Coclustering CD45 with CD4 or CD8 Alters the Phosphorylation and Kinase Activity of p56ck
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Summary
Antibody-mediated CD4 crosslinking results in increased tyrosine phosphorylation and tyrosine kinase activity of the associated p56ck. Treatment with anti-CD4 and anti-Ig also induced the phosphorylation of p56ck in a CD45- mutant cell line, indicating that the increase in phosphotyrosine content of p56ck is not the result of being sequestered from CD45 protein tyrosine phosphatase (PTPase). Antibody-mediated coclustering of CD45 with CD4 inhibited the anti-CD4-induced phosphorylation of p56ck on tyrosine and the concomitant increase in vitro kinase activity. Similar results were obtained when CD45 was coclustered with CD8 on cytotoxic T cell lines. These observations provide strong evidence that p56ck is a substrate for CD45 in vivo and provide an essay to study the regulation and specificity of CD45 PTPase activity.

Materials and Methods

Cell Lines and Antibodies. The CD45+ parental and CD45- mutant SAKRTLS 12.1 cell lines (4), the CD4+ Th hybridoma AODH 7.1 (10), and the IL-2-dependent CD8+ cytotoxic cell line L3A1 (11) are previously described murine lymphoid cell lines. L3A1 is a variant that has been maintained in IL-2 without antigen stimulation but still maintains its antigen specificity and cytotoxic function. The mAb 13/2.3 is specific for murine CD45 (12). The mAbs R171.2, directed against the transferrin receptor (TR), GK1.5, directed against CD4, and 53.6.72, directed against CD8, were obtained through the American Type Culture Collection (Rockville, MD).

Antibody Clustering and Immunoprecipitations. Cells were incubated at 107/ml in serum-free DMEM in the presence of 5-10 μg/ml antibody for 30 min on ice. Cells were then washed once in serum-free DMEM and resuspended in DMEM at 4 x 107/ml. 10 μl of rabbit anti-rat Ig antisera (United States Biochemicals Corp., Cleveland, OH) was added, and the cells were incubated for 2-15 min at 37°C. For blots of total cell lysates, the cells were pelleted and lysed in boiling 0.5% SDS in 10 mM Tris-HCl (pH 8.0). DNA in the samples was then sheared by passing several times through a 22-gauge needle. An equal volume of 2× Laemmli SDS-electrophoresis sample buffer was added, and the sample was boiled again. For immunoprecipitations, the cells were lysed in 1% NP-40/10 mM Tris-HCl (pH 8.0)/150 mM NaCl. Lysates were clarified by centrifugation for 10 min in a microfuge and then immunoprecipitates prepared with an anti-p56ck antiserum (13), generously provided by Dr. Bart Sefton (The Salk Institute). Immunoprecipitates were subsequently washed three times with 0.5% NP-40 in the same buffer and twice with buffer alone.

Immunoblotting. Samples containing 1-2 x 106 cell equivalents were subjected to electrophoresis on an SDS/9.5% polyacrylamide gel. Proteins were then transferred to Immobilon-P (Millipore Continental Water Systems, Bedford, MA) in 20% methanol/20 mM Tris base/96 mM glycine for 2-3 h at 60 V in a transfer apparatus (Bio-Rad Laboratories, Richmond, CA). Immunoblotting was performed with rabbit anti-p56ck antiserum (13) or affinity-purified rabbit antiphosphotyrosine antiserum (4) and detected with 125I-labeled protein A (ICN, Irvine, CA). Prestained SDS electropho-
resis molecular size markers (Sigma Chemical Co., St. Louis, MO) were used as standards.

**Kinase Assays.** Immunoprecipitated samples prepared as described above were incubated in kinase buffer (10 mM Pipes, pH 7.2/10 mM MnCl₂) containing 5 μCi of γ-[³²P]ATP (ICN) for 10–20 min at 30°C. To stop the reaction, an equal volume of 2× reducing Laemmli SDS-electrophoresis sample buffer was added, and the samples were immediately boiled for 2 min. The samples were subjected to electrophoresis on an SDS/10% polyacrylamide gel. Gels were stained in Coomassie blue, dried, and exposed to X-omat film for 10–30 min at room temperature.

**Results**

**CD45 Is not Required for Phosphorylation of p56kk Induced by CD4 or CD8 Clustering.** As previously reported (8, 9), crosslinking of CD4 on the cell surface with an anti-CD4 mAb and second anti-Ig antibody leads to a rapid increase in the tyrosine phosphorylation of p56kk, as detected by immunoblotting of total cell proteins (data not shown) or anti-p56kk immunoprecipitates with antiphosphotyrosine antibodies (Fig. 1 A). If CD45 were normally to play a regulatory role in maintaining a low basal level of phosphorylation of p56kk, then clustering of CD4/p56kk or CD8/p56kk complexes might sequester p56kk from the CD45 PTPase, and lead to the observed increase in the phosphotyrosine content of p56kk. To investigate this possibility, CD4 was clustered on the surface of CD45- mutant and CD45+ parental SAKRTLS 12.1 cells, and the effects on the phosphorylation of p56kk were analyzed. As shown in Fig. 1, antibody crosslinking of CD4 on the surface of SAKRTLS 12.1 CD45 cells induced a similar increase in the phosphotyrosine content of p56kk and its kinase activity, as in the CD45+ parental cells. This indicates CD45 is not required to observe this effect and that CD4 mAb crosslinking is not merely sequestering p56kk from the CD45 phosphatase domains, thus inducing an increase in phosphotyrosine content.

**Coclustering of CD45 with CD4 Inhibits CD4-induced Phosphorylation of p56kk.** We then determined if coclustering CD45 with CD4 in these SAKRTLS 12.1 cell lines had any effect on the increased tyrosine phosphorylation of p56kk induced by CD4 clustering. Fig. 1 A clearly demonstrates that when CD45 is cocrosslinked with CD4, there is no longer an increase in tyrosine phosphorylation. Immunoblotting of replicate samples of immunoprecipitates with anti-p56kk antibodies confirmed that they contained equal amounts of p56kk (data not shown). The change in phosphotyrosine content of p56kk was also associated with a concomitant increase in kinase activity of p56kk, as determined by in vitro autophosphorylation (Fig. 1 B). A similar though less dramatic increase in the ability of p56kk to phosphorylate enolase in vitro was also observed (data not shown). Clustering of CD45 alone had no direct effect on either the tyrosine phosphorylation of p56kk nor its in vitro kinase activity. However, cocrosslinking of CD45 with CD4 completely abolished the increase in phosphorylation on tyrosine of p56kk and the increase in kinase activity induced by anti-CD4 mAbs alone. This effect was specific, as coclustering of CD4 with the transferrin receptor did not reduce the anti-CD4 mAb-induced increase in phosphorylation of p56kk (data not shown) or the associated increase in in vitro kinase activity (Fig. 1 B).

The observation that CD45 can inhibit the phosphorylation on tyrosine of p56kk stimulated by CD4 crosslinking is not restricted to SAKRTLS 12.1 cells. As shown in Fig. 2 A, the same result was obtained if CD4 and CD45 were crosslinked on AODH 7.1 cells, a Th hybridoma, and two other CD4+ functional Th cell lines. In addition, cocrosslinking of CD8 with CD45 on the cytotoxic T cell line L3AI also inhibits the phosphorylation of p56kk induced by crosslinking with CD8 mAbs alone (Fig. 2 B). Similar results were obtained with three other cytotoxic T cell lines (data not shown).
It is possible that coclustering CD4 or CD8 with CD45 could inhibit phosphorylation by an indirect mechanism such as steric hindrance, and not by the action of CD45 PTPase. To investigate this issue, we first induced phosphorylation of p56\(^{kk}\) on tyrosine by crosslinking with anti-CD4 mAbs alone and subsequently coclustered CD45 to determine whether CD45 can dephosphorylate p56\(^{kk}\) under these circumstances. The results of such an experiment are shown in Fig. 3. AODH 7.1 cells were treated at 4°C for 30 min with anti-CD4 mAb, washed, and incubated with anti-Ig antibodies at 37°C for an additional 2 min. One replicate sample of cells was then immediately boiled in electrophoresis sample buffer (Fig. 3, lane 2), another incubated for an additional 13 min at 37°C (lane 4), and a third incubated for 13 min at 37°C after the addition of anti-CD45 mAb (lane 6). Other controls included cells incubated for a total of 15 min for 37°C with anti-Ig alone (Fig. 3, lane 1), or with anti-CD45 and anti-Ig (lane 7). Treatment with anti-CD4 mAb and anti-Ig for 2 min at 37°C induced a dramatic increase in phosphorylation of p56\(^{kk}\) (Fig. 3, lane 2) that was sustained during the 15-min incubation (lane 4). Addition of anti-CD45 mAbs at 2 min, however, almost completely reversed the increase in phosphorylation of p56\(^{kk}\) induced by clustering of CD4/p56\(^{kk}\) complexes. This result strongly suggests that coclustering of CD45 with CD4 leads directly to the dephosphorylation of p56\(^{kk}\). Conversely, coclustering of CD4 and CD45 neither induces phosphorylation of CD45 on tyrosine that could be detected by immunoblotting with antiphosphotyrosine antibodies nor alters the in vitro PTPase activity of CD45, indicating that p56\(^{kk}\) does not act reciprocally on CD45 (data not shown).

Discussion
The demonstration that clustering of CD45 with CD4/p56\(^{kk}\) and CD8/p56\(^{kk}\) complexes results in the dephosphorylation of p56\(^{kk}\) is important for several reasons. First, it provides additional evidence that p56\(^{kk}\) can serve as a substrate for CD45 in vivo. Second, it raises the possibility that one mechanism by which the action of CD45 may be regulated during T cell activation or other physiological responses is by redistribution in the cell membrane that brings CD45 into close proximity with a specific substrate. Such a rearrangement might involve molecular interactions between the external domain of CD45 and other cell membrane molecules either on the same cell or an adjacent cell. Finally, antibody-mediated coclustering of CD45 with CD4 or other cell membrane molecules provides an in vivo assay to investigate the specificity of CD45 PTPase activity and its regulation. For example, crosslinking of CD45 with the receptor for epidermal growth factor (EGF) expressed in a human Burkitt's lymphoma cell line (14) does not inhibit the autophosphorylation of the receptor induced by EGF binding (Ostergaard, H.L., R. Taetle, S. Smedrud, and I. Trowbridge, unpublished results). Further, crosslinking of CD45 with the IL-2-R does not inhibit the increased protein tyrosine phosphorylation induced by IL-2 binding (D. Shackelford, personal communication). Both of these results indicate that coclustering of CD45 with phosphotyrosine-containing proteins is not sufficient for their dephosphorylation and suggest that CD45 has restricted substrate specificity in vivo.
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