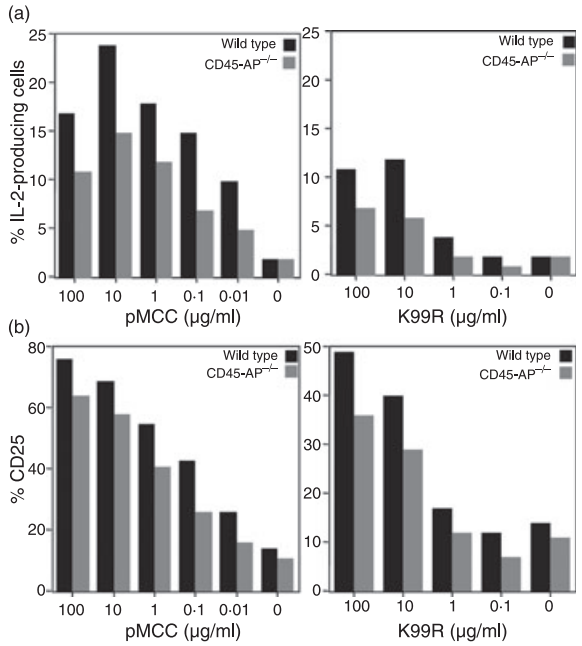

Figure 4. CD45-associated protein (CD45-AP) is required for optimal interleukin (IL)-2 production following peptide stimulation. (a) Purified $CD4^+$ T cells from wild-type or CD45-AP-deficient and T-cell receptor transgenic mice were stimulated with agonist (pMCC) or partial agonist (K99R) peptide as in Fig. 3. After approximately 36 hr of stimulation, the cells were harvested and examined for IL-2 production by flow cytometry. Analysis was performed by gating on $CD4^+$ T cells, and the percentage of CD4 T cells producing IL-2 is displayed. (b) Cells were stimulated as in (a), and then analysed for CD25 expression after approximately 16 hr of stimulation. The data are representative of two independent experiments.

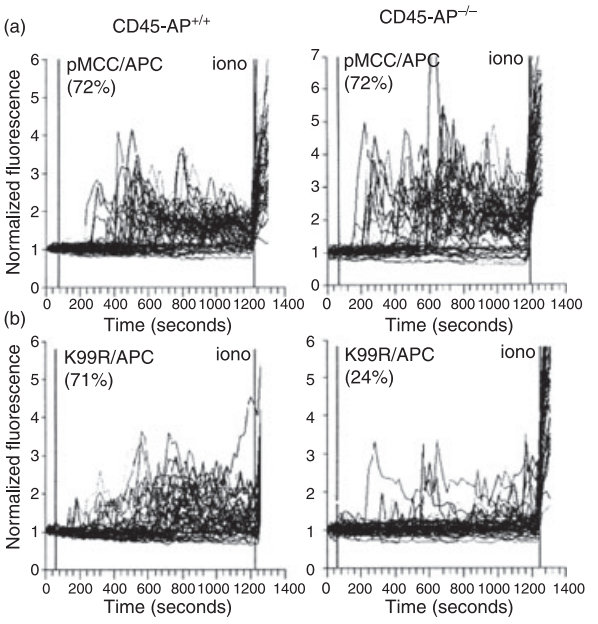

Figure 5. Decrease in calcium mobilization in response to partial agonist peptide ligand in CD45-associated protein (CD45-AP)-deficient T cells. Purified $CD4^+$ T cells from CD45-AP-deficient and wild-type and T-cell receptor transgenic animals were loaded with the calcium-sensitive fluorochrome Fluo-3, and stimulated with B10.A(5R) antigen-presenting cells (APCs) pulsed with 100 μg/ml of either agonist peptide (pMCC) or a partial agonist peptide ligand (K99R). Changes in the fluorescence intensity of individual cells were detected using a video laser cytometer. Each graph indicates the pattern of calcium mobilization in a field of 40–50 cells, where each line represents the average fluorescence intensity of an individual T cell over time. The per cent responding cells is indicated in parentheses for each group. The initial vertical line indicates the addition of APCs pulsed with peptide, and the second line indicates the addition of ionomycin (666 ng/ml). The data are representative of two independent experiments.

for optimal IL-2 production following physiological stimulation with peptide ligands.

CD45-AP regulation of early TCR-dependent signalling events following peptide stimulation

In order to characterize the role of CD45-AP in regulating early TCR signalling events following physiological peptide stimulation, purified CD4 T cells from wild-type and CD45-AP-deficient mice were stimulated with APCs pulsed with either the high-potency agonist peptide (pMCC) or the low-potency partial agonist peptide (K99R). Similarly to the proliferation data, there was a significant decrease in the percentage of cells undergoing an increase in calcium mobilization upon stimulation with the partial agonist peptide (Fig. 5b), while calcium mobilization in response to the agonist peptide (pMCC) was not significantly affected by the absence of CD45-AP (Fig. 5a).

As activation of PLC-γ is closely linked to increases in calcium mobilization following TCR ligation, we also examined PLC-γ tyrosine phosphorylation following peptide stimulation in wild-type and CD45-AP-deficient T cells. Similarly to the calcium data, we found no reproducible difference in PLC-γ phosphorylation upon comparison of wild-type or CD45-AP-deficient T cells following stimulation with different doses of agonist peptide as defined by densitometric analysis (Fig. 6a). There was also no difference in total tyrosine phosphorylation, zeta-associated protein (ZAP)-70 phosphorylation or linker of activated T cells (LAT) tyrosine phosphorylation between wild-type and CD45-AP-deficient T cells following stimulation with different doses of agonist peptide (data not shown). Upon stimulation with the partial agonist peptide, K99R, there was low but detectable induction of PLC-γ tyrosine phosphorylation in both wild-type and CD45-AP-deficient cells compared with unstimulated cells. As the level of induction was relatively low, differences between the wild-type and CD45-AP-deficient cells as defined by densitometry are imprecise. Nevertheless,

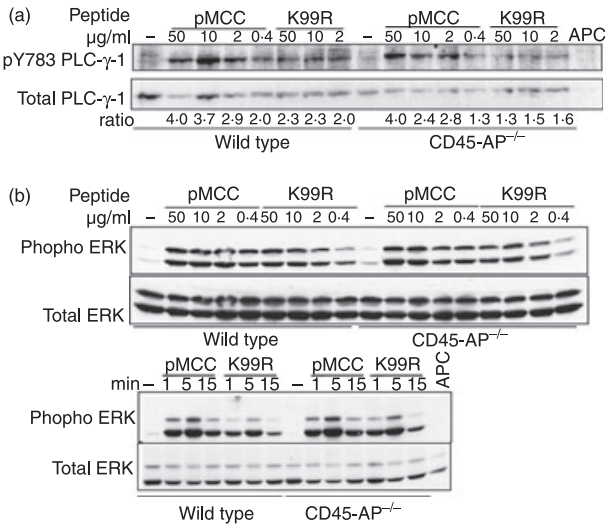

Figure 6. Regulation of phospholipase C gamma-1 (PLCγ-1) and extracellular signal-regulated kinase (ERK)-1/2 phosphorylation by CD45-associated protein (CD45-AP). Purified $CD4^+$ T cells from CD45-AP-deficient and wild-type and T-cell receptor transgenic animals were stimulated with B10.A(5R) antigen-presenting cells (APCs) pulsed with the indicated doses of either agonist peptide (pMCC) or partial agonist peptide (K99R), or with APCs with no specific peptide added (–), and cell extracts were analysed by western blot for pY-783 PLC-γ-1 (a) or phospho ERK-1/2 (b). To ensure equal loading, the same blot was stripped and re-probed for total PLC-γ or total ERK. In (a), densitometric values for increases in PLC-γ phosphorylation compared with unstimulated cells are indicated. All values are normalized for total PLC-γ protein. The lane labelled 'APC' contains cell lysates from APCs only. The data are representative of three independent experiments.

densitometric analysis revealed a small decrease in PLC-γ phosphorylation in CD45-AP-deficient T cells compared with wild-type T cells upon stimulation with the partial agonist peptide (Fig. 6a).

The partial agonist peptide used in these studies does not induce detectable ZAP-70 or LAT phosphorylation;[23] however, ERK phosphorylation is readily detectable, as has been described in other situations of low-potency TCR stimulation.[25,26] Therefore, we next examined the requirement for CD45-AP for ERK activation following partial agonist peptide stimulation (Fig. 6b). In contrast to the calcium mobilization data, phosphorylation of ERK1/2 was similar in wild-type and CD45-AP-deficient cells upon stimulation with different doses of either the partial agonist peptide K99R or the agonist peptide pMCC (Fig. 6b). As the duration of ERK activation is a critical parameter in regulating cell activation and differentiation, we also examined the kinetics of ERK phophorylation in wild-type and CD45-AP-deficient cells. As shown in Fig. 6(b), we were unable to detect any requirement for CD45-AP for ERK activation with either high- or low-potency peptides regardless of dose or time-point examined.

In total, the defects in proliferation and calcium mobilization found in the CD45-AP-deficient T cells suggest that CD45-AP is required for efficient T-cell activation primarily in situations of weak TCR stimulation.

CD45-AP promotes CD45–TCR and CD45–lck association

We previously correlated the ability of CD45 to associate with CD4 and the CD3/TCR complex with increased sensitivity of the TCR to low-potency stimuli.[6,10] These data suggest that the maintenance of a basal complex of CD45 with the TCR and/or CD4/lck serves to prime the TCR to respond optimally to ligand recognition.[27] As CD45-AP also seems to promote TCR sensitivity, we examined the role of CD45-AP in mediating CD45–TCR association in co-immunoprecipitation experiments. In these experiments, primary CD4 T cells were surface-biotinylated and CD45 or the TCR was immunoprecipitated. As shown in Fig. 7(a), CD45-AP-deficient T cells have a decreased association of surface-labelled CD45 with the TCR complex following CD45 or TCR-β immunoprecipitation. The decrease in the association of biotinylated CD45 with TCR-ζ upon immunoprecipitation with anti-TCR-β suggests that decreased CD45–TCR association in the CD45-AP-deficient cells represents a decrease in surface membrane association. The identification of these bands as CD45 was confirmed using CD45-deficient cell lines. Furthermore, similar decreases in CD45 co-immunoprecipitated with the TCR were also seen in CD45-AP-deficient cells upon direct western blot analysis for CD45 (data not shown). In total, these data suggest that the CD45–TCR association is in part indirect and is mediated or stabilized by CD45-AP.

In addition to a basal association of CD45 with the TCR, we previously found that the association of CD45 with the CD3/TCR complex is differentially regulated following stimulation with agonist and partial agonist peptides. The CD45–TCR association is sustained or stabilized following stimulation with a strong agonist peptide, but the association is destabilized following stimulation with a partial agonist peptide.[6] In order to determine if stimulation with peptide would promote CD45–TCR association in the CD45-AP-deficient T cells, similar experiments were carried out in the current study. As shown in Figs 7(b) and (c), CD45–TCR association was diminished by approximately 50% in the CD45-AP-deficient cells both before and after stimulation with the agonist peptide.

In addition to promoting the association of CD45 with the CD3/TCR complex, previous studies in tumour cell lines, or heterologous transfection systems, have indicated that CD45-AP may play a role in promoting or stabilizing

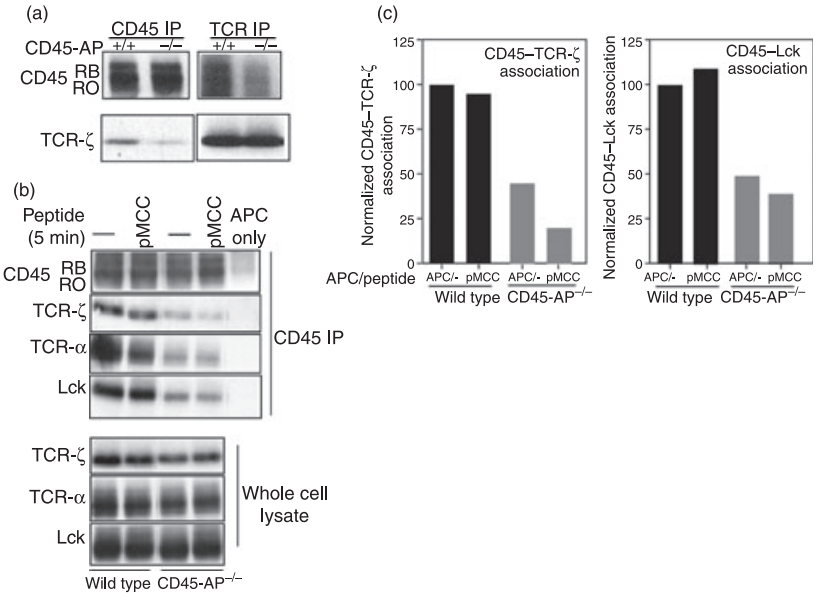

Figure 7. CD45-associated protein (CD45-AP) promotes association of CD45 with the T-cell receptor (TCR) complex and with Lck. (a) Purified $CD4^+$ T cells were isolated from wild-type or CD45-AP-deficient mice and cells were surface-biotinylated and immunoprecipitated with either anti-CD45 (30-F11) or anti-TCR-β (H57). Immunoprecipitated proteins were separated using 6·5–16% gradient sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and then blotted for either biotinylated CD45 using avidin-coupled horseradish peroxidase or TCR-ζ using specific rabbit antiserum. (b) Purified wild-type or CD45-AP-deficient and transgenic T cells were stimulated with the agonist peptide pMCC, as in Fig. 6, for 5 min, then lysed and immunoprecipitated with anti-CD45. Association of TCR-ζ, TCR-α and lck with CD45 was then assessed by western blot. Prior to immunoprecipitation, a portion of the whole cell lysate was kept aside to control for total levels of TCR-ζ, TCR-α and lck as indicated in the bottom panels. (c) Densitometric analysis of the amount of TCR-ζ or lck co-immunoprecipitated with CD45 in (b) is indicated. Data are corrected for the amount of CD45 that was immunoprecipitated under each condition and then displayed normalized to the basal level of association upon stimulation with antigen-presenting cells with no exogenous specific peptide. The data are representative of more than three independent experiments. APC, antigen-presenting cell; IP, immunoprecipitation.

CD45–Lck association.[13,15,16,28] However, experiments examining CD45–Lck association in primary cells from CD45-AP-deficient mice have yielded contradictory results. In the studies by Matsuda *et al.* there was an approximately 50% loss in CD45–Lck association.[17] In contrast, subsequent reports failed to find a significant defect in CD45–Lck association in CD45-AP-deficient thymocytes.[18,19]

Importantly, the Ding *et al.*[19] and Kung *et al.*[18] papers utilized thymocytes to evaluate CD45–Lck association, while Matsuda *et al.*[17] utilized total splenic T cells. As there is evidence that thymocytes and mature T cells differ in the organization of TCR-associated signalling pathways, this may explain the discrepant results.[27] In addition, as Lck may be distributed differently in CD8 and CD4 T cells, it is important to evaluate the role of CD45-AP in purified T-cell subsets. Thus, in order to address the role of CD45-AP in regulating CD45–Lck association in purified primary CD4 T cells, before and after T-cell stimulation, we assessed CD45–Lck association in the same experiment, shown in Fig. 7(b). Similarly to the association with TCR-α and TCR-ζ, the CD45–Lck association is diminished by approximately 50% in unstimulated cells as well as 5 min after peptide stimulation. Thus, our data are consistent with a role for CD45-AP in regulating the association of both the TCR and Lck with CD45 before and after peptide stimulation in primary CD4 T cells.

Discussion

In the current report we have examined the role of CD45-AP in regulating CD4 T-cell activation. CD45-AP directly interacts with CD45 and is postulated to play a role in mediating CD45–substrate interaction.[11,12] However, the physiological role of CD45-AP during $CD4^+$ T-cell activation has remained unclear. Previous studies utilizing CD45-AP-deficient mice have reported apparently contradictory results regarding the role of CD45-AP in regulating primary T-lymphocyte activation. These differences may be caused by differences in gene targeting strategies, but are also probably caused by differences in the cell populations analysed and/or the assay systems used to evaluate T-cell activation. In particular, detailed analysis of CD4 T-cell responses to peptide stimulation have not been previously reported. In the present study, we focused on the role of CD45-AP using TCR transgenic CD4 T cells isolated from CD45-AP-deficient mice, and assessed T-cell activation following physiological stimulation with either agonist or partial agonist peptide ligand.

Using this system, we found that CD4 T cells from CD45-AP-deficient mice had a decreased sensitivity to low-potency stimulation. In the absence of CD45-AP there was a decrease in proliferation following stimulation with low doses of anti-CD3 in conjunction with anti-CD28 (Fig. 1). We noted that decreased proliferation was not as evident upon stimulation with anti-CD3 in the absence of anti-CD28. This was probably a result of the increased doses of anti-CD3 required to generate a response in the absence of costimulatory signals even in wild-type mice, and thus comparison of the response to low-potency stimulation with anti-TCR was not possible. Alternatively, it is possible that CD45-AP is particularly important for regulation of CD4 T-cell responses only in conjunction with costimulatory signals and/or is important for CD28-specific signal transduction. However, no differences in Akt activation, which is promoted by CD28-dependent signals, were detected, suggesting that there was a defect in responsiveness to low doses of anti-CD3.

Consistent with the hypothesis that CD45-AP is required to enhance sensitivity to low-potency signals to the TCR, CD45-AP-deficient TCR transgenic cells also exhibited a decrease in proliferation and IL-2 production following stimulation with a partial agonist peptide. In contrast, proliferation in response to a strong agonist peptide was not significantly affected in the CD45-AP-deficient T cells, although a partial defect in IL-2 production was evident in intracellular cytokine analysis. It is unclear why we did not observe a decrease in proliferation upon stimulation with low doses of the agonist peptide pMCC, despite a decrease in IL-2 production. This may have been because the amount of IL-2 produced by individual cells in response to agonist peptide stimulation is in excess of the amount required for optimal proliferation, and thus the modest decrease in IL-2 production seen in the absence of CD45-AP does not have an impact on the proliferation assay. In contrast, the amount of IL-2 produced in response to partial agonist peptide stimulation may be more limiting, and thus decreased production may have a greater impact. In addition, it is possible that other factors also contribute to the defect in proliferation seen in the CD45-AP-deficient cells upon partial agonist peptide stimulation independently of IL-2 production.

In parallel with the role of CD45-AP in regulating proliferation and IL-2 production, the CD45-AP-deficient T cells were also less efficiently stimulated to undergo an increase in intracellular calcium in response to low-avidity peptides, while the response to high-avidity peptides was intact (Fig. 5). In total, these data suggest that CD45-AP plays a role in tuning TCR signalling thresholds.

Previously published studies found that the association of CD45 with CD4 and/or the CD3/TCR complex correlated with enhanced T-cell activation.[4,7,10] Our current data support a role for CD45-AP in facilitating basal CD45 interaction with lck and the CD3/TCR complex (Fig. 7). In co-immunoprecipation experiments there was a significant decrease in the constitutive association of CD45 with Lck and the CD3 complex in unstimulated $CD4^+$ T cells. Thus, one mechanism by which CD45-AP may promote T-cell activation is facilitation of interactions of CD45 with substrates such as Lck prior to TCR signalling. This may help maintain a pool of relatively active Lck and enhance the efficiency of generating and/or sustaining TCR-mediated signal transduction events.

Although this is the first report indicating that CD45-AP plays a role in regulating CD45 association with the TCR, there are conflicting reports about the role of CD45-AP in regulating CD45–lck association in co-immunoprecipitation experiments in primary T cells.[17–19] As mentioned previously, one explanation for the contrast in results is that there may be differences in the role of CD45-AP in regulating CD45–lck association in thymocytes and in mature T cells. Another possible explanation for the differences in results is that CD45–lck association is thought to be particularly enriched within lipid raft membrane microdomains.[29] The use of different detergents and/or differences in immunoprecipitation procedures may alter the accessibility or stability of CD45–lck complexes to immunoprecipitation in the different studies.

In addition to regulating CD45–substrate interactions, CD45-AP may also play a role in directly regulating CD45 phosphatase activity. CD45-AP has recently been shown to inhibit CD45 dimerization and to promote CD45 phosphatase activity when transfected into a CD45-AP-deficient T-cell lymphoma.[30] It is not yet known how CD45 dimerization affects access to substrates. Our current data support a model in which inhibition of CD45 dimerization by CD45-AP may facilitate enhanced interaction of CD45 monomers with Lck and/or the CD3/TCR complex. In order to fully characterize the regulation of CD45 activity, it will be important to assess the stability of CD45 homodimers and dynamic regulation of CD45 phosphatase activity in different membrane compartments and in different CD45-containing signalling complexes before and during T-cell activation.

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