Reconstitution of Interactions between Protein-tyrosine Phosphatase CD45 and Tyrosine-protein Kinase p56<sup>lk</sup> in Nonlymphoid Cells*

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To further understand the functional interactions between CD45 and p56<sup>lk</sup> in T-cells, we stably reconstituted their expression in a nonlymphoid system. The results of our analyses demonstrated that CD45 could dephosphorylate tyrosine 505 of p56<sup>lk</sup> in NIH 3T3 fibroblasts. As is the case for T-cells, removal of the unique domain of p56<sup>lk</sup> interfered with dephosphorylation of tyrosine 505 in fibroblasts, further stressing the importance of this region in the interactions between CD45 and p56<sup>lk</sup>. The ability of CD45 to dephosphorylate tyrosine 505 in NIH 3T3 cells was also greatly influenced by the catalytic activity of p56<sup>lk</sup>. Indeed, whereas CD45 provoked dephosphorylation of kinase-defective Lck molecules in this system, it failed to stably dephosphorylate kinase-active p56<sup>lk</sup> polypeptides. Finally, our studies showed that CD45 was also able to inhibit the oncogenic potential of a constitutively activated version of p56<sup>lk</sup> in NIH 3T3 cells. This effect did not require the Lck unique domain and apparently resulted from selective dephosphorylation of substrates of activated p56<sup>lk</sup> in fibroblasts. In addition to providing insights into the nature and regulation of the interactions between CD45 and p56<sup>lk</sup> in T-cells, these results indicated that CD45 clearly has the capacity to both positively and negatively regulate p56<sup>lk</sup>-mediated functions in vivo.

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*p56<sup>lk</sup> is a lymphocyte-specific member of the Src family of tyrosine-protein kinases (Refs. 1–3; reviewed in Refs. 4 and 5). Unlike the other members of the Src family, it bears several independent structural domains that include, from the amino terminus to the carboxyl terminus: (i) sites of myristylation (glycine 2) and palmitylation (cysteines 3 and/or 5), involved in targetting to cellular membranes (Ref. 6; reviewed in Ref. 7); (ii) a unique domain of roughly 50 amino acids, which mediates binding to the CD4 and CD8 T-cell surface antigens (8–12); (iii) Src homology (SH)1 3 and SH2 domains, capable of interacting with proline-rich motifs and phosphotyrosine-containing sequences, respectively (reviewed in Ref. 13); (iv) a kinase domain, including sites for ATP-binding, phosphotransfer (lysine 273) and autophosphorylation (tyrosine 394) and (v) a negative regulatory domain, encompassing the major site of in vivo tyrosine phosphorylation, tyrosine 505.

The catalytic activity of p56<sup>lk</sup> is primarily regulated by phosphorylation of tyrosines 394 and 505 (reviewed in Ref. 5). Phosphorylation at tyrosine 394 activates the catalytic function of p56<sup>lk</sup>, by provoking a conformational change in the kinase domain (14). Conversely, phosphorylation at tyrosine 505 inhibits the enzymatic activity of Lck (15–17), presumably by allowing an intramolecular interaction between the carboxyl-terminal end and the SH2 domain of the enzyme. Accumulating data indicate that tyrosine 505 phosphorylation is mediated by p50<sup>csk</sup>, a tyrosine-protein kinase expressed in all cell types (17–19; reviewed in Ref. 20). However, tyrosine 505 can also be a site of autophosphorylation. This possibility is suggested by the findings that p56<sup>lk</sup> could undergo phosphorylation at tyrosine 505 in bacteria (which lack endogenous tyrosine-protein kinases) or during in vitro kinase reactions (21, 22). Nontheless, autophosphorylation may not be a prominent component of tyrosine 505 phosphorylation in mammalian cells, because a kinase-defective version of p56<sup>lk</sup> (lysine 273 to arginine 273 (Arg<sup>273</sup>) Lck) was still extensively phosphorylated at this site in transfected NIH 3T3 fibroblasts (19).

Contrary to Lck polypeptides expressed in NIH 3T3 cells, those isolated from T-lymphocytes are poorly phosphorylated at tyrosine 505 (23). This difference is seemingly consequent to the action of CD45, a transmembrane protein-tyrosine phosphatase selectively expressed in nucleated hemopoietic cells (for a review, see Ref. 24). This notion is supported by the observation that Lck polypeptides immunoprecipitated from CD45-deficient T-cells exhibited a marked (8–10-fold) increase in tyrosine 505 occupancy, when compared with p56<sup>lk</sup> molecules recovered from their CD45-positive counterparts (25–29). In contrast, expression of CD45 had little or no effect on the tyrosine phosphorylation of two other Src family kinases, p59<sup>fyn</sup> and p60<sup>fyn</sup> (26). The lack of constitutive dephosphorylation of tyrosine 505 in CD45-negative T-cells is thought to explain the inability of these cells to become activated upon stimulation with antigen or anti-T-cell receptor antibodies (30–33).

Over the past few years, significant efforts have been directed toward understanding the mechanism(s) by which CD45 causes selective dephosphorylation of p56<sup>lk</sup> in T-cells. Because a small fraction of Lck molecules can be co-immunoprecipitated with CD45 in mild detergent lysates of T-cells (34, 35), it is likely that these two molecules are in close proximity in the cell. Interestingly, we demonstrated that deletion of the unique...
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**RESULTS**

**Expression of CD45 and Lck Polypeptides in NIH 3T3 Cells**—cDNAs encoding wild-type p56\(^{lck}\), the CD45-negative (Lck-positive) variant p56\(^{lck-}\), and the CD45-positive variant p56\(^{lck+}\) were introduced into NIH 3T3 fibroblasts. CD45-negative NIH 3T3 cells expressing either p56\(^{lck-}\) or p56\(^{lck+}\) were stained with anti-CD45 mAb M1.89.18.7 and fluorescein isothiocyanate-conjugated goat anti-rat IgG. Immunofluorescence was detected by FACScan analysis. In panel A, neomycin-resistant NIH 3T3 cells were also stained with anti-CD45 mAb (dotted line). C, anti-CD45 immunoblot. Cells were lysed in nonionic detergent-containing buffer, and CD45 was immunoprecipitated (I.P.) from 1 mg of total cell proteins using anti-CD45 mAb M1.89.18.7. CD45 expression was then measured by immunoblotting with a polyclonal rabbit anti-CD45 serum. The position of CD45 is shown on the left, and those of prestained molecular mass markers (in kDa) are shown on the right. Exposure was 15 h.

**MATERIALS AND METHODS**

**Cells**—NIH 3T3 mouse fibroblasts and \(\phi\)-2 packaging cells were grown in alpha-methyl essential medium (41) containing 10% fetal calf serum and antibiotics. Derivatives expressing the neomycin phosphate-transferase were grown in the additional presence of G418 (0.5 \(\mu\)g/ml), whereas cells expressing the puromycin resistance gene were propagated in medium supplemented with puromycin (1 \(\mu\)g/ml).

**Antibodies**—Anti-mouse CD45 rat monoclonal antibody (mAb) M1.89.18.7 was obtained from the American Type Culture Collection. Purified antibodies were used in our experiments. Rabbit anti-CD45 polyclonal antibodies were generated against the carboxy-terminal tail of CD45 and were kindly provided by Dr. Phil Branton (McGill University, Montreal, Quebec, Canada). Rabbit antisera directed against the tyrosine having the capacity to bind both CD45 and p56\(^{lck}\) were kindly provided by Dr. Phil Branton (McGill University, Montreal, Quebec, Canada). Rabbit antisera directed against annexin II (Upstate Biotechnology Inc., Lake Placid, NY) or annexin II (43) significantly reduced activity, cells were treated for 10 min at 37 °C with the protein-tyrosine phosphatase inhibitor pervanadate (1:50, v/v) as described elsewhere (8), with the exception of CD45 immunoprecipitations, which were performed using mAb M1.89.18.7 (10 \(\mu\)g) coupled to protein G-Sepharose (Pharmacia Biotech Inc.). Following immunoblotting, immunoreactive products were detected by autoradiography and quantitated using a PhosphorImager (BAS 2000, Fuji). Peptide mapping with cyanogen bromide was also performed as reported previously (19).

**Concentration**—Cell concentration studies were conducted according to a previously described protocol (23).

**Cell Transformation Assays**—To examine focus formation, NIH 3T3 derivatives (10\(^5\) or 10\(^4\) cells) were seeded in 6-well Costar plates with 10\(^5\) neomycin-resistant NIH 3T3 cells. Foci were counted after 9 days of growth. For growth in soft agar, 2 \(\times\) 10\(^5\) cells were plated in medium containing 0.3% agar as described elsewhere (15). Colony formation was monitored for 2 weeks. All assays were done in duplicate and were repeated at least three times.

**Generation of CD45-positive Variants of NIH 3T3 Fibroblasts**—To further dissect the regulation of p56\(^{lck}\) by CD45 in T-cells, we wished to recreate the expression of these two molecules in a nonlymphoid mammalian cell system. To this end, mouse NIH 3T3 fibroblasts were chosen, because they do not normally express either polypeptide. As a first step, NIH 3T3 variants expressing the isoform of CD45 predominantly contained in mature T-cells, CD45 R0/T200 (24), were generated as outlined under “Materials and Methods.” Two CD45-positive NIH 3T3 clones (clones 2 and 31) were selected for further studies. As depicted in Fig. 1 (A and B), FACScan analyses showed that these two cell lines expressed easily appreciable amounts of CD45 at their surface. To ensure that full-length CD45 polypeptides were expressed in these cells, CD45 was immunoprecipitated with mAb M1.89.18.7 and subsequently immunoblotted with a polyclonal rabbit anti-CD45 serum (Fig. 1C).
1C). This experiment revealed that the two clones contained a 180-kDa immunoreactive polypeptide (lanes 2 and 3) that comigrated with the CD45 molecule immunoprecipitated from the mouse T-cell line Bl-141 (lane 4). The CD45-positive fibroblasts expressed approximately 10 times lower amounts of CD45 than Bl-141 T-cells.

**CD45 Can Induce Dephosphorylation of p56<sup>lck</sup>** in NIH 3T3 Cells—Next, CD45-positive and CD45-negative NIH 3T3 cells were infected with retroviruses encoding either wild-type or kinase-inactive (Arg273<sup>R</sup>) p56<sup>lck</sup> molecules. Kinase-defective Lck molecules were included in these studies to eliminate the possibility of compensatory autophosphorylation at tyrosine 505, as discussed elsewhere (19, 23). Although the results reported herein primarily concerned CD45-positive clone 31, similar results were obtained with clone 2 (data not shown).

The impact of CD45 on the tyrosine phosphorylation of p56<sup>lck</sup> in NIH 3T3 cells was first examined (Fig. 2). Lck polypeptides were recovered by immunoprecipitation, and their phosphotyrosine content was assessed by immunoblotting with anti-phosphotyrosine mAb 4G10 (Fig. 2A, top panel). Moreover, the abundance of p56<sup>lck</sup> was verified by immunoblotting of parallel anti-Lck immunoprecipitates with an antiserum generated against the Lck SH3 region (middle panel). This was taken into consideration when determining the relative extents of Lck tyrosine phosphorylation in these various cell lines. Finally, the levels of CD45 were monitored by immunoblotting of anti-CD45 immunoprecipitates with rabbit anti-CD45 antibodies (bottom panel).

In keeping with our previous report (19), wild-type p56<sup>lck</sup> (Fig. 2A, lane 2) and Arg273<sup>p56<sup>lck</sup></sup> (lane 3) were tyrosine phosphorylated to similar extents in NIH 3T3 cells lacking CD45. However, although tyrosine phosphorylation of wild-type p56<sup>lck</sup> was not decreased by expression of CD45 (lane 4), the phosphotyrosine content of kinase-inactive Lck molecules was reduced 5-fold (lane 5). The differential impact of CD45 on wild-type and Arg273<sup>p56<sup>lck</sup></sup> was not caused by variations in the expression levels of either Lck or CD45, because the two cell lines contained comparable quantities of these two products (middle and bottom panels).

To identify the sites of tyrosine phosphorylation on these Lck molecules, peptide mapping studies were conducted using cyanogen bromide. Cells were metabolically labeled with 32P<i>o</i>, and Lck polypeptides were recovered by immunoprecipitation. Following cleavage with cyanogen bromide, phosphorylated peptides were resolved by gel electrophoresis and detected by autoradiography (Fig. 2B). The positions of C1 (which contains amino-terminal sites of serine, threonine, and tyrosine phosphorylation), C2 (which encompasses tyrosine 394), and C3 (which bears tyrosine 505) are shown on the left. The migrations of prestonated molecular mass markers are indicated in kDa on the right. Exposure was 2 days.

**Effects of CD45 expression on p56<sup>lck</sup> tyrosine phosphorylation in NIH 3T3 cells**. A, anti-phosphotyrosine immunoblot. The extent of tyrosine phosphorylation of either wild-type (W) or kinase-inactive Arg273<sup>R</sup> (R) Lck polypeptides in the presence (+) or the absence (−) of CD45 was assessed by anti-phosphotyrosine (α-P-tyr) immunoblotting of Lck immunoprecipitates (top). The abundance of Lck was measured by immunoblotting of parallel immunoprecipitants with an anti-Lck serum (middle), whereas that of CD45 was verified by immunoblotting of anti-CD45 immunoprecipitates with a rabbit anti-CD45 serum (bottom). The positions of Lck and CD45 are indicated on the left. Exposures were 36 (top), 4 (middle panel), and 36 h (bottom panel). I.P., immunoprecipitation. B, peptide mapping studies. Phosphotyrosine sites were evaluated by peptide mapping of 32P<sub>i</sub>-labeled p56<sup>lck</sup> using cyanogen bromide. The position of the C3 fragment (which bears tyrosine 505) is shown on the left. Exposure was 22 h. I.P., immunoprecipitation. B, peptide mapping studies. Sites of phosphorylation were defined by cyanogen bromide cleavage analyses as described in the legend of Fig. 2B. The positions of C1 (which contains amino-terminal sites of serine, threonine, and tyrosine phosphorylation), C2 (which encompasses tyrosine 394), and C3 (which bears tyrosine 505) are shown on the left. The migrations of prestonated molecular mass markers are indicated in kDa on the right. Exposure was 15 h.

**Effects of the protein-tyrosine phosphatase inhibitor pervanadate on Lck tyrosine phosphorylation in CD45-positive NIH 3T3 cells**. A, anti-phosphotyrosine immunoblot. CD45-positive NIH 3T3 cells expressing either wild-type (W) or kinase-inactive Arg273<sup>R</sup> (R) Lck were incubated for 10 min at 37°C in the presence (+) or the absence (−) of pervanadate (PV). Lck tyrosine phosphorylation was monitored as outlined in the legend of Fig. 2A. The position of p56<sup>lck</sup> is shown on the left. Exposure was 22 h. I.P., immunoprecipitation. B, peptide mapping studies. Sites of phosphorylation were defined by cyanogen bromide cleavage analyses as described in the legend of Fig. 2B. The positions of C1 (which contains amino-terminal sites of serine, threonine, and tyrosine phosphorylation), C2 (which encompasses tyrosine 394), and C3 (which bears tyrosine 505) are shown on the left. The migrations of prestonated molecular mass markers are indicated in kDa on the right. Exposure was 15 h.
Lck are shown on the I.P. in detergent-containing buffer, Lck polypeptides were recovered by immunoprecipitation (lane 4). In addition, the phosphatase inhibitor provoked phosphorylation of both wild-type (lane 3) and kinase-defective (lane 4) p56\textsuperscript{Lck} within the tyrosine 394-containing C2 fragment, in keeping with earlier studies (19, 48, 49). Although the basis for phosphorylation of kinase-inactive Lck molecules at the "autophosphorylation" site has not yet been elucidated, this finding suggests that another cellular tyrosine-protein kinase can transphosphorylate tyrosine 394 in vivo (19, 48, 49).

The Unique Domain of p56\textsuperscript{Lck} Is Necessary for CD45-mediated Dephosphorylation of Tyrosine 505 in NIH 3T3 Cells—To verify that dephosphorylation of tyrosine 505 in NIH 3T3 cells occurred via a mechanism similar to that existing in T-cells, we examined whether the unique domain of p56\textsuperscript{Lck} was also necessary for the effect of CD45 in fibroblasts. Arg\textsuperscript{273} Lck polypeptides lacking residues 16–62 (∆16–62R273 Lck) were introduced in CD45-negative and CD45-positive NIH 3T3 cells. Anti-phosphotyrosine immunoblotting of anti-Lck immunoprecipitates (Fig. 4A, top panel) established that ∆16–62R273 Lck (lane 3) was tyrosine phosphorylated to nearly the same extent as Arg\textsuperscript{273} p56\textsuperscript{Lck} (lane 1) in CD45-negative cells. In cells expressing CD45, however, the tyrosine phosphorylation of ∆16–62R273 Lck (lane 4) was only reduced 1.5-fold, in comparison with the ∆-fold decrease noted above for Arg\textsuperscript{273} Lck (lane 2; Fig. 2A, lanes 3 and 5). Complementary peptide mapping analyses confirmed that, unlike Arg\textsuperscript{273} Lck (Fig. 4B, lane 2), ∆16–62R273 Lck remained prominently phosphorylated at tyrosine 505 in CD45-positive cells (lane 4).

Because CD45 is a membrane-bound phosphatase, we wished to ensure that the association of p56\textsuperscript{Lck} with membranes in NIH 3T3 cells was not altered by deletion of the unique region. After incubation in hypotonic buffer, cells were mechanically lysed in hypotonic buffer, and lysates were separated into particulate (P100) and cytosolic (S) fractions by differential centrifugation. After extraction in detergent-containing buffer, Lck polypeptides were recovered by immunoprecipitation (I.P.) and detected by immunoblotting. The positions of Lck are shown on the left. Exposure was 13 h.

Fig. 4. Impact of deletion of the unique domain on Lck tyrosine phosphorylation in NIH 3T3 cells. A, anti-phosphotyrosine immunoblot. NIH 3T3 cell lines expressing either Arg\textsuperscript{273} (R) or ∆16–62R273 (∆U-R) Lck in the presence (+) or the absence (−) of CD45 were lysed in nonionic detergent-containing buffer. The extent of Lck tyrosine phosphorylation was monitored by anti-phosphotyrosine immunoblotting of anti-Lck immunoprecipitates (top panel). The abundance of Lck was also determined by immunoblotting of parallel immunoprecipitates with an antiserum directed against the SH3 domain of p56\textsuperscript{Lck} (bottom panel), whereas the levels of CD45 were verified by immunoblotting of anti-CD45 immunoprecipitates with a rabbit anti-CD45 serum. The positions of Lck and CD45 are indicated on the left. Exposures were 28 (top panel) and 15 h (middle and bottom panels). B, peptide mapping studies. Sites of phosphorylation were defined by cyanogen bromide cleavage analyses as described in the legend of Fig. 2B. The position of the C3 fragment, which encompasses tyrosine 505, is shown on the left. The migrations of prestained molecular mass markers are indicated in kDa on the right. Exposure was 2 days. C, cell fractionation studies. Cell were mechanically lysed in hypotonic buffer, and lysates were separated into particulate (P) and cytosolic (S) fractions by differential centrifugation. After extraction in detergent-containing buffer, Lck polypeptides were recovered by immunoprecipitation (I.P.) and detected by immunoblotting. The positions of Lck are shown on the left. Exposure was 13 h.
rulation of several intracellular proteins (15, 16). This biochemical modification leads to oncogenic cellular transformation, with its characteristic morphological alterations and the ability to form foci in monolayers and grow in semi-solid medium. To test whether CD45 could also regulate activated p56\(^{lck}\) molecules, CD45-positive NIH 3T3 cells were infected with retroviral transformation by Phe\(^{505}\) p56\(^{lck}\). CD45-negative neomycin-resistant NIH 3T3 cells (Neo) were used as control recipient. Immunoblot analyses showed that all infected cells expressed comparable amounts of Phe\(^{505}\) p56\(^{lck}\) (data not shown; see Fig. 7A, bottom panel).

Whereas fibroblasts expressing either the neomycin phosphotransferase alone (Fig. 5A) or CD45 alone (Fig. 5B) were flat and possessed short plasma membrane extensions, those containing Phe\(^{505}\) p56\(^{lck}\) without CD45 (Fig. 5C) were rounded and refractile and displayed multiple neuronal-like processes (15, 16). By contrast, cells expressing Phe\(^{505}\) Lck and CD45 (Fig. 5D) exhibited an intermediate morphology, being less rounded, less refractile, and showing fewer processes than cells containing activated Lck alone (Fig. 5C). The ability of these cells to form foci in monolayer cultures and colonies in soft agar was also assessed (Table I). Like cells containing Phe\(^{505}\) Lck alone, cells expressing Phe\(^{505}\) Lck and CD45 could form foci in monolayers. However, the foci were five times smaller than those generated by Phe\(^{505}\) Lck-expressing cells. Furthermore, although fibroblasts expressing Phe\(^{505}\) p56\(^{lck}\) formed large colonies in soft agar, cells expressing Phe\(^{505}\) Lck and CD45 failed to grow under this condition.

To elucidate the mechanism by which CD45 prevented oncogenic transformation by Phe\(^{505}\) p56\(^{lck}\), we studied its impact on the accumulation of phosphotyrosine-containing proteins (Fig. 6). Anti-phosphotyrosine immunoblotting of total cell lysates demonstrated that cells expressing Phe\(^{505}\) Lck and CD45 (Fig. 6A, lane 4) contained lower amounts of phosphotyrosine-containing proteins than cells expressing Phe\(^{505}\) p56\(^{lck}\) alone (lane 3). This diminution especially affected polypeptides of 140, 120, and 36 kDa. Individual substrates were also recovered by immunoprecipitation with specific antibodies, and their phosphotyrosine content was determined by anti-phosphotyrosine immunoblotting (Figs. 6, B–D). In keeping with the analysis of total cell lysates (Fig. 6A), tyrosine phosphorylation of the 36-kDa annexin II was absent in cells expressing Phe\(^{505}\) Lck and CD45 (Fig. 6B, lane 4) in contrast to cells containing Phe\(^{505}\) Lck alone (lane 3). However, CD45 had little or no impact on the extent of tyrosine phosphorylation of cortactin (Fig. 6C), as well as on that of GAP and its associated p190 and p62 (Fig. 6D). We were not able to determine the identity of the 140- and 120-kDa substrates that appeared to be tightly regulated by CD45 in Phe\(^{505}\) p56\(^{lck}\)-expressing cells (Fig. 6A, lane 4).

We wanted to determine whether the effect of CD45 on Phe\(^{505}\) Lck-expressing cells was due to dephosphorylation of Phe\(^{505}\) p56\(^{lck}\) or to dephosphorylation of downstream targets. To this end, the phosphotyrosine content of Phe\(^{505}\) Lck was first examined by anti-phosphotyrosine immunoblotting of anti-Lck immunoprecipitates (Fig. 7A). This study showed that expression of CD45 reduced the extent of tyrosine phosphorylation of Phe\(^{505}\) p56\(^{lck}\) by ~2-fold. To ascertain whether these...
changes were due to dephosphorylation of tyrosine 394, the positive regulatory site of p56<sup>Ick</sup>, peptide mapping studies were conducted (Fig. 7B). These analyses failed to show any reduction in phosphorylation of the C2 fragment of p56<sup>Ick</sup>, which contains tyrosine 394. Although a small decrease in C1 phosphorylation could be seen in CD45-positive cells in this experiment (Fig. 7B, lane 2), a similar change was not observed in other experiments (data not shown).

These findings raised the possibility that CD45 did not act by dephosphorylating Phe<sup>505</sup> p56<sup>Ick</sup> but rather by dephosphorylating its substrates. To help support this idea, the impact of CD45 on transformation by a variant of Phe<sup>505</sup> Lck lacking the unique domain was tested (Fig. 8 and Table II). Because the unique domain plays an important role in the CD45-mediated dephosphorylation of p56<sup>Ick</sup> (this report and Ref. 23), we reasoned that removal of this domain should have no impact on the effect of CD45 if it were due to dephosphorylation of downstream substrates. Thus, CD45-positive and CD45-negative NIH 3T3 cells were infected with retroviruses encoding Δ16–62F505 Lck. Cells containing Δ16–62F505 Lck without CD45 were morphologically transformed in a manner analogous to Phe<sup>505</sup> Lck-expressing cells (Table II and data not shown). Moreover, these cells were capable of growing in soft agar, albeit with a slightly lower efficiency than Phe<sup>505</sup> Lck-expressing cells containing Δ16–62F505 Lck and CD45. These findings indicated that CD45 did not act by dephosphorylating Phe<sup>505</sup> p56<sup>Ick</sup> in nonlymphoid cells.
mass markers are shown on the right bottom panel and 24 h (panel). The positions of the major tyrosine phosphorylation substrates dephosphorylation of tyrosine 505 in NIH 3T3 cells. Thus, CD45 inhibited Phe505 Lck-mediated transformation by a similar to that taking place in T-cells. (CD45) or antibodies directed against the SH3 region of Lck (probed by immunoblotting with either anti-phosphotyrosine antibodies, the unique region of p56
111
lck
16–62F505 Lck 10.4
12760
FIG. 8. Effect of CD45 on tyrosine protein phosphorylation in Δ16–62F505 Lck-expressing NIH 3T3 cells. Total cell lysates were probed by immunoblotting with either anti-phosphotyrosine antibodies (top panel) or antibodies directed against the SH3 region of Lck (bottom panel). The positions of the major tyrosine phosphorylation substrates as well as of Lck are indicated on the left. Those of prestained molecular mass markers are shown on the right in kDa. Exposures were 3 (top panel) and 24 h (bottom panel).

| Cell line        | Growth in soft agar | Morphologya |
|------------------|---------------------|-------------|
| Neo              | 0                   | 0           |
| CD45+ clone 31   | 0                   | 0           |
| Phe505 p56Δk     | 16.4b               | +++         |
| CD45+ clone 31 + Phe505 p56Δk | 0 | +       |
| Δ16–62F505 Lck   | 10.4c               | +++         |
| CD45+ clone 31 + Δ16–62 F505 Lck | 0 | +       |

a –, similar to parental NIH 3T3 cells; +, some rounding up with few visible cellular processes; ++++, rounded, refractile, with multiple neuronal-like processes.
b Colonies of 0.5–1.0 mm. c Colonies of 0.2–0.3 mm.

expressing Phe505 p56Δk (lane 4). Hence, these data indicated that CD45 inhibited Phe505 Lck-mediated transformation by a process independent of the Lck domain.

DISCUSSION

Herein, we report the first successful attempt at reconstituting the CD45-mediated regulation of p56Δk in a nonlymphoid system. The results of our experiments showed that expression of the R0 isoform of CD45 in NIH 3T3 fibroblasts caused a ~5-fold decrease in the extent of tyrosine 505 phosphorylation of kinase-defective (Arg273) p56Δk molecules. This effect was most likely due to the phosphatase activity of CD45, because treatment with pervanadate, a protein-tyrosine phosphatase inhibitor, restored phosphorylation of the carboxyl-terminal tyrosine of Lck in CD45-positive NIH 3T3 cells. As demonstrated for BI-141 T-cells (23), the unique region of p56Δk was also found to play an important role in the CD45-induced dephosphorylation of tyrosine 505 in NIH 3T3 cells. Thus, dephosphorylation of the inhibitory tyrosine of p56Δk by CD45 can occur in a nonlymphoid cellular system by a mechanism similar to that taking place in T-cells.

These findings implied that no other lymphoid-specific components are absolutely necessary for the CD45-mediated dephosphorylation of p56Δk. Specifically, they also demonstrated that CD45-associated protein, a protein that can associate with both CD45 and p56Δk (37–41) and is not expressed in NIH 3T3 cells, is not critical for the action of CD45 on p56Δk. This idea was further supported by our finding that enforced expression of CD45-associated protein in NIH 3T3 cells did not modify the ability of CD45 to dephosphorylate wild-type, Arg273, or Phe505 Lck molecules. Nevertheless, it is likely that additional features or processes enhance the ability of CD45 to dephosphorylate tyrosine 505 in T-cells. Indeed, although CD45 provoked dephosphorylation of kinase-inactive Lck molecules in fibroblasts, it failed to stably dephosphorylate wild-type Lck polypeptides. In contrast, the two forms of Lck were equally well dephosphorylated in T-cells (23). The higher amounts of CD45 typically present in T-cells may explain this difference. Possibly, greater amounts of CD45 are needed for stable dephosphorylation of kinase-active Lck polypeptides in vivo. Alternatively, the presence of other yet unidentified molecules interacting with CD45, Lck or both, may contribute to more efficient dephosphorylation of Lck in T-cells.

These findings provided clear evidence that the catalytic activity of p56Δk can influence the ability of CD45 to stably dephosphorylate tyrosine 505. It is conceivable that the enzymatic function of p56Δk antagonizes the effect of CD45 by allowing autophosphorylation at tyrosine 505 (21, 22). Alternatively, the enzymatic activity of Lck may facilitate the recruitment of p50Δk, the tyrosine-protein kinase normally responsible for phosphorylating tyrosine 505 (Refs. 16–19). Lastly, it is possible that active Lck molecules can modify the function of CD45 and reduce its ability to dephosphorylate tyrosine 505. Regardless of the mechanism underlying this phenomenon, these observations raised the interesting possibility that activation of p56Δk may diminish the capacity of CD45 to dephosphorylate tyrosine 505 in T-cells, thereby providing a potential negative feedback mechanism. This concept may explain the earlier finding that CD44-mediated activation of p56Δk in T-cells led to a paradoxical augmentation of tyrosine 505 phosphorylation, in addition to the expected increase in tyrosine 394 phosphorylation (50, 51).

The impact of CD45 on an activated version of p56Δk (Phe505 Lck) was also evaluated. Contrary to cells containing Phe505 p56Δk alone, cells harboring Phe505 p56Δk and CD45 exhibited a less transformed morphology, formed smaller foci in monolayer cultures, and were unable to grow in soft agar. These biological effects were accompanied by a noticeable reduction in the extent of tyrosine protein phosphorylation induced by Phe505 p56Δk. Several findings indicated that the impact of CD45 in this context was primarily caused by dephosphorylation of downstream signaling targets rather than dephosphorylation of Phe505 p56Δk. First, the phosphotyrosine content of Phe505 p56Δk was only minimally reduced (less than ~2-fold) in CD45-positive NIH 3T3 cells. Second, CD45 did not cause a global reduction of tyrosine protein phosphorylation in Phe505 p56Δk-expressing cells. Instead, it seemed to regulate a specific set of tyrosine phosphorylation substrates. These included annexin II, as well as polypeptides of 140 and 120 kDa of yet undetermined identity. In contrast, CD45 had no or little effect on substrates such as cortactin and GAP, as well as the GAP-associated p190 and p62. Third, removal of the Lck unique domain had no consequence on the ability of CD45 to inhibit transformation by Phe505 Lck. Because the unique region was crucial for CD45-mediated dephosphorylation of Lck in other systems (this report and Ref. 23), we feel that this observation provided a most compelling indication that CD45 reduced

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The biological characteristics of NIH 3T3 derivatives were analyzed to determine the impact of CD45 on the growth in soft agar and the morphology of the colonies. The data are presented in Table II.

Table II: Biological characteristics of NIH 3T3 derivatives

| Cell line | Growth in soft agar | Morphologya |
|-----------|---------------------|-------------|
| Neo       | 0                   | 0           |
| CD45+ clone 31 | 0 | 0          |
| Phe505 p56Δk | 16.4b | +++        |
| CD45+ clone 31 + Phe505 p56Δk | 0 | +         |
| Δ16–62F505 Lck | 10.4c | +++       |
| CD45+ clone 31 + Δ16–62 F505 Lck | 0 | +         |

a –, similar to parental NIH 3T3 cells; +, some rounding up with few visible cellular processes; ++++, rounded, refractile, with multiple neuronal-like processes.

b Colonies of 0.5–1.0 mm. c Colonies of 0.2–0.3 mm.
transformation by Phe505 Lck by acting on downstream signaling events.

These results suggested that CD45 may also have the ability to negatively regulate Lck-mediated signals in T-cells. This notion is consistent with the observation that antibody-mediated co-aggregation of CD45 and the T-cell antigen receptor prevented T-cell receptor-induced intracellular tyrosine protein phosphorylation and T-cell activation (52). Similarly, it supports the finding that some CD45-negative T-cell lines (such as YAC-N1) contained elevated levels of phosphorytrose-containing proteins prior to T-cell receptor stimulation (29, 53). Hence, CD45 may provide inhibitory signals in T-cells, in addition to its aforementioned positive regulatory role in unstimulated T-cells. Obviously, if these two opposite effects were to occur physiologically, it is expected that they would be differentially regulated at the various stages of T-cell activation.

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