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Immunology

Protein-tyrosine-phosphatase CD45 is phosphorylated transiently on tyrosine upon activation of Jurkat T cells

(transmembrane protein/phytohemagglutinin/phenylarsine oxide)

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ABSTRACT **The leukocyte common antigen (CD45) is an abundant lymphocyte surface antigen that has been reported to be involved in signaling through the T-cell antigen receptor. CD45 is a transmembrane protein-tyrosine-phosphatase. An internal segment comprises two domains each of which is homologous to other protein-tyrosine-phosphatases; the extracellular segment has the hallmarks of a ligand-binding motif. Since tyrosine phosphorylation is an early signal resulting from stimulation of the T-cell antigen receptor and CD45 is required for proper activation through the receptor, we explored whether CD45 might be regulated by tyrosine phosphorylation. Treatment of a T-cell leukemia line (Jurkat) with either phytohemagglutinin or anti-CD3 antibodies induced phosphorylation of tyrosine residues in CD45; treatment with phorbol 12-myristate 13-acetate did not. Phosphorylation of CD45 was transient, disappearing within 40 min after phytohemagglutinin treatment. The requirement for stringent conditions of phosphatase inhibition suggests that CD45 is capable of autodephosphorylation *in vivo*. These observations support recent reports indicating CD45 is involved in an early step in the T-cell activation cascade. They also suggest that phosphorylation/dephosphorylation of tyrosine residues in CD45 should be explored further as a possible regulatory mechanism.**

The role of protein-tyrosine kinases (PTKs) in signal transduction and cell proliferation (1, 2) attests to the importance of phosphorylation of tyrosine residues, despite the observation that it accounts for only a small percentage of the total phosphate associated with protein *in vivo* (3). Strict control over the level of phosphorylation, particularly of tyrosine residues, seems to be necessary for normal cell growth, since overexpression or mutation of endogenous PTKs often leads to transformation of the affected cells (2). Dephosphorylation by protein-tyrosine-phosphatases (PTPases) provides one mechanism for controlling the level of phosphotyrosine. The recent discovery of at least 15 PTPases (4, 5), as well as identification of 27 different PCR fragments from *Styela plicata* (6) that are homologous to known PTPase domains, suggests that the PTPases are as diverse and as important for control of phosphotyrosine levels as are the PTKs. As an example, the protein-serine/threonine kinase $p34^{cdc2}$, which controls the transition from the G_2 phase of the cell cycle into mitosis, is activated by the dephosphorylation of a tyrosine residue in its ATP-binding site (7). In addition, a PTPase has been identified as the pathogenic agent of the plague (8), while another is encoded by the vaccinia viral genome (9). Such observations suggest that PTPases will be identified as critical elements in the control of a diverse array of fundamental cellular processes. Thus, characterization of the mechanisms that control their activity will be an essential step in understanding the physiological role of reversible protein tyrosine phosphorylation.

The stimulation of T lymphocytes by antigen, by antibodies to the T-cell antigen receptor (TCR)/CD3 complex, or by mitogenic lectins results in hydrolysis of inositolphospholipids, mobilization of Ca^{2+} from intracellular stores, cytokine secretion, and proliferation (10–12). There is substantial evidence that the TCR-mediated activation of T cells induces a rapid increase in PTK activity that leads to increased phosphorylation of the ζ chain of the TCR (13) and several other intracellular targets (14, 15). The PTKs $p56^{lck}$ (16), which is associated with the CD4 or CD8 cell surface antigens, and $p59^{fyn}$ (17, 18), which may be associated with the TCR, may be involved in these early tyrosine phosphorylation events, but their precise roles have not yet been established.

In fact, recent findings indicate that a transmembrane PTPase, CD45 (19, 20), is required for induction of the early tyrosine phosphorylation of proteins and for effective coupling of the TCR to the phosphatidylinositol pathway (21), suggesting that antigen-induced dephosphorylation is required for activation of PTKs. The involvement of both PTKs and PTPases in T-cell activation pathways raises the possibility of direct regulatory interactions between the two opposing activities. An example of this type of regulation is provided by CD45 and $p56^{lck}$, where dephosphorylation of the kinase *in vitro* results in activation (22). Given the requirement for both PTPases and PTKs for complete T-cell activation, we sought evidence that CD45 might be directly regulated by the PTKs. Using phospho amino acid analysis and anti-phosphotyrosine antibodies, we have demonstrated that CD45 itself is phosphorylated on tyrosine residues *in vivo* following stimulation with either the mitogenic lectin phytohemagglutinin (PHA) or anti-CD3 antibodies. Our findings show that CD45 in T cells is a target for an as yet unknown PTK. This observation suggests that reversible phosphorylation of tyrosine residues in CD45 may be of regulatory significance.

MATERIALS AND METHODS

Cell Cultures and Extractions. The human acute T-cell leukemia cell line Jurkat (clone E6-1, ATCC TIB 152), was grown at 37°C in RPMI 1640 with 10% newborn calf serum. Before treatment, the cells were concentrated to 1–5 × 10^7 cells per ml in 10 ml of RPMI 1640 medium. The cells were stimulated with either PHA-L (5 μg/ml; Sigma), phorbol 12-myristate 13-acetate (PMA, 5 μg/ml; Sigma), both PHA and PMA, or anti-CD3 antibody (G19-4, 5 μg/ml). The PTPase inhibitor phenylarsine oxide (PhAsO, Sigma), dissolved in dimethyl sulfoxide, was added to the cells 10 min

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Abbreviations: PHA, phytohemagglutinin; PhAsO, phenylarsine oxide; PMA, phorbol 12-myristate 13-acetate; PTK, protein-tyrosine kinase; PTPase, protein-tyrosine-phosphatase; TCR, T-cell antigen receptor; PVDF, poly(vinylidene difluoride).

before extraction with buffer A [1% (vol/vol) Nonidet P-40/10 mM EDTA/50 mM Tris·HCl, pH 7.5/1 mM phenylmethanesulfonyl fluoride/1 mM benzamidine/0.001% aprotinin/0.001% pepstatin/0.001% leupeptin] containing 100 μM iodoacetate and 100 μM PhAsO and titrated to pH 1.5 with HCl. The extracts were homogenized and insoluble cell debris was pelleted at 10,000 rpm in a Sorvall SS-34 rotor.

^{32}P Labeling. Jurkat cells were washed for 30 min in phosphate-free medium and then centrifuged and resuspended to a concentration of 1–5 × 10^8 cells per ml in 2 ml of phosphate-free medium. The cells were incubated with $KH_2{}^{32}PO_4$ (500 μCi/ml; 1 μCi = 37 kBq) for 4 hr at 37°C; stimulation and extraction were as described above.

Immunoprecipitations and Immunoblotting. Fifty micrograms of the appropriate antibody was incubated with cell extracts (adjusted to pH 7.5) for 2–12 hr at 4°C. Protein A-Sepharose (5 mg, Pharmacia) was used to precipitate the primary antibody and the proteins bound to it. The precipitates were washed twice in 10 ml of buffer A, twice in 10 ml of buffer A/500 mM NaCl, and twice in 10 ml of 50 mM Tris·HCl, pH 7.5/200 μM Na_3VO_4/100 μM PhAsO. The samples were solubilized in SDS sample buffer and subjected to electrophoresis in an SDS/7.5% polyacrylamide gel (23). Following transfer to poly(vinylidene difluoride) (PVDF) membrane (Millipore), blots were incubated with anti-CD45 antibody (monoclonals 9.4 and Gap 8.3 or polyclonal 7671) or anti-phosphotyrosine monoclonal antibody 4G-10 (Upstate Biotechnology). The blots were developed with goat anti-mouse or goat anti-rabbit secondary antibody complexed to horseradish peroxidase (Vector Laboratories).

Phospho Amino Acid Analysis. Solubilized anti-CD45 immunoprecipitates prepared from ^{32}P-labeled cells were subjected to a second round of immunoprecipitation by first precipitating proteins with acetone (to remove SDS) and repeating the immunoprecipitation and wash procedure with a different anti-CD45 antibody. After SDS/PAGE and immunoblotting, the bands detected with anti-CD45 antibody were excised for phospho amino acid analysis (24).

PTPase Assays. CD45 was immunoprecipitated from extracts of Jurkat cells lysed in 1% Nonidet P-40/50 mM Hepes/0.1% 2-mercaptoethanol/100 mM NaCl. The precipitates were washed four times in the same buffer without 2-mercaptoethanol, to remove any residual reducing agent. The reducing agents and PhAsO were added to separate aliquots as indicated before PTPase assay as described previously (25).

***In Vitro* Phosphorylation.** CD45 was phosphorylated *in vitro* by v-abl (gift from Curt Diltz) and $pp60^{c\text{-}src}$ (Oncogene Science, Manhasset, NY) with the protocol supplied by Oncogene Science. In brief, the appropriate kinase was incubated for 10 hr at 30°C in 50 mM Hepes, pH 7.5/0.1 mM EDTA/0.15% Brij 35/0.1% 2-mercaptoethanol/0.01% bovine serum albumin/10 mM $MgCl_2$/0.1 mM ATP (200 μCi of [γ-^{32}P]ATP per ml). Na_3VO_4 (1 mM) was included in the assay buffer to prevent autodephosphorylation. Phosphorylation was detected by autoradiograms of Western blots performed for each assay.

RESULTS AND DISCUSSION

The discovery of potent inhibitors of the PTPases (26), most notably orthovanadate (Na_3VO_4), has facilitated the detection of phosphotyrosine in proteins. Pretreatment of cells with PhAsO has been shown to enhance recoveries of phosphotyrosine (27) but to have no apparent effect on the activity of two PTKs, $p56^{lck}$ and $p59^{fyn}$, *in vitro* (15). CD45 was observed to be phosphorylated by v-abl and $pp60^{c\text{-}src}$ *in vitro*, but only in the presence of PTPase inhibitors such as Na_3VO_4 (data not shown), suggesting that CD45 can autodephosphorylate *in vitro*. Inclusion of PTPase inhibitors in the extraction solvent was not sufficient to allow detection of significant levels of phosphotyrosine in CD45 from PHA-treated Jurkat cells (Fig. 1, lanes A and B). However, phosphotyrosine was clearly observed in CD45 when T cells were pretreated with PhAsO prior to PHA stimulation and extraction (lane D), suggesting that a PTPase inhibitor is required to block autodephosphorylation *in vivo* as well. These results indicate that phosphorylation of CD45 on tyrosine residues is induced by PHA treatment.

To explore the mechanism by which PhAsO enhances the detection of phosphotyrosine, we examined the effect of PhAsO on isolated CD45. Since the PTPases are inhibited by reagents that modify thiols (28) and PhAsO reacts with vicinal sulfhydryls as well as sulfhydryls proximal to hydroxyls or carboxyls (29), PhAsO may inhibit the PTPases in this manner. Garcia-Morales *et al.* (15) have reported that CD45 is inhibited by PhAsO in the absence of reducing agents. However, the activity of CD45 is lost rapidly in the absence of reducing agents even without PhAsO present (Fig. 2). In the presence of 0.1% 2-mercaptoethanol, CD45 retains its activity and is not inhibited by PhAsO (Fig. 2). Apparently, 2-mercaptoethanol can compete with CD45 for PhAsO. Since glutathione (≈5 mM) is the major intracellular reducing agent (30), we tested its ability to stabilize CD45 *in vitro* and to interfere with inhibition by PhAsO. Glutathione does protect the activity of CD45, but it does not prevent inhibition of CD45 by PhAsO (Fig. 2). Thus, these data suggest that PhAsO can inhibit CD45 *in vivo*.

Fig. 3 demonstrates that the anti-phosphotyrosine antibodies recognize phosphotyrosine and not some other feature of CD45. Two bands were observed on a Western blot with anti-phosphotyrosine monoclonal antibody; these correspond to those detected by anti-CD45 antibodies (Fig. 1). Binding of anti-phosphotyrosine antibody to CD45 was completely blocked by phosphotyrosine, whereas phosphoserine and phosphothreonine were without effect. The anti-phosphotyrosine antibody no longer recognized CD45 after pretreatment of the Western blots with active CD45. Thus CD45, which is specific for phosphotyrosine residues *in vitro* (31), is capable of dephosphorylating the denatured CD45 molecule. These controls demonstrate that the anti-phosphotyrosine antibody specifically detects phosphotyrosine in CD45. Furthermore, phospho amino acid analysis confirmed the presence of phosphotyrosine in twice-immunoprecipitated CD45 from PHA-treated cells (Fig. 3*B*)

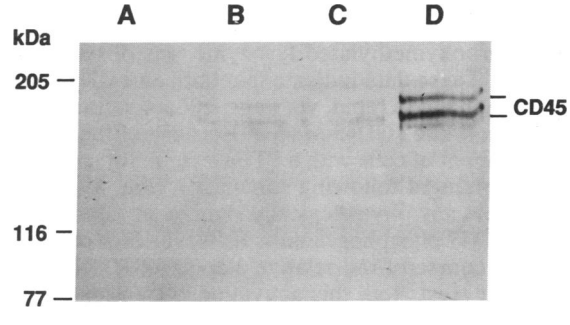

FIG. 1. Pretreatment of cells with PhAsO is required for optimal detection of phosphotyrosine in CD45. Jurkat cells (5 × 10^7) were treated with PHA (5 μg/ml) and extracted as indicated. CD45 was immunoprecipitated with monoclonal antibody 9.4 and analyzed by SDS/PAGE. Protein bands were then transferred to PVDF membrane and immunoblotted with an anti-phosphotyrosine antibody. Lane A, 200 μM Na_3VO_4/buffer A; lane B, extraction with PTPase inhibitor buffer (buffer A/200 μM Na_3VO_4/100 μM iodoacetic acid/100 μM PhAsO; titrated to pH 1.5 with HCl); lane C, 37 μM PhAsO added 10 min before extraction (concurrent with PHA treatment) with buffer A/200 μM Na_3VO_4; lane D, 37 μM PhAsO pretreatment (as in lane C), and extraction with PTPase inhibitor buffer.

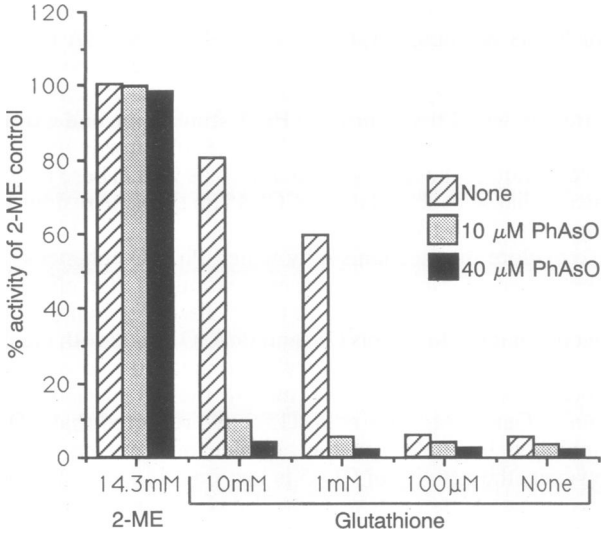

FIG. 2. Effects of PhAsO on CD45 in the presence of glutathione. PTPase activity of CD45 precipitated from 5×10^6 Jurkat cells was measured to determine the effect of PhAsO on CD45 activity in the presence of 14.3 mM (0.1%, vol/vol) 2-mercaptoethanol (2-ME) or various concentrations of glutathione. CD45 was immunoprecipitated and washed extensively in 50 mM Hepes, pH 7.2/0.1% Triton X-100 to remove any residual reducing agent. The indicated reducing agents were added back to separate aliquots of CD45, followed by addition of PhAsO, as indicated. Activity was measured relative to the activity of CD45 in 14.3 mM 2-ME without PhAsO present.

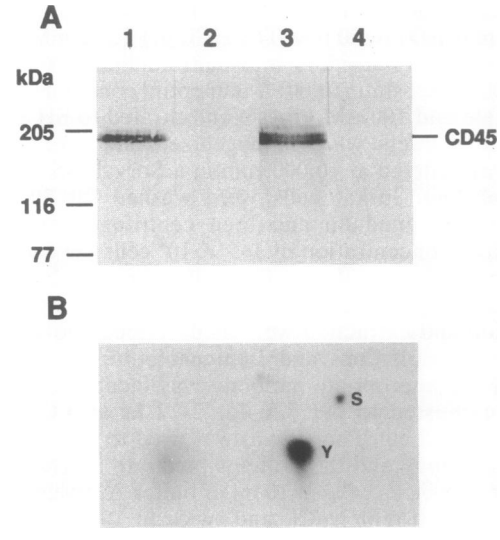

FIG. 3. PHA-induced phosphorylation of CD45. (*A*) Western blots of extracts of PHA-stimulated Jurkat cells. Jurkat cells were activated and extracted as in Fig. 2. Each lane contained the protein immunoprecipitated, with monoclonal antibody 9.4, from $\approx 5 \times 10^7$ Jurkat cells. Four identical immunoprecipitates were subjected to electrophoresis and transferred to PVDF membrane. The lanes were separated in order to assess the specificity of the antibody by several types of controls. Lane 1, anti-phosphotyrosine antibody; lane 2; blot was pretreated for 10 min at 37°C with 50 μg of active CD45, then immunoblotted with anti-phosphotyrosine antibody; lane 3, anti-phosphotyrosine antibody plus 5 mM phosphoserine and 5 mM phosphothreonine; lane 4, anti-phosphotyrosine antibody and 1 mM phosphotyrosine. (*B*) Phospho amino acid analysis of CD45 isolated from PHA- and PMA-treated Jurkat cells. Jurkat cells (5×10^8) were ^{32}P-labeled and then treated with PHA (5 μg/ml), PMA (5 μg/ml), and PhAsO (37 μM) 10 min prior to extraction in PTPase inhibitor buffer (see Fig. 1). The extracts were twice immunoprecipitated as described in *Materials and Methods*, first with anti-CD45 monoclonal antibody 9.4 then with anti-CD45 polyclonal antibody 7671. The washed pellet was subjected to electrophoresis and transferred to PVDF. Two bands detected by immunoblotting with another CD45-specific monoclonal antibody, Gap 8.3, were excised for phospho amino acid analysis. Positions of phospho amino acid standards (S, phosphoserine; Y, phosphotyrosine) as detected by ninhydrin staining are indicated.

and established the lack of phosphotyrosine in CD45 from untreated cells.

To probe the physiological significance of this phosphorylation, a time course of phosphorylation of CD45 was examined. Phosphotyrosine was observed in CD45 within 5 min of PHA treatment and was no longer detected after 40 min (Fig. 4). Similar results were observed following treatment of the cells with anti-CD3 antibodies and with a mixture of PHA and PMA (data not shown); however, PMA treatment alone did not lead to detectable phosphorylation of CD45 on tyrosine residues. The effect of this phosphorylation on CD45 activity is not readily assessed since isolation of phosphorylated CD45 requires inhibition of activity. To address the possibility that PHA has a direct effect on CD45, the PTPase activity of antibody-purified CD45 was assayed in the presence and absence of PHA. No significant effect of PHA 5–50 μg/ml on PTPase activity toward myelin basic protein or reduced, carboxymethylated lysozyme was observed (data not shown). These data indicate that both anti-CD3 antibodies and PHA induce a rapid, yet transient, activation of a PTK that phosphorylates CD45 *in vivo*. Because of the necessity for pretreatment of cells with a PTPase inhibitor, the amount of ^{32}P incorporated following stimulation (Fig. 4) cannot be used to obtain any physiologically significant estimate of the extent of CD45 phosphorylation. However Fig. 4 does provide a time course of the relative activity of PTK(s) toward CD45 and suggests that this activation is transient.

PHA binds to the TCR as well as other surface antigens, including CD3, CD2, and CD45 (11). Activation of T cells with PHA induces proliferation, stimulates synthesis of interleukin 2 and its receptor (11), and rapidly increases PTK activity toward several substrates. Although PHA may interact with multiple cell surface antigens, T-cell responses to PHA resemble those observed with agents that act directly upon the TCR complex. T cells treated with PTK inhibitors (13) and T cells lacking CD45 (21) fail to elicit phosphatidylinositol-derived second messengers, implying that both kinases and phosphatases play an active role in T-cell activation. In fact, Koretzky *et al.* (32) have demonstrated that CD45 appears to be required for activation of at least one PTK during T-cell activation. The transient phosphorylation of CD45 was observed following stimulation with either PHA or anti-CD3 antibodies but not with phorbol ester treatment. Thus we conclude that CD45 is a physiologically relevant substrate for a T-cell PTK and that the nature of the stimulus that causes phosphorylation of CD45 is consisent with its direct involvement in signaling through the TCR complex.

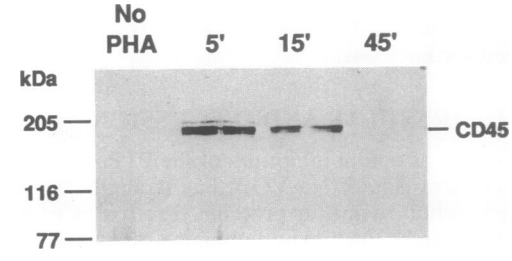

FIG. 4. Time course for PHA-induced phosphorylation of CD45. Aliquots of 5×10^8 cells were treated with PhAsO 10 min before extraction, independent of the time of activation. PHA (5 μg/ml) was added 5, 15, or 40 min before extraction. Western blots of anti-CD45 immunoprecipitates were immunostained with anti-phosphotyrosine antibodies.

The requirement for both PTKs and PTPases in the early stages of T-cell activation, the ability of CD45 to activate $p56^{lck}$ by dephosphorylation *in vitro*, and now the observation that CD45 is phosphorylated and then rapidly dephosphorylated (in the absence of inhibitors) during T-cell activation raise the intriguing possibility that the PTKs and PTPases actually cooperate to transduce a signal. Traditionally it has been proposed that the kinase transduces a signal and then eventually the phosphatase shuts it off. This model is gradually giving way to one in which there is a more dynamic relationship between the PTKs and PTPases where both are required to transduce a signal and there is continual bilateral feedback between the two processes.

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