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CD45 modulates T cell receptor/CD3-induced activation of human thymocytes via regulation of tyrosine phosphorylation*

Stimulation of thymocytes or mature T cells via the T cell receptor (TcR)/CD3 complex activates a cascade of processes inducing cells to enter the cell cycle. A key step is the activation of phosphatidylinositol-specific phospholipase C (PI-PLC) within seconds following TcR/CD3 stimulation, an event which is strongly enhanced by co-ligation of the CD4 (or CD8) accessory molecule with TcR/CD3. In contrast, co-ligation of CD45 inhibits the same TcR/CD3 responses. The machinery which couples the TcR/CD3 complex, CD4, and CD45 to PI-PLC appears to involve regulation of tyrosine phosphorylation, as the TcR/CD3 and CD4 receptors are associated with the tyrosine kinases $p59^{fyn}$ and $p56^{lck}$, respectively, and CD45 has intrinsic tyrosine phosphatase activity. Here, we have examined the ability of CD45 to regulate signal transduction via TcR/CD3 in human thymocytes. Co-cross-linking CD45 to the TcR/CD3 complex strongly suppressed the tyrosine phosphorylation of several intracellular substrates normally seen following TcR/CD3 stimulation. This effect of CD45 was associated with inhibition of a rise in intracellular calcium following TcR/CD3 ligation. Since TcR/CD3 stimulation of mature T cells induces tyrosine phosphorylation of PLCγ1, we investigated this phenomenon in thymocytes, and asked whether ligation of CD45 might regulate this process. By immunoprecipitation we found that TcR/CD3 stimulation induced tyrosine phosphorylation of PLCγ1, an effect which was enhanced by co-cross-linking CD4 to TcR/CD3. In contrast, co-ligation of CD45 strongly blocked PLCγ1 phosphorylation induced by either stimulus. Consistent with previous findings in mature T cells, CD45 cross-linking was able to partially inhibit TcR/CD3-induced thymocyte proliferation when interleukin 2 was used as a second signal, but almost completely (80%–90%) blocked proliferation when anti-CD28 mAb was used as the second signal, suggesting that CD45 cross-linking may be able to block interleukin 2 production via the CD28 pathway. These effects of CD45 on TcR/CD3 signaling and proliferation in thymocytes point towards a potential role for this pathway in thymic selection.

1 Introduction

Activation of either mature T lymphocytes or immature thymocytes can occur via engagement of a number of cell surface antigens including the TcR/CD3 complex, CD2, and CD28 [1–11]. While the intracellular second messengers of the CD28 pathway have not been fully elucidated, the signals transduced by stimulation of TcR/CD3 and CD2 are remarkably similar. A key element in these pathways is the activation of phosphatidylisositol-specific phospholipase C (PI-PLC), which catalyzes the cleavage of phosphoinositol bisphosphate into diacylglycerol and inositol trisphosphate [12–14]. These second messengers in turn activate protein kinase C and induce a rapid rise in intracellular calcium. This is followed by IL 2R expression, and IL 2-dependent cell division [15]. Recent studies have suggested that activation of PI-PLC might itself be mediated via the prior activation of protein tyrosine kinases following stimulation of TcR/CD3 and/or CD2 [16]. Indeed, induction of tyrosine kinase activity is the earliest signal detected following stimulation of the TcR/CD3 complex [6]. The tyrosine kinase $p59^{fyn}$ has been shown to associate with TcR/CD3, and is a likely candidate for this activity [17]; the identity of a CD2-associated kinase remains unknown.

Several other findings also highlight the importance of protein tyrosine phosphorylation in T cell activation. First, stimulation of TcR/CD3 has been shown to induce tyrosine phosphorylation of PLCγ1 in mature T cells and T cell lines [18–20], and this has been proposed to couple receptor engagement to induction of PI-PLC activity [18–23]. Second, co-ligation of the TcR/CD3 complex with the CD4 (or CD8) molecule markedly enhances TcR/CD3-induced protein tyrosine phosphorylation, presumably by activating the CD4 (or CD8) associated $p56^{lck}$ tyrosine kinase [16]. This effect is correlated with augmentation of TcR/CD3-induced PI-PLC activation, calcium flux, and enhancement of proliferation in both mature T cells and thymocytes [11,

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Abbreviation: **PI-PLC:** Phosphatidylinositol-specific phospholipase C

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16]. Third, specific inhibitors of tyrosine phosphorylation can block TcR/CD3-induced responses associated with PI-PLC activation [24, 25]. Fourth, among the cellular substrates phosphorylated is the CD3 ζ chain, a polypeptide which appears to be an important regulatory element of the TcR/CD3 complex [26].

The CD45 molecule, present on all hematopoetic cells, is a protein tyrosine phosphatase which is capable of regulating signal transduction and functional responses [27–31]. CD45 has been shown to be physically associated with both TcR/CD3 [32] and CD2 [33], and in T lymphocytes CD45 ligation inhibits inositol phosphate production, calcium flux, and proliferation initiated by activation of either pathway [28, 30, 31]. The ability of CD45 to modulate signals transduced by TcR/CD3 or CD2 correlates with its ability to inhibit tyrosine phosphorylation of a number of intracellular substrates, including proteins of molecular weights 150, 36, and 35-kDa [30]. This suggests that the tyrosine phosphatase activity of CD45 may act by blocking key signal transduction steps which couple TcR/CD3 stimulation to T cell activation.

We have recently reported that co-stimulation of TcR/CD3 with either CD4 or CD8 using bispecific antibody heteroconjugates augmented TcR/CD3 signals (assessed by calcium mobilization and tyrosine phosphorylation) in both mature CD4/CD8 single-positive thymocytes and immature $CD4^+CD8^+$ thymocytes [11]. Subsequent cell proliferation could be induced by the provision of a second signal in the form of either IL 2 or anti-CD28 mAb, whereas in the absence of CD4 or CD8 co-stimulation, only IL 2 supported proliferation by soluble anti-CD3 mAb. This requirement for exogenous IL 2 or CD28 mAb to induce proliferation of TcR/CD3-stimulated thymocytes made this a useful system in which to explore further the regulation of TcR/CD3 signal transduction via the CD45 molecule, as well as to study the potential function of CD45 in thymic ontogeny.

Here, we report that co-cross-linking CD45 to TcR/CD3 strongly inhibits protein tyrosine phosphorylation in response to TcR/CD3 stimulation in both mature CD4/CD8 single-positive and immature $CD4^+CD8^+$ thymocytes. This effect is associated with inhibition of TcR/CD3-induced calcium mobilization. Co-engagement of CD4 with TcR/CD3, a maneuver which greatly enhances TcR/CD3-induced responses in thymocytes, is not able to overcome the inhibitory influence of CD45 on either tyrosine phosphorylation or calcium mobilization in double-positive thymocytes. Immunoprecipitation with a PLCγ1-specific antibody further demonstrates that tyrosine phosphorylation of PLCγ1, normally induced by TcR/CD3 stimulation and augmented by CD4 co-ligation, is almost completely inhibited by simultaneous co-cross-linking of CD45. CD45 co-cross-linking also partially inhibited thymocyte proliferation induced by cross-linking CD3, or CD3 plus CD4, in the presence of IL 2. However, CD45 almost completely suppressed thymocyte proliferation when anti-CD28 mAb was used in place of IL 2, suggesting that cross-linking of CD45 to CD28 may also block the CD28 signal transduction pathway. Together, these results indicate that CD45 might play an important role in thymic selection through its ability to inhibit TcR/CD3 and CD28 signals.

2 Materials and methods

2.1 mAb and reagents

The mouse anti-human mAb 9.3 (anti-CD28, IgG_{2a}), G19-4 (anti-CD3, IgG_1), G17-2 (anti-CD4, IgG_1), 9.4 (anti-CD45 framework determinant, IgG_{2a}), and 9.6 (anti-CD2, IgG_{2a}), and the goat-anti mouse mAb 187.1 (anti-κ) were produced as previously described [34–38]. mAb to PLCγ1 were a generous gift of S. G. Rhee [39]. FITC- and PE-conjugated nonspecific isotype-matched control antibodies were purchased from Coulter Immunology (Hialeah, FL). FITC- and PE-labeled goat anti-mouse Ab were purchased from Tago (Burlingame, CA). The heteroconjugate mAb CD3xCD4 was prepared by chemical cross-linking as previously described [40]. mAb were conjugated with biotin using biotin-succinimide (Sigma Chemical Co., St. Louis, MO) as described [41]. Avidin was purchased from Sigma, and human IL 2 was obtained from Calbiochem (San Diego, CA). The IL 2 was supplied as a solution containing 640 half-maximal U/ml, and lot 801720 was used.

2.2 Cells

Thymic tissue was obtained as surgical pathology specimens from children under the age of 3 who underwent routine thymectomy at the time of cardiothoracic surgery. The $CD3^+$ human T cell line CEM (clone CEM.6) has been previously described [42].

2.3 Isolation of thymocytes

Thymic tissue was gently expressed through a nylon mesh to obtain a single-cell suspension, and mononuclear cells were isolated using Ficoll-paque density gradient centrifugation. Preparations of thymocytes enriched for $CD4^+CD8^+$ cells (85%–95% purity) were obtained by selecting for the $CD28^{-/dull}$ fraction of thymocytes, as CD28 is expressed at high surface density only on single-positive cells [10, 11]. Unfractionated cells were incubated with 2 μg/ml of anti-CD28 mAb for 1 h at 4 °C. The cells were washed three times, and incubated with goat anti-mouse immunoglobulin-coated magnetic beads (Advanced Magnetics Institute, Cambridge, MA) for 30 min on a rocker at 20 °C. The beads were then collected using a magnet and the separation procedure was repeated on the unbound cell fraction. The remaining unbound cells were >99% $CD2^+$, and >97% were $CD28^{-/dull}$ by indirect immunofluorescence. When stained for CD4 and CD8, these cells were a mean of 2% $CD4^-CD8^-$, 86% $CD4^+CD8^+$, 4% $CD4^+CD8^-$, and 8% $CD4^-CD8^+$. The majority of $CD4^-CD8^+$ cells were $CD3^{-/dull}$, and thus represented the immature stage of thymocytes in transition to $CD4^+CD8^+$ cells. Overall, less than 8% of the negatively selected cells were $CD3^{+(bright)}$.

2.4 Analysis of cell surface phenotype

Cells (1×10^6) were washed twice, mixed with a saturating amount of the appropriate FITC- or PE-conjugated antibody, and suspended in 100 μl of a solution of 50% fetal calf

serum and 50% PBS containing 0.1% sodium azide. Cells were incubated for 45 min at 4 °C, washed twice with cold PBS, and resuspended in 0.5 ml PBS with 1% formaldehyde for flow cytometric analysis. When unlabeled mAb were used, cells were first incubated as indicated above with the unlabeled antibody, washed twice, and stained in a similar fashion with FITC-conjugated goat anti-mouse Ab prior to fixation in 1% formaldehyde. Fluorescent analyses were performed on a FACScan (Becton Dickinson, Mountain View, CA).

2.5 Cell culture

Thymocytes were cultured in complete medium consisting of RPMI 1640, 10^5 U/l penicillin, 100 μg/l streptomycin, 5 mM Hepes, 2 mM L-glutamine, and 10% fetal calf serum. All products were purchased from Gibco Laboratories (Grand Island, NY). Cells were cultured at a density of 1×10^6/ml. When specified, the medium was supplemented with IL 2 using a 1 : 10 dilution of the stock solution to yield a final concentration of 64 half-maximal U/ml. The concentration of anti-CD28 mAb used in cell cultures was always 1 μg/ml. Anti-CD3, -CD4, and -CD45 mAb were used at a concentration of 10 μg/ml, and when indicated, they were cross-linked with 40 μg/ml of goat anti-mouse mAb.

2.6 Proliferative assays

Cells were cultured for 4 days in complete medium in 96-well round-bottom microtiter plates at 10^5 cells/well in a total volume of 0.2 ml. Proliferation, measured as DNA synthesis, was determined by adding 1 μCi of [^{3}H]thymidine (ICN Radiochemicals, Irvine, CA) to each well for the last 18 h of culture, after which plates were harvested with a PHD 200 Cell Harvesting System (Cambridge Technologies, Cambridge, MA). All assays were performed in quadruplicate.

2.7 Measurement of intracellular calcium

Intracellular calcium was measured using a protocol that has been previously described [43]. Briefly, unstimulated cells were loaded with indo-1 for 45 min at room temperature and washed to remove excess indo-1. The cells were then warmed to 37 °C, and basal intracellular calcium measurements determined as the mean indo-1 violet/blue fluorescence ratio. Cells were stimulated with 10 μg/ml of the indicated mAb, biotinylated, and/or conjugate antibodies and the mean indo-1 violet/blue fluorescence ratio was calculated as a function of time. When indicated, avidin was used to cross-link biotinylated mAb at a 4 : 1 ratio of avidin : biotin-mAb. In addition, responding cells were defined as those cells with indo-1 ratios 2 standard deviations above the ratio for control (unstimulated) cells. $CD28^+$ and $CD28^{-/dull}$ cells were separately analyzed by staining with PE-conjugated 9.3 (anti-CD28) and gating on the appropriate population.

2.8 Determination of protein tyrosine phosphorylation

For this analysis, 10^7 cells were suspended in 1 ml of medium in microfuge tubes. At time = − 10 min, 10 μg/ml of each biotinylated mAb was added, and the mAb were cross-linked at time = 0 min by the addition of 40 μg/ml of avidin. At the indicated time points, the cells were rapidly pelleted, the culture medium was removed, and 150 μl of SDS sample buffer containing 25 mM dithiothreitol and 50 μM sodium orthovanadate, was added to the cells. The samples were vortexed, boiled for 5 min, and stored at − 70 °C. The lysates were then subjected to SDS-PAGE on 10% gels as described [44]. The proteins were transferred to PVDF (Millipore, Bedford, MA) and the filters probed first with 0.5 μg/ml of a purified rabbit anti-phosphotyrosine antiserum [45], and subsequently with ^{125}I-staphylococcal protein A (ICN Radiochemicals). The filters were then exposed to X-ray film.

2.9 Immunoprecipitation of PLCγ1

Fresh unfractionated thymocytes were incubated at 10^7 cells/ml with biotinylated mAb to CD4, CD3, and/or CD45 at 5 μg/ml for 5 min at 37 °C. Avidin was added to crosslink mAb at a 5 : 1 (wt/wt) ratio. Cells were lysed in 0.5 ml modified RIPA buffer (1% NP40, 0.25% Na-deoxycholate, 150 mM Nacl, 50 mM Tris, pH 7.5) supplemented with proteinase and phosphatase inhibitors as previously described [46, 47]. Nuclei were removed by centrifugation at 14000 × g for 10 min, and 1 μg mAb to PLCγ1 [39] was added to clarified lysates and incubated for 1–2 h at 0 °C. Immune complexes were recovered by the addition of 50 μl rabbit anti-mouse Ig-coated Sepharose (1 h incubation), and the beads were washed three times in modified RIPA buffer and once in buffer without detergents. Samples were boiled 5 min in SDS-PAGE sample buffer, subjected to SDS-PAGE on an 8% gel, transferred to nitrocellulose and immunoblotted with anti-phosphotyrosine as indicated above.

3 Results

3.1 Effects of CD45 cross-linking on protein tyrosine phosphorylation

We first examined the effects of CD45 cross-linking on tyrosine phosphorylation using immunoblotting (Fig. 1). As previously reported, cross-linking TcR/CD3 on thymocytes induced tyrosine phosphorylation of numerous substrates, including those of relative molecular mass 170, 150, 135, 100, 80, 76, 71, 36, and 35-kDa [11], and co-ligation of CD4 with CD3 strongly enhanced this response on all substrates. In contrast to these effects of CD4, co-ligation of CD45 with CD3 inhibited the phosphorylation of most substrates, particularly those of 150, 100, 36, and 35-kDa. When CD3, CD4, and CD45 were simultaneously cross-linked, the "CD45 effect" was dominant. That is, the pattern of tyrosine phosphorylation which was observed most resembled that of CD3xCD45, rather than CD3xCD4. Only the 100- and 35-kDa substrates were phosphorylated after CD3xCD4xCD45 ligation, and this was a much weaker response than was seen after CD3xCD4. Interestingly, although CD4 stimulation does not result in calcium mobilization, IL 2R expression or IL 2-dependent proliferation in thymocytes ([11], and data not shown), cross-linking CD4 alone induced a pattern of tyrosine phosphorylation similar to that seen with CD3

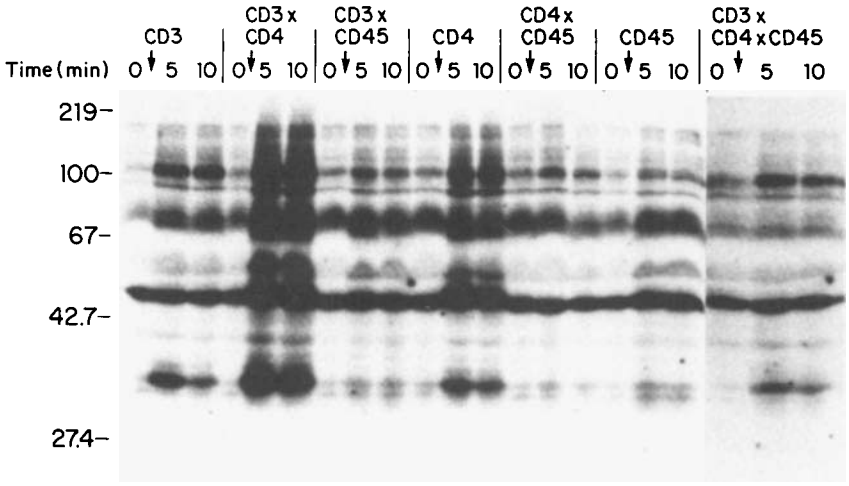

Figure 1. Opposing effects of CD4 and CD45 on TcR/CD3-induced tyrosine phosphorylation. Unfractionated thymocytes (1×10^7/lane) were cultured in microfuge tubes. At time = − 10 min, 10 μg/ml of the indicated biotin-conjugated mAb CD3 (G19-4, IgG_1), CD4 (G17-2, IgG_1), and/or CD45 (9.4, IgG_{2a}) were added to the medium. The mAb were cross-linked at time = 0 min (indicated by arrow) by the addition of 40 μg/ml of avidin. Cells were lysed at the indicated time points, and subjected to SDS-PAGE. Following transfer, the filters were incubated with anti-phosphotyrosine antibody followed by ^{125}I-labeled protein A. Isolated double-positive thymocytes responded identically to the stimuli used, although the signal strength was slightly lower (data not shown).

cross-linking. This was also blocked by co-ligation of CD45 with CD4.

3.2 Effects of CD45 cross-linking on TckR/CD3-induced calcium flux in thymocytes

In mature T cells, a rise in intracellular calcium in response to TcR/CD3 stimulation occurs temporally after activation of a TcR/CD3-associated tyrosine kinase, and is blocked by inhibitors of tyrosine phosphorylation or by co-cross-linking CD45 with TcR/CD3 [16, 24, 25, 30, 31]. Therefore, we next examined the effects of CD45 engagement on thymocyte calcium mobilization. We have previously shown that mature $CD3^+$ CD4/CD8 single-positive thymocytes express high levels of the CD28 accessory molecule, whereas immature $CD3^+CD4^+CD8^+$ (double-positive) thymocytes are $CD28^{-/dim}$ [10, 11]. In these studies, we utilized this differential expression of CD28 to examine separately mature single-positive and immature, double-positive thymocytes by gating on $CD28^{+(bright)}$ and $CD28^{-/dim}$ cells, respectively. Staining of cells with CD28 mAb for gating had no effect on baseline calcium levels or on the response to subsequent stimuli ([11], and data not shown). Cross-linking of the TcR/CD3 complex using biotin-anti-CD3 mAb plus avidin induced a rise in intracellular calcium in both populations of thymocytes (Fig. 2). Consistent with previous data by ourselves and others, a higher response was observed in single-positive cells, both in terms of mean calcium flux (Fig. 2) and percent cells responding (48%–55% of $CD28^{-/dim}$ cells *vs.* 92%–95% of $CD28^{+(bright)}$ cells) [11, 48]. To examine the effect of CD45, the anti-framework mAb 9.4 was used. A complete inhibition of calcium flux was seen when CD45 was cross-linked to CD3, in agreement with previous reports in thymocytes [48] and with our own findings in mature T cells [30]. We next used CD3xCD4 co-cross-linking as a primary stimulus. While the percentage of cells responding to CD3xCD4 (49%–55% of $CD28^{-/dim}$ cells *vs.* 96%–99% of $CD28^{+(bright)}$ cells) was similar to that for CD3 cross-linking, co-ligation of CD4 augmented the mean calcium flux and/or timing of the response in both populations of cells. In this regard, the double-positive thymocytes were significantly more dependent on CD4 co-cross-linking then were the single-positive cells. Furthermore, while co-ligation of CD45 also strongly suppressed the calcium

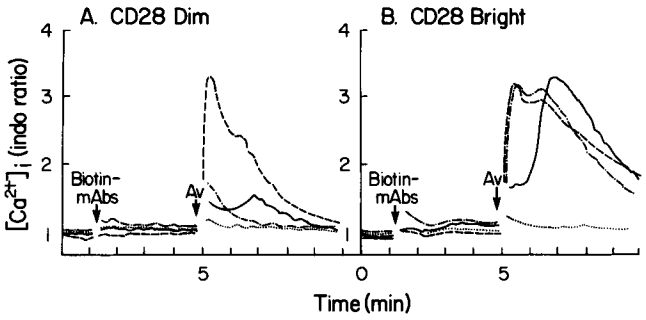

Figure 2. Effect of co-cross-linking CD45 and/or CD4 to TcR/CD3 on calcium mobilization in immature and mature thymocytes. Unfractionated thymocytes were loaded with indo-1, washed, and stained with PE-labeled anti-CD28 mAb. Electronic gating was used to define $CD28^{dim}$ cells (left panels) and $CD28^{bright}$ cells (right panels). At time = 1.5 min (first arrow) 10 μg/ml of the following biotinylated mAb were added: CD3 (solid line ——); CD3 + CD45 (dotted line ·····); CD3 + CD4 (dashed line – – –); CD3 + CD4 + CD45 (dash-dot – · – · –). These were cross-linked by the addition of avidin (at a 4 : 1 weight/weight ratio) at time = 5 min (second arrow). Results are expressed as mean intracellular calcium concentration (indo ratio of 1.0 = 131 nM, 2.0 = 223 nM, 3 = 338 nM).

response to CD3xCD4 cross-linking in immature thymocytes, this maneuver had no effect on single-positive thymocytes with respect to either the magnitude of calcium flux (Fig. 2) or the percentage of cells responding (data not shown). Separate ligation of CD45 15 min prior to cross-linking CD3 or CD3xCD4 blunted the calcium flux in both thymocyte subpopulations in each of two experiments (data not shown), although the inhibitory effects were much smaller than that observed with co-cross-linking (Fig. 2).

3.3 Tyrosine phosphorylation of PLCγ1

Our previous work and the data presented above demonstrate that co-ligation of CD4 or CD45 have opposite effects on TcR/CD3-induced tyrosine phosphorylation (as assessed by immunoblotting of whole cell extracts), calcium mobilization and inositol phosphate hydrolysis, *i.e.* enhancement by CD4 and inhibition by CD45 [11, 16, 30]. Since these processes are thought to occur by activation of PLC

following TcR/CD3 stimulation, it was of interest to examine whether the regulatory effects of CD4 and CD45 might be exerted directly upon PLC through their associated tyrosine kinase and phosphatase activities. Both unseparated and isolated double-positive thymocytes were stimulated by cross-linking these mAb alone or in combination, after which PLCγ1 was immunoprecipitated with an isozyme-specific mAb. The immunoprecipitate was subjected to SDS-PAGE, followed by transfer to a filter and immunoblotting with anti-phosphotyrosine mAb (Fig. 3, panel A). PLCγ1 did not exhibit any detectable tyrosine phosphorylation in unstimulated thymocytes, however tyrosine phosphorylation was readily induced following stimulation via TcR/CD3. This effect of TcR/CD3 stimulation was strongly enhanced by cross-linking CD4 to CD3, and inhibited by cross-linking CD45 to CD3. When the three cell surface antigens were simultaneously cross-linked, the effects of CD45 appeared to dominate those of CD4, as tyrosine phosphorylation of PLCγ1 was reduced to barely detectable levels. Although CD4 cross-linking alone is able to induce tyrosine phosphorylation of several intracellular substrates (Fig. 1), this method of stimulation did not phosphorylate PLCγ1 (Fig. 3).

The experiment in Fig. 3 also demonstrates the previously noted affects of CD4 and/or CD45 co-cross-linking on tyrosine phosphorylation of pp35 and pp36, as these two proteins are also immunoprecipitated with the anti-PLCγ1 mAb. Similar to PLCγ1, CD4 enhanced, and CD45 inhibited, TcR/CD3-induced phosphorylation of these substrates, and when used together the CD45 effect predominated. Three sets of bands, one above pp35/36 and two below, are seen at equal intensity in all lanes. These represent nonspecific binding of the second-step ^{125}I-labeled protein A reagent to the anti-PLCγ1 mAb used for immunoprecipitation. No bands were seen in control precipitations performed without anti-PLCγ1 mAb (Fig. 3, panel B, lanes 3, 4, and 7). Panel B also shows that PLCγ1 can be precipitated by anti-PLCγ1 Ab even in the absence of CD3 stimulation (lane 5), but that under these circumstances, PLCγ1 is not tyrosine phosphorylated (lane 1).

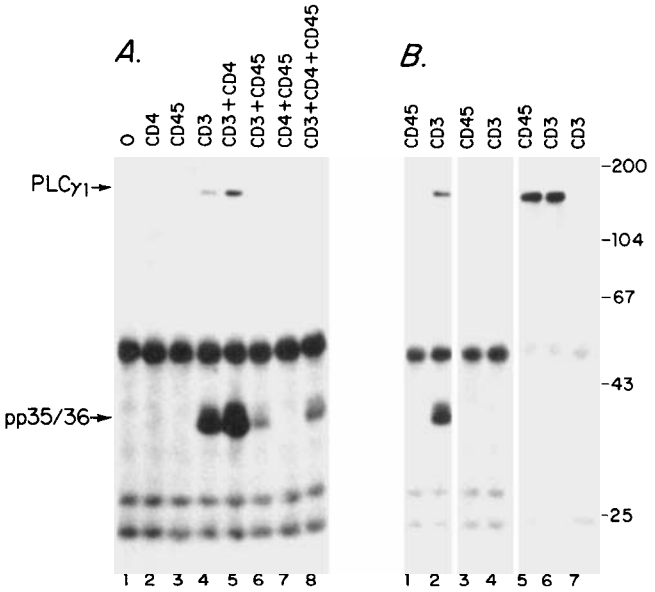

Figure 3. Regulation of PLCγ1 tyrosine phosphorylation by CD3, CD4, and CD45. (A) Unfractionated thymocytes (1×10^7/lane) were incubated in microfuge tubes in control medium or with 5 μg/ml of biotinylated mAb to CD3, CD4, and/or CD45. Cells were incubated for 5 min at 37 °C, after which avidin was added at a final concentration of 25 μg/ml. Cells were lysed after 30 s, and the postnuclear lysates were immunoprecipitated with anti-PLCγ1 mAb as indicated in Sect. 2.9. The samples were then analyzed by SDS-PAGE and immunoblotting with anti-phosphotyrosine antibody as per the legend for Fig. 1. Identical results were obtained when isolated $CD28^{-/dim}$ cells were used (data not shown). (B) CEM cells were stimulated with the indicated mAb as in (A). Lanes 1, 2, 5, and 6 were immunoprecipitated with anti-PLCγ1 mAb; lanes 3, 4, and 7 were control precipitations done without anti-PLCγ1 mAb. Following SDS-PAGE lanes 1–4 were blotted with anti-phosphotyrosine Ab. Lanes 5, 6 and 7 were blotted with anti-PLCγ1 mAb.

3.4 Effects of CD4 and CD45 co-cross-linking on thymocyte proliferation

Although mature T cells exhibit IL 2-dependent proliferation following TcR/CD3 cross-linking by immobilized mAb, thymocytes possess a poorly characterized defect in IL 2 production, such that proliferation following TcR/CD3 cross-linking requires a second signal [10]. This signal can be provided either in the form of exogenous IL 2, or through stimulation of the CD28 pathway which induces endogenous IL 2 production [10]. This requirement for a second signal in thymocytes provided us with an opportunity to dissect further the inhibitory effects of CD45 ligation.

Our previous work on mature T cells showed that anti-CD45 mAb in solution had no inhibitory effect on T cell proliferation induced by immobilized anti-CD3 mAb. In contrast, co-immobilizing CD45 with CD3 or CD3 plus CD4 partially blocked proliferation (30%–75%), an effect which was not overcome with exogenous IL 2 [30, 31]. In our current studies of thymocytes we utilized soluble mAb alone or in combination, and provided additional cross-

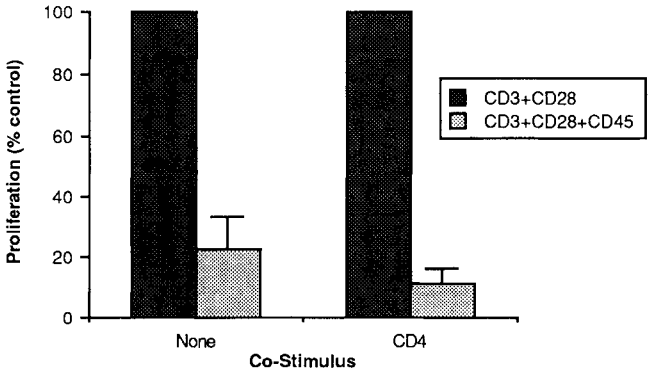

Figure 4. Inhibition of thymocyte proliferation by CD45. Unfractionated thymocytes were cultured for 96 h in microtiter plates at 10^5 cells/well. Anti-CD3, anti-CD4, and anti-CD45 mAb were used at 10 μg/ml, and anti-CD28 mAb at 1 μg/ml. All wells contained the goat anti-mouse mAb 187.1 at 40 μg/ml. Tritiated thymidine was added to the cultures 18 h before harvesting. Results are expressed as percent control ± SD, and are the mean of three experiments (100% = 43 959 cpm for CD3 + CD28 and 33 351 cpm for CD3 + CD4 + CD28). No significant proliferation was observed in the absence of CD3 stimulation or anti-CD28 co-stimulation.

linking by adding a goat-mouse mAb (Fig. 4). As expected, cross-linking CD3 in this fashion was ineffective at inducing proliferation; however, a proliferative response was easily induced in the presence of anti-CD28 mAb. Cross-linking CD45 with CD3 plus CD28 strongly suppressed proliferation, and co-cross-linking CD4 failed to overcome this inhibitory effect of CD45. In contrast, when IL 2 was used as a second signal in place of CD28 mAb a much smaller and variable inhibitory effect of CD45 was observed (10%–50%) (data not shown).

4 Discussion

Here, we have found that CD4 and CD45 regulate TcR/CD3-induced tyrosine phosphorylation of PLCγ1. Previous work in other systems has shown that stimulation of surface molecules with intrinsic tyrosine kinase activity, such as the receptor for epidermal growth factor (EGF) or platelet-derived growth factor (PDGF), is accompanied by tyrosine phosphorylation of PLCγ1 and activation of PI-PLC [22, 23]. It was, therefore, proposed that EGF- and PDGF-induced activation of the inositol phospholipid pathway occurs as a consequence of tyrosine phosphorylation of PLCγ1, as this has previously been shown to increase the catalytic activity of this enzyme [22]. Recent work has confirmed that PLCγ1 is tyrosine phosphorylated following TcR/CD3 stimulation of mature T cells and T cell lines [18–20]. Our data shows that PLCγ1 is similarly regulated via TcR/CD3 in immature thymocytes, that signals transduced by the CD4 and CD45 accessory molecules modulate this process, and that these effects correlate with the ability of these surface molecules to initiate, enhance, or inhibit cell activation.

PLCγ1, a 150-kDa protein, is not seen on anti-phosphotyrosine immunoblotting of whole cell lysates (Fig. 1); however, it is readily visualized by immunoprecipitation (Fig. 4), when using up to tenfold greater cell equivalents of precipitated protein. Two other protein substrates, pp35 and pp36 were also immunoprecipitated using the antibody to PLCγ1, and were regulated in the same manner as PLCγ1. Tyrosine phosphorylation of these substrates in mature T cells has been previously noted to be regulated by CD45 [30]. The data presented here indicates that these substrates are associated with PI-PLC, and that tyrosine phosphorylation of pp35 or pp36 might regulate PLC activity.

Interestingly, while CD45 cross-linking to TcR/CD3 blocked the TcR/CD3-induced calcium response in all thymocytes, co-ligation of CD4 to TcR/CD3 conferred resistance to the inhibitory effects of CD45 on the mature single-positive cells, but not on the double-positive thymic subpopulation. This is consistent with previously noted distinctions in TcR/CD3 signalling in immature *vs.* mature thymocytes [49]. Whether basis for these observations is inherent structural differences in the compositions of the TcR/CD3, CD4, and CD45 molecules, in their physical relationship to each other, and/or in their coupling to intracellular signal transducing molecules such as $p56^{lck}$ is not known. In any event, the dominant effect of CD45 (*vs.* CD4) on calcium flux in double-positive thymocytes is consistent with the ability of CD45 to block tyrosine phosphorylation of PLCγ1 in cells stimulated with CD3 plus CD4.

These studies have also suggested a potentially important functional interaction between CD28 and CD45. In mature T cells, the induction of calcium mobilization and proliferation via cross-linking CD28 can be almost totally inhibited by co-cross-linking CD45 [31]. In this study, since we utilized thymocytes in place of mature T cells proliferation was not observed in response to TcR/CD3 stimulation alone, but required a second signal such as IL 2 or anti-CD28 mAb. Consistent with our previous studies in mature T cells, CD45 engagement partially inhibited proliferation to anti-TcR/CD3 plus anti-CD4 plus IL 2 (10%–50%). In contrast to those results, a much stronger suppressive effect (80%–90%) was seen when cells were stimulated with anti-CD28 in place of IL 2. As with tyrosine phosphorylation of PLCγ1, co-cross-linking CD4 was not able to overcome the inhibitory effect of CD45. One potential explanation of this is that ligation of CD45 can directly regulate the CD28 pathway. Although stimulation of the CD28 antigen has been well documented to enhance IL 2 production [9], the early intracellular signals activated through this pathway remain poorly characterized. Initial work indicates that cross-linking CD28 can induce protein tyrosine phosphorylation in TcR/CD3-activated T cells via an as yet undetermined pathway (Vandenberghe, P. et al., submitted), and that tyrosine kinase inhibition may block T cell stimulation induced by phorbol ester plus anti-CD28 mAb [50]. If induction of tyrosine phosphorylation mediates the CD28 pathway, then CD45 might act by dephosphorylating the same substrates, or perhaps by regulating a CD28-associated tyrosine kinase. While IL 2 binding to the high-affinity IL 2R can also induce tyrosine phosphorylation [51, 52], the substrates are distinct from those induced by CD28, and CD45 cross-linking does not regulate this process directly [52]. Furthermore, CD28 cross-linking can initiate inositol phosphate hydrolysis in resting T cells [43], whereas IL 2 does not induce PI-PLC activity [43] or tyrosine phosphorylation of PLCγ1 (data not shown). Thus, CD28 cross-linking, such as was used in our studies, activates a pathway of tyrosine phosphorylation and cell activation distinct from that of IL 2. The ability of CD45 cross-linking to almost completely block CD28-induced proliferation (as opposed to partial inhibition of IL 2-dependent proliferation) suggests a functional interaction between these two accessory molecules which may be an important regulatory event in T cell development or activation.

In summary, our data shows that the CD45 can modulate PI-PLC activity in human thymocytes, and that this effect is correlated with regulation of tyrosine phosphorylation of PLCγ1. While CD45 cross-linking blocked TcR/CD3 signals in both mature, single-positive and immature, double-positive thymocytes, only double-positive thymocytes remained sensitive to CD45 when TcR/CD3 was cross-linked to CD4. Since thymic selection occurs following TcR/CD3 engagement on a double-positive thymocyte by an MHC molecule present on a thymic stromal cell [52], this interaction likely involves co-ligation of CD4 (or CD8) by non-polymorphic MHC determinants. The ability of CD45 to regulate this signal suggests that CD45 could function to block the TcR-mediated events which result in both positive and negative selection of thymocytes [53, 54]. Ultimate determination of the role of CD45 during thymic ontogeny (and T cell activation) awaits identification and localization of the natural ligand(s) for CD45 and its isoforms.

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