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Activators of Protein Kinase C Induce Dissociation of CD4, But Not CD8, from p56^{lck}

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The CD4 and CD8 T cell receptor accessory molecules can both be isolated from T lymphocytes in association with p56^{lck}, a membrane-associated, cytoplasmic tyrosine protein kinase that is expressed exclusively in lymphoid cells. The enzymatic activity of p56^{lck} may therefore be regulated by CD4 and CD8 and be important in antigen-induced T cell activation. Exposure of human T cells and some mouse T cells to the tumor promoter 12-O-tetradecanoyl phorbol-13-acetate (TPA), an activator of protein kinase C, caused the dissociation of p56^{lck} and CD4. Activation of protein kinase C may therefore interrupt regulation of p56^{lck} by CD4 and alter the ability of p56^{lck} to interact with polypeptide substrates. In contrast, exposure of cells to TPA did not cause dissociation of p56^{lck} and CD8. Regulation of p56^{lck} by CD4 may therefore differ from regulation by CD8.

THE p56^{lck} PROTEIN IS A MEMBER OF the src family of cellular tyrosine protein kinases (*1*). It is found in essentially all T cells (*1*), where it is presumed to bind to the cytoplasmic face of the plasma membrane. A continuing question has been whether the protein kinases of this family are regulated directly by proteins expressed on the cell surface. The finding that p56^{lck} is physically associated with the T cell accessory molecules CD4 and CD8 (*2*) suggests that its activity may be regulated by these molecules during T cell activation. This is of potential significance in the control of lymphoid cell proliferation and development because expression of constitutively activated p56^{lck} can induce unregulated proliferation of fibroblasts (*3*). In addition, it has been reported that cross-linking of CD4 with antibodies increases the in vitro tyrosine protein kinase activity of p56^{lck} (*4*).

The CD4, CD8, and p56^{lck} proteins all undergo phosphorylation on serine when lymphoid cells are treated with phorbol esters (*5–8*) that are activators of protein kinase C. The functional consequences of these induced phosphorylations are not well understood, although the phosphorylation of CD4 may induce the internalization of this molecule (*5, 6, 9*). We now compare the properties of the complex of CD4 and p56^{lck} with those of the complex of CD8 and p56^{lck}, with particular emphasis on the effects of activation of protein kinase C.

We studied the interaction of CD4 and CD8 with p56^{lck} in HPB-MLT, a human leukemia T cell line (*10*) that expresses both CD4 and CD8 (Fig. 1). At least 30% of p56^{lck} is in association with CD4 and at least 10% is in association with CD8 in this cell line (Fig. 1C). An immunoprecipitate prepared from these cells with the OKT4 antibody to CD4 (anti-CD4) was assayed for protein kinase activity by incubation with [γ-^{32}P]-labeled adenosine triphosphate (ATP). Incorporation of ^{32}P into p56^{lck} (*11*) (Fig. 1A, lane 3) and into an exogenous substrate, acid-denatured enolase (Fig. 1B, lane 3), was observed. In contrast, no labeling of p56^{lck} (Fig. 1A, lane 4) and much reduced labeling of enolase (Fig. 1B, lane 4) were detected when a similar immunoprecipitate from cells that had been exposed to 12-O-tetradecanoyl phorbol-13-acetate (TPA) for 15 min was examined, the level of immunoprecipitated kinase activity being reduced seven- to tenfold. This effect of TPA was probably due to activation of protein kinase C. Treatment of cells with dimethyl sulfoxide (DMSO) (the solvent in which TPA was dissolved) or with 4-β-phorbol (an analog of TPA that does not activate protein kinase C) was without effect (*12*). Conversely, treatment with the synthetic activator of protein kinase C, 1-oleoyl-2-acetylglycerol, had an effect similar to that of TPA (*12*).

The reduced p56^{lck} protein kinase activity associated with CD4 in cells treated with TPA was not caused by loss of p56^{lck} after TPA treatment. Neither the precipitation of p56^{lck} with a rabbit antiserum to p56^{lck} (anti-p56^{lck}) (*13*) (Fig. 1A, lane 8) nor the protein kinase activity of p56^{lck} (Fig. 1B, lane 8) was inhibited by treatment with TPA. However, the gel mobility of p56^{lck} was altered markedly. Approximately 30 to 50% of the p56^{lck} from TPA-treated cells that became labeled with ^{32}P in vitro migrated with an apparent molecular size of 60 to 65 kD (Fig. 1A, lane 8). The retarded gel mobility results from the increased phosphorylation of the protein on serine (*14*) and threonine (*15*) residues. The reduced precipitation of p56^{lck} by OKT4 was also not due to inhibition of the precipitation of CD4 by TPA treatment. Exposure of cells for 10 min to TPA did not affect the immunoprecipitation of CD4 that had been labeled by cell-surface iodination (*12*).

Analysis of the same immunoprecipitates by protein blotting with anti-p56^{lck} antibodies showed that the precipitation of p56^{lck} by OKT4 was undetectable after the exposure of the cells to TPA (Fig. 1C, lane 4). The lack of labeling of p56^{lck} during incubation with ATP in vitro therefore was not the result of inactivation of the protein but rather the result of dissociation of the complex of CD4 and p56^{lck}. We have also examined the effect of the treatment of T cells with TPA on the association of p56^{lck} with CD4 in two other human leukemia T cell lines, VB (*16*) and D-CEM. In both, treatment with TPA induced an almost complete dissociation of CD4 and p56^{lck} in less than 30 min (*12*).

Identical protein kinase assays of immunoprecipitates prepared from HPB-MLT cells with the OKT8 antibody to CD8 (anti-CD8) showed relatively weak labeling of p56^{lck} by autophosphorylation (Fig. 1A, lane 5). Protein blot analysis with anti-p56^{lck} antibodies showed that p56^{lck} was present in the anti-CD8 immunoprecipitates (Fig. 1C, lane 5) at a threefold lower concentration than in OKT4 immunoprecipitates (Fig. 1C, lane 3). The p56^{lck} associated with CD8 was, however, active in the phosphorylation of enolase (Fig. 1B, lane

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5). In contrast to the results with anti-CD4 antibodies, the amount of $p56^{lck}$ in immunoprecipitates prepared from TPA-treated HPB-MLT cells with antibodies to CD8 was undiminished as measured by either protein blotting (Fig. 1C, lane 6) or by enolase phosphorylation in vitro (Fig. 1B, lane 6).

Treatment of murine thymocytes with TPA induced the rapid dissociation of CD4 and $p56^{lck}$ but had no detectable effect on the complex of CD8 and $p56^{lck}$ (Fig. 2). Similar data were obtained (*12*) with the $CD4^+$-$CD8^+$ AKR1 murine leukemia T cell line (*17*). The ability of TPA to dissociate the CD4-$p56^{lck}$ complex is therefore not restricted to cells of human origin or cells that have been established as cell lines. Dissociation of CD4 and $p56^{lck}$ was not however observed in all murine T cells. L2 (*18*) and D10 (*19*) are both antigen-responsive $CD4^+$-$CD8^-$ murine T cell lines, and TPA did not induce the dissociation of $p56^{lck}$ from CD4 in either cell line (*12*). Little dissociation was also seen after TPA treatment of the $CD4^+$-$CD8^-$ murine SAKRTLS 12.1 T cell line (*17*) (Fig. 3, lanes 1 and 2).

Most of the $p56^{lck}$ bound to CD4 in SAKRTLS 12.1 cells and to CD8 in murine L3 lymphocytes [antigen-responsive $CD4^-$-$CD8^+$ cells (*20*)] was found to undergo TPA-induced phosphorylation (Fig. 3). This demonstrates that neither CD4 nor CD8 blocks TPA-induced phosphorylation of bound $p56^{lck}$ and that the phosphorylation of $p56^{lck}$ is not sufficient to cause dissociation. Rather, this finding suggests that dissociation results from some other TPA-induced event, perhaps the phosphorylation of CD4 or of some third cellular component.

We therefore investigated whether the ability of TPA to induce the internalization of CD4 correlated with its ability to induce the dissociation of CD4 and $p56^{lck}$ and found that it did. TPA induced the internalization of CD4, as monitored by flow cytometry, in all three human cell lines (HPB-MLT, VB, and D-CEM) and in a murine line (AKR1), in which CD4 and $p56^{lck}$ underwent dissociation (*21*). Additionally, TPA causes rapid internalization of CD4 in murine thymocytes (*22*). We found the kinetics of dissociation and internalization were similar, being detectable in 10 min and essentially complete in 30 min (*21*). In contrast, TPA did not induce the internalization of CD4 in the three murine cell lines L2, D10, and SAKRTLS 12.1, in which it did not induce dissociation (*21*). Internalization of CD4 in response to TPA appears to require the phosphorylation of CD4 (*9*). Attempts to determine whether the phosphorylation of CD4 in response to TPA differs in cells in which it is internalized and in cells in which it is not internalized have produced inconclusive results (*23*).

The effect of TPA on T lymphocytes mimics some events that occur during T cell activation. Activation of protein kinase C occurs in T cells stimulated by antibody to CD3 (*24, 25*), and T cell activation induces the phosphorylation of $p56^{lck}$ in a similar

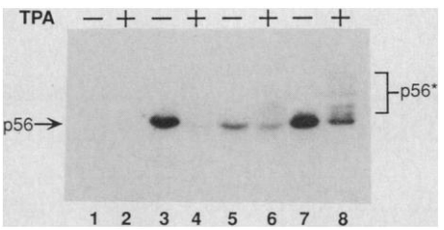

Fig. 2. The effect of TPA on the association of $p56^{lck}$ with CD4 and CD8 in murine thymocytes. Cells teased from a thymus of a 3-week-old male BALB/c KE mouse were separated from connective tissue by filtration through nylon mesh. Immunoprecipitates were prepared from 10^7 cells with rabbit antibodies to rat immunoglobulin (anti–rat Ig) (Cappel) alone (lanes 1 and 2), rat antibody GK1.5 (anti-CD4) plus rabbit anti–rat Ig (lanes 3 and 4), rat antibody 53.6.72 (anti-CD8) plus rabbit anti–rat Ig (lanes 5 and 6), or rabbit anti-$p56^{lck}$ (lanes 7 and 8) before and after treatment of the cells with TPA (100 ng/ml) for 30 min. $p56^{lck}$ concentrations were determined by protein blotting as described in the legend to Fig. 1. The several forms of p56* in the TPA-treated thymocytes are indicated by a bracket. −, Immunoprecipitates from untreated cells; +, immunoprecipitates from TPA-treated cells.

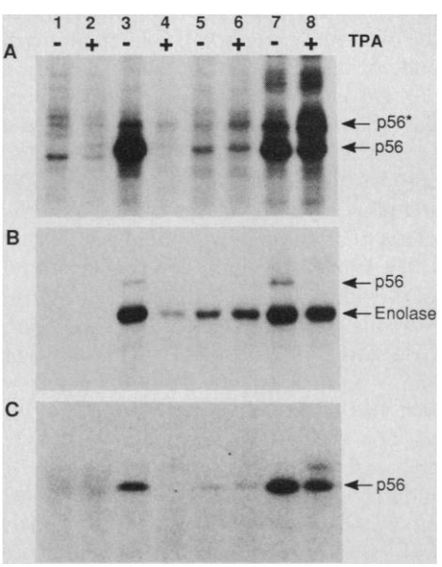

Fig. 1. The effect of TPA on the association of CD4 and CD8 with $p56^{lck}$ in human T cells. (**A**) $p56^{lck}$ labeled by autophosphorylation. (**B**) Phosphorylation of enolase. (**C**) Quantification of precipitated $p56^{lck}$ by protein blotting. Immunoprecipitates were prepared from 2×10^6 human HPB-MLT cells with either T40-25.2, a rat antibody to the human T cell receptor (*29*) (lanes 1 and 2), OKT4 (lanes 3 and 4), OKT8 (lanes 5 and 6), or rabbit anti-$p56^{lck}$ (lanes 7 and 8) before and after exposure of the cells for 15 min to TPA (100 ng/ml in DMSO; final concentration of DMSO was 0.1%). Cells were lysed, and immunoprecipitates were washed three times in a solution of 3% Nonidet P-40, 20 mM tris-HCl (*p*H 8.4 at 4°C), 150 m*M* NaCl, 1 m*M* phenylmethylsulfonyl fluoride, 2 m*M* EDTA, 200 μ*M* Na_3VO_4, and 50 m*M* NaF and then washed twice in a solution of 150 m*M* NaCl and 50 m*M* tris-HCl (*p*H 7.2 at 4°C). Immunoprecipitation was performed with excess antibody. Antibodies were bound to fixed *Staphylococcus aureus* bacteria before incubation with the lysates. After washing, the immunoprecipitates were divided into three parts. Autophosphorylation of $p56^{lck}$ was assayed by incubation of immunoprecipitates for 10 min at 30°C with 5 μCi of [γ-^{32}P]ATP (4500 Ci/mmol) (ICN) in 10 m*M* 1,4-piperazinediethanesulfonic acid (Pipes), *p*H 7.1, and 10 m*M* $MnCl_2$. The samples were then dissolved in SDS sample buffer. The phosphorylation of enolase was assayed as above with 2.5 μg of acid-denatured enolase and 40 m*M* Pipes-NaOH. Immunoprecipitates for protein-blot analysis were dissolved directly in SDS sample buffer. All samples were analyzed by SDS–polyacrylamide gel electrophoresis under reducing conditions on 15% acrylamide gels. Gels containing ^{32}P-labeled proteins were dried and subjected to autoradiography. The unlabeled samples were transferred electrophoretically to Immoblion (Millipore) (*30*), and the amount of $p56^{lck}$ in each sample was quantified by incubation of the filter sequentially with anti-$p56^{lck}$ and ^{125}I-labeled protein A. The blot was then subjected to fluorography at −70°C with an intensifying screen and film that had been sensitized. p56* is the major modified form of $p56^{lck}$ that appears after activation of protein kinase C. −, Immunoprecipitates from untreated cells; +, immunoprecipitates from TPA-treated cells.

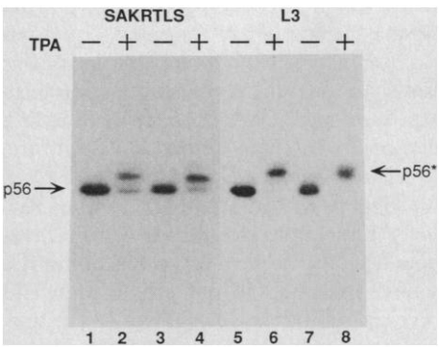

Fig. 3. The increased serine phosphorylation of $p56^{lck}$ induced by TPA is not sufficient to cause dissociation from CD4 and CD8. Immunoprecipitates were prepared from 2×10^6 SAKRTLS 12.1 cells with either GK1.5 (anti-CD4) plus rabbit anti–rat Ig (lanes 1 and 2) or rabbit anti-$p56^{lck}$ (lanes 3 and 4) before and after treatment of the cells for 15 min with TPA (100 ng/ml) as described in Fig. 2. Immunoprecipitates were also prepared from 2×10^6 L3 cells with either 53.6.72 (anti-CD8) plus rabbit anti–rat Ig (lanes 5 and 6) or rabbit anti-$p56^{lck}$ (lanes 7 and 8) both before and after treatment with TPA. $p56^{lck}$ present in the immunoprecipitates was visualized by protein blotting with anti-$p56^{lck}$ as described in Fig. 1. −, Immunoprecipitates from untreated cells; +, immunoprecipitates from TPA-treated cells.

manner to that seen in the presence of TPA (*26*). The dissociation of CD4 and p56^{lck} that accompanies protein kinase C activation could be important during either T cell activation by antigen or T cell maturation in the thymus. Dissociation of CD4 and p56^{lck} could have two effects: It might terminate regulation of the enzymatic activity of p56^{lck} by CD4 (*4*), or it might alter the ability of p56^{lck} to interact with specific polypeptide substrates.

Since dissociation of CD4 and p56^{lck} appears to occur in concert with TPA-induced internalization of CD4, the differences between the properties of T cells that are induced to internalize CD4 by TPA and those that are not might be revealing as to the role of the dissociation of CD4 and p56^{lck} in T cell function and development. Unfortunately, generalizations are difficult to make. Although TPA-induced internalization of CD4 is reported to occur in most human T cell lines (*5, 27, 28*), in human peripheral blood lymphocytes (*6*), in murine thymocytes (*22*), and in some murine CD4$^+$-CD8$^+$ cell lines such as AKR1 (*12*), it does not occur in a number of mature, antigen-responsive murine T cells (*12, 29*).

The association of p56^{lck} with both CD4 and CD8 is unlikely to be coincidental. The implication is that p56^{lck} is important in the development or function, or both, of major histocompatibility complex class I–restricted and class II–restricted T cells. The properties of p56^{lck} when it is bound to CD4 differ somewhat from those of the protein when it is bound to CD8. When bound to CD4, p56^{lck} will undergo autophosphorylation in vitro and, in many types of T cells, dissociate when protein kinase C is activated. In contrast, when bound to CD8, p56^{lck} undergoes less vigorous autophosphorylation in vitro and does not dissociate when protein kinase C is activated. It is possible that the regulation of p56^{lck} by CD4 and by CD8 will thus be found to differ.

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Recombinant 47-Kilodalton Cytosol Factor Restores NADPH Oxidase in Chronic Granulomatous Disease

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A 47-kilodalton neutrophil cytosol factor (NCF-47k), required for activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase superoxide ($O_2^{\cdot-}$) production, is absent in most patients with autosomal recessive chronic granulomatous disease (AR-CGD). NCF-47k cDNAs were cloned from an expression library. The largest clone predicted a 41.9-kD protein that contained an arginine and serine-rich COOH-terminal domain with potential protein kinase C phosphorylation sites. A 33–amino acid segment of NCF-47k shared 49% identity with *ras* p21 guanosine triphosphatase activating protein. Recombinant NCF-47k restored $O_2^{\cdot-}$-producing activity to AR-CGD neutrophil cytosol in a cell-free assay. Production of active recombinant NCF-47k will enable functional regions of this molecule to be mapped.

ACTIVATED PHAGOCYTIC CELLS PRODUCE superoxide anion ($O_2^{\cdot-}$), which is converted to hydrogen peroxide and other microbicidal oxygen products. $O_2^{\cdot-}$ generation requires activation of a latent NADPH oxidase. A membrane-bound cytochrome b_{558} (*1*), and both 47-kD and 65-kD neutrophil cytosol factors (NCF-47k and NCF-65k) (*2–4*), are necessary for activation of the NADPH oxidase. It is unclear how NADPH oxidase is activated, how many additional components are required, or how all of the components interact to achieve a functional enzyme complex.

Several genetic forms of chronic granulomatous disease (CGD) have been identified, in which phagocytic cells are defective in cytochrome b_{558} (*1*), NCF-47k, or NCF-65k (*2, 3*). We have isolated cDNA clones that encode NCF-47k and demonstrated that recombinant NCF-47k (rNCF-47k) restores $O_2^{\cdot-}$-producing activity to NCF-47k–deficient AR-CGD polymorphonuclear neutrophil (PMN) cytosol in a cell-free reconstitution system.

We obtained NCF-47k cDNA clones using rabbit antiserum B-1, which recognizes NCF-47k and NCF-65k (*3*), to screen a Lambda-ZAP expression library of cDNA inserts derived from differentiated HL-60 cells (*5, 6*). NCF-47k cDNA clones were identified based on the ability of their recombinant fusion protein to inhibit B-1 antibody detection of NCF-47k on immu-

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