MOLECULAR AND CELLULAR BIOLOGY, Oct. 1988, p. 4353–4361
0270-7306/88/104353-09$02.00/0

Vol. 8, No. 10

Alterations of the Lymphocyte-Specific Protein Tyrosine Kinase ($p56^{lck}$) during T-Cell Activation

ANDRÉ VEILLETTE,[1]* IVAN D. HORAK,[1] EVA M. HORAK,[2] MICHAEL A. BOOKMAN,[2] AND JOSEPH B. BOLEN[1]

Laboratory of Tumor Virus Biology[1] and Medicine Branch,[2] National Cancer Institute, Bethesda, Maryland 20892

Received 2 June 1988/Accepted 12 July 1988

The lymphocyte-specific tyrosine protein kinase $p56^{lck}$ is abundantly expressed in L3T4$^+$ (CD4$^+$) and Lyt-2$^+$ (CD8$^+$) T-lymphocytes, where it is predominantly phosphorylated in vivo on the carboxy-terminal tyrosine residue 505 (Y-505). Upon exposure to activating signals (mitogenic lectins, antibodies to the T-cell receptor), the $p56^{lck}$ expressed in normal cloned murine T-cells is modified into a product which migrates at approximately 59 kilodaltons on sodium dodecyl sulfate-polyacrylamide gels and which possesses several amino-terminal serine phosphorylations. The changes in both mobility and amino-terminal phosphorylation can be reproduced by known activators of protein kinase C (4 α-phorbol 12 β-myristate, dioctanoylglycerol), suggesting that this signal transduction pathway (or related pathways) mediates at least part of these events. Interestingly, agents raising intracellular calcium (such as A23187) cause the appearance of several of these amino-terminal phosphorylation changes but do not cause the pronounced shift in electrophoretic mobility. These data suggest that at least two serine kinase systems are implicated in the alterations of $p56^{lck}$ associated with T-cell activation and that the *lck* gene product plays a critical role in normal T-cell physiology.

T-lymphocyte activation represents a series of programmed biochemical alterations which culminate in the expression of either helper-inducer or cytotoxic-suppressor T-cell functions. While the phenotypic properties of activated T-cells are relatively well described (for a review, see reference 33), less is known about the early molecular events which initiate this process. However, it has been established that during this response the T-lymphocytes undergo a rapid increase in phosphatidylinositol-4,5-bisphosphate hydrolysis which results in the stimulation of protein kinase C (PKC) by diacylglycerol and elevation of intracellular calcium levels as at least a partial consequence of D-inositol-1,4,5-triphosphate production (33). These observations have fostered the view that the cellular functions influenced by altered calcium concentrations and phosphorylation of PKC substrates help mediate the process of T-lymphocyte activation.

lck is a member of the *src* family of tyrosine-specific protein kinase genes abundantly expressed in T-lymphocytes (20, 30). The relatively high levels of *lck* transcripts detected in normal T-lymphocytes coupled with elevated *lck* expression in some murine T-cell tumors (22, 32) has led to the concept that the *lck* gene product, $p56^{lck}$, plays an important role in T-cell growth and development. In contrast with polypeptide growth factor receptors, $p56^{lck}$ does not possess extracellular or transmembrane domains (for a review, see J. A. Cooper, *in* B. Kemp and P. F. Alewood, ed., *Peptides and Protein Phosphorylation*, in press), thereby prohibiting direct interactions with external factors. However, like growth factor receptors, $p56^{lck}$ and the other members of the *src* family of tyrosine kinases are associated with plasma membranes, where they are thought to potentially modify and/or amplify cellular signal transduction responses to proliferative and differentiation stimuli. Consistent with this idea is the observation that $pp60^{c\text{-}src}$ is rapidly phosphorylated and its protein kinase activity is transiently activated in fibroblasts exposed to platelet-derived growth factor (27). Additionally, $pp60^{c\text{-}src}$ has been shown to be phosphorylated by PKC after treatment of fibroblasts with certain tumor promoters (10) or exposure of the human T-cell leukemia cell line Jurkat to antibodies to the CD3/T-cell receptor complex (15).

Because $p56^{lck}$ represents the most abundant member of the *src* family of tyrosine protein kinases to be expressed in T-lymphocytes, we analyzed the effects of different activating signals on the biophysical and biochemical properties of $p56^{lck}$ in normal cloned murine T cells. Our results demonstrate that $p56^{lck}$ is abundantly expressed in both L3T4$^+$ (CD4$^+$) and Lyt-2$^+$ (CD8$^+$) cloned T-cells, where it is predominantly phosphorylated in vivo on tyrosine (Y) 505 with little or no detectable occupancy of Y-394, the major site of in vitro phosphorylation. After exposure of T-cells to activating signals, the Lck protein is rapidly modified into a series of products which demonstrate reduced electrophoretic mobility and multiple new amino-terminal serine phosphorylations. These alterations in Lck phosphorylation and gel mobility could also be induced by treatment of the T-cells with activators of PKC. However, treatment of the cells with calcium ionophores, which resulted in the appearance of the majority of the amino-terminal $p56^{lck}$ phosphorylations, did not induce the altered electrophoretic mobility. We interpret our results to suggest that at least two serine protein kinase pathways are implicated in the alterations of the *lck* gene product during T-cell activation.

MATERIALS AND METHODS

Cells. C8 and B10 are cloned lines of L3T4$^+$ helper-inducer class II major histocompatibility complex (MHC)-restricted T-lymphocytes derived from C57BL/6 (B6) mice immunized with the RBL5 tumor and are specific for viral envelope glycoprotein gp70 (2). Clones were maintained by cyclic stimulation with irradiated (30 Gy) B6 spleen cells and inactivated Friend virus as a source of gp70 antigen. 1B6 is a Lyt-2$^+$ class I MHC-restricted cytotoxic T-lymphocyte clone derived from B6 mice after immunization with FBL3 tumor cells (21). 1B6 was maintained by cyclic stimulation

* Corresponding author.

with irradiated (30 Gy) B6 spleen cells and irradiated (100 Gy) FBL3 tumor cells. Culture medium consisted of a 1:1 mixture of Eagle-Hanks amino acids and RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 μg of gentamicin per ml, 100 U of penicillin per ml, and 5×10^{-5} M 2-mercaptoethanol (ER medium). For bulk expansion, cells were recovered 48 h after activation and replated in ER medium containing 100 U of human recombinant interleukin-2 (IL-2) (kindly provided by the Cetus Corporation, Emeryville, Calif.) per ml. Resting T-cells were harvested 5 to 7 days later by Ficoll-Hypaque density gradient centrifugation. RBL5 and FBL3 are Friend-Moloney-Rauscher virus-induced T-cell leukemia lines derived from B6 mice (9). EL4 is a chemically induced T-cell leukemia of B6 mice (9). YAC-1 cells were derived from a lymphoma induced by inoculation of the Moloney murine leukemia virus into a newborn A/Sn mouse. LSTRA (kindly provided by Bart Sefton, Salk Institute, La Jolla, Calif.) is a Moloney murine leukemia virus-induced T-cell thymoma line induced in BALB/c mice (4). All tumor cells other than LSTRA were propagated in ER medium. LSTRA cells were propagated in Dulbecco modified Eagle medium supplemented with 20% fetal calf serum, 50 mM L-glutamine, gentamicin, penicillin, and 5×10^{-5} M 2-mercaptoethanol.

Treatments. For short-term experiments (Lck protein analysis, gamma interferon mRNA determination), T-cell clones were treated for 30 min with the following agents (alone or in combination): concanavalin A (con A) (final concentration, 25 μg/ml) (Pharmacia), phytohemagglutinin (5 μg/ml) (Sigma Chemical Co.), ionomycin (in dimethyl sulfoxide [DMSO]; final concentration, 2.5 μM) (Calbiochem-Behring), A23187 (in DMSO; final concentration, 2.5 μM) (Calbiochem-Behring), dioctanoylglycerol (diC8) (in DMSO; 100 μM) (Sigma), and 4 α-phorbol 12 β-myristate (PMA) (in ethanol; final concentration, 8 nM or 50 ng/ml) (Sigma). The final concentrations (vol/vol) of ethanol and DMSO were less than 0.1%. No detectable effects on lymphocyte function or $p56^{lck}$ were observed when ethanol or DMSO alone was used. To examine the effect of antibodies to the T-cell receptor complex, T-cells were incubated for 30 min on ice with culture supernatant from 145-2C11 hybridoma cells recognizing a component of the epsilon subunit of T3 (17) (kindly provided by Jonathan Ashwell, National Institutes of Health). Cells were then washed with phosphate-buffered saline and incubated for 30 min at 37°C in ER medium containing 10 μg of rabbit anti-hamster immunoglobulin G (Organon Teknika) per ml. Control experiments were conducted by using the hybridoma supernatant or the cross-linking antibody alone. Similar experiments were conducted by using a rat antibody against L3T4 (GK1.5 [34]; kindly provided by Sandra Bridges, National Institutes of Health) by using rabbit anti-rat immunoglobulin G (Organon Teknika) as the cross-linking second antibody.

For long-term stimulation experiments, the following ranges of dose or concentration were used: con A, 0.2 to 25 μg/ml; PMA, 0.4 to 50 ng/ml; A23187, 0.02 to 2.5 μM; IL-2, 1 to 1,000 U/ml.

Immunoblotting. Cells were lysed in a buffer containing 1% Nonidet P-40 (50 mM Tris [pH 8.0], 2 mM EDTA) supplemented with the protease inhibitors leupeptin and aprotinin (each at a concentration of 10 μg/ml), the phosphotyrosyl phosphatase inhibitor sodium orthovanadate (100 μM), and the serine phosphatase inhibitor sodium fluoride (50 mM). These conditions were found to extract Lck proteins as efficiently as radioimmune precipitation assay buffer or sodium dodecyl sulfate (SDS) boiling (our published data). Equivalent amounts of cellular lysates (20 μg) were denatured in SDS-loading buffer and resolved on 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels. The proteins were then transferred onto nitrocellulose, and Lck detection and quantitation were performed as described previously (31) by using a polyclonal rabbit antiserum generated against a synthetic peptide corresponding to residues 39 to 58 of the murine $p56^{lck}$ sequence (22, 32).

Metabolic labeling and immunoprecipitation. Cells were labeled for 4 to 18 h in phosphate-free RPMI 1640 containing 2% dialyzed fetal calf serum and supplemented with 1.0 mCi of $^{32}P_i$ (Amersham Corp.; carrier free) per ml. Long periods of labeling (18 h) were associated with a significant reduction of cell viability, and in most experiments the labeling time was limited to 4 h (our unpublished data). The cells were then treated with the agents indicated above for 30 to 60 min in the presence of radioactive label. The cells were subsequently washed in phosphate-buffered saline and lysed as described above. The Lck proteins were immunoprecipitated as previously described (31) and resolved on 8% SDS-PAGE gels, and the labeled proteins were detected by autoradiography.

Peptide mapping and phosphoamino acid analysis. Radiolabeled proteins were electroeluted from the gel and precipitated with cold trichloroacetic acid. For tryptic peptide mapping, the proteins were oxidized with performic acid and exhaustively digested with tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (Worthington Biochemicals) (13), and the peptide fragments were resolved by electrophoresis (1,000 V for 27 min) in a buffer containing 1% ammonium carbonate (pH 8.9). The plates were dried, and the second dimension analysis was conducted by ascending chromatography (5 h) in a solution containing *n*-butanol (75 volumes), pyridine (50 volumes), acetic acid (15 volumes), and water (60 volumes). The radioactive tryptic fragments were localized by autoradiography. Cyanogen bromide cleavage and phosphoamino acid analysis were performed as described elsewhere (1, 13).

Immune complex kinase assays. Autophosphorylation and exogenous substrate phosphorylation (casein, enolase) reactions were performed as described previously (31).

Thymidine incorporation. Proliferation assays were performed in 96-well plates with 2×10^4 resting cells per well in a final volume of 200 μl. The cells were incubated in the presence of the appropriate agent(s) for 60 to 72 h at 37°C with 1 μCi of [^{3}H]thymidine per well added during the last 16 h. Cells were lysed, harvested onto glass fiber filters, washed, and counted for incorporated [^{3}H]thymidine.

Determination of gamma interferon mRNA expression. Cells were treated for 6 h with the regimens mentioned previously. They were subsequently washed in phosphate-buffered saline and lysed in guanidinium isothiocyanate, and RNA was isolated by ultracentrifugation as described previously (6). Total cellular RNA (5 μg) was denatured and separated on 1.1% agarose–2.2 M formaldehyde gels. Equal loading of RNA was assessed by ethidium bromide staining. Northern (RNA) blotting to nitrocellulose filter was performed as described previously (8). The filter was subsequently hybridized to a synthetic end-labeled antisense oligonucleotide corresponding to the first 51 coding nucleotides of the murine gamma interferon cDNA sequence (11). The hybridization and washing conditions used were as described previously (8).

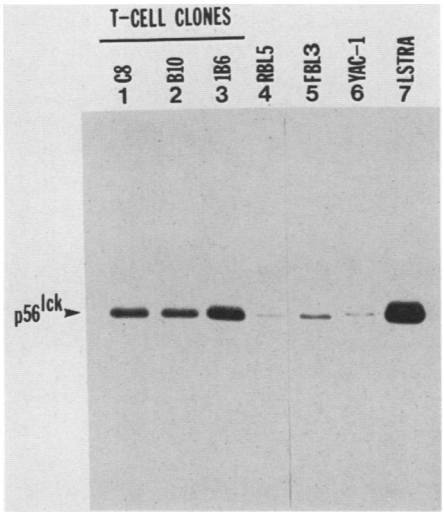


FIG. 1. Expression of $p56^{lck}$ in murine T-cells. Various murine T-cell clones or lines were harvested, washed, and lysed in detergent containing buffer. Equivalent amounts of denatured cellular lysates (20 μg) were separated on 8% SDS-PAGE gels and analyzed by Lck immunoblot as described in Materials and Methods. Lanes: 1, C8 clone ($L3T4^+$); 2, B10 clone ($L3T4^+$); 3, 1B6 clone ($Lyt\text{-}2^+$); 4, RBL5; 5, FBL3; 6, YAC-1; 7, LSTRA. Exposure, 6 h. The position of $p56^{lck}$ is indicated.

RESULTS

Expression of $p56^{lck}$ in murine T-lymphocytes. The results of immunoblotting experiments analyzing the levels of $p56^{lck}$ expressed in a variety of normal and transformed murine T-cell lines are shown in Fig. 1. An immunoreactive 56-kilodalton (kDa) protein corresponding to the *lck* gene product was detectable in all cells examined. High levels of $p56^{lck}$ could be observed in both $L3T4^+$ (Fig. 1, lanes 1 and 2) and $Lyt\text{-}2^+$ (lane 3) T-cell clones. These antigen-specific T-cell clones retain most of the characteristics of normal T-lymphocytes including IL-2 dependence and the ability to be activated and to display a helper-inducer or cytotoxic-suppressor phenotype (2, 12, 23). Lower levels of Lck protein were observed in a variety of chemically and virally transformed murine T-cell lines (Fig. 1, lanes 4 to 6). The lower levels of $p56^{lck}$ seen in these immature T-cell lines are consistent with the previous observation that T-cells arrested at the early stages of thymic maturation express lower levels of *lck* transcripts (14). LSTRA cells (Fig. 1, lane 7) were found to have the highest levels of Lck protein (approximately 5- to 10-fold higher than those seen in the normal T-cell clones). This Moloney murine leukemia virus-induced thymoma cell line is known to overexpress $p56^{lck}$ because of altered transcriptional (22, 32) and translational (21) controls. These results demonstrate that $p56^{lck}$ is abundantly expressed in all cloned normal T-lymphocytes and, to a lesser degree, in all the transformed T-cell lines we examined.

Pattern of in vivo phosphorylation of $p56^{lck}$ in normal T-cells. To analyze the posttranslational regulatory events involved in potentially controlling the activity of $p56^{lck}$ in normal T-cells, we evaluated the pattern of $p56^{lck}$ in vivo phosphorylation by using three different antigen-specific murine T-cell clones (C8, B10, and 1B6) (Fig. 2A). For these experiments, resting lymphocytes were metabolically labeled for 4 h with $^{32}P_i$ and the purified radiolabeled $p56^{lck}$ was analyzed by tryptic peptide mapping. Evaluation of $L3T4^+$ (C8 and B10; Fig. 2A, panels 1 and 2, respectively) and $Lyt\text{-}2^+$ (1B6; panel 3) clones revealed that $p56^{lck}$ is phosphorylated in vivo on a single major phosphotyrosine-containing tryptic fragment (peptide 2). This fragment is different from the peptide containing the major site of in vitro tyrosine phosphorylation (Y394, peptide 1) (Fig. 2B). Others have shown that the tyrosine residue phosphorylated in peptide 2 in vivo is Y-505 (19). Low levels of Y-394 phosphorylation (less than 5% of the extent of Y-505 phosphorylation) were also occasionally observed (Fig. 2A, panel 3, and data not shown). These results were independent of the duration of metabolic labeling (4 h versus 18 h) and were not significantly affected by the conditions used for cell lysis and immunoprecipitation (data not shown). The general location of the tyrosine phosphorylation sites to the carboxy terminus were confirmed independently by cyanogen bromide cleavage of in vivo $^{32}P_i$-labeled $p56^{lck}$ (data not shown). In addition to Y-505, the normal murine T-cells were found to be variably phosphorylated on two other tryptic fragments containing phosphoserine (peptides 5 and 6). These two peptides were found to be derived from the amino-terminal 28-kDa C1 cyanogen bromide fragment (31) (data not shown).

These results differed significantly from those obtained in similar analyses of LSTRA cells. We found that the $p56^{lck}$ expressed in LSTRA cells is significantly phosphorylated on Y-394 (peptide 1) in vivo (Fig. 2C). On the basis of tryptic peptide analysis, the Y-394 occupancy in the LSTRA cells is approximately 50% of that of Y-505. These results are in agreement with those obtained previously by others using antisera directed against the carboxy-terminal portion of the molecule (3, 19). Coupled with the fact that the LSTRA cells possess elevated levels of total cellular phosphotyrosine (4) (a feature characteristic of cells transformed by viral tyrosine kinases), these data suggest that $p56^{lck}$ might have participated in the oncogenesis of this thymoma cell line.

In summary, the $p56^{lck}$ expressed in normal T-lymphocytes is phosphorylated in vivo principally on Y-505 with little or no detectable phosphorylation of Y-394. By analogy with $pp60^{c\text{-}src}$ (7), these data suggest that the enzymatic activity of the *lck* gene product is repressed in the resting T-cells.

Effect of activating signals on $p56^{lck}$. At least two early signals are thought to be critical in T-cell activation: the stimulation of PKC and an increase in intracellular calcium (33). These signals are promptly induced upon the interaction of T-lymphocytes with the appropriate antigen and compatible antigen-presenting accessory cell. In contrast with purified peripheral blood T-lymphocytes, significant activation of normal T-cell clones can be obtained in vitro by treatment with single classes of agents such as mitogenic lectins (12). In addition, treatment with cross-linked antibodies to selected T-cell surface proteins has been shown to mimic interactions of both the antigen and antigen-presenting cell with the appropriate surface recognition molecules, thereby eliminating the requirement of antigen-presenting cells in the activation assays (18, 24, 33). Thus, because of the potential difficulties in analyzing Lck in multiple cell populations if the T-cell activation assays were conducted in the presence of accessory cells, we used these alternative stimulatory modes with single agents to study the alterations of the *lck* gene product during activation in the normal T-cell clones.

Exposure of the helper-inducer C8 cells to the mitogenic lectin con A led to a significant degree of T-cell activation as measured by increased [^{3}H]thymidine incorporation and

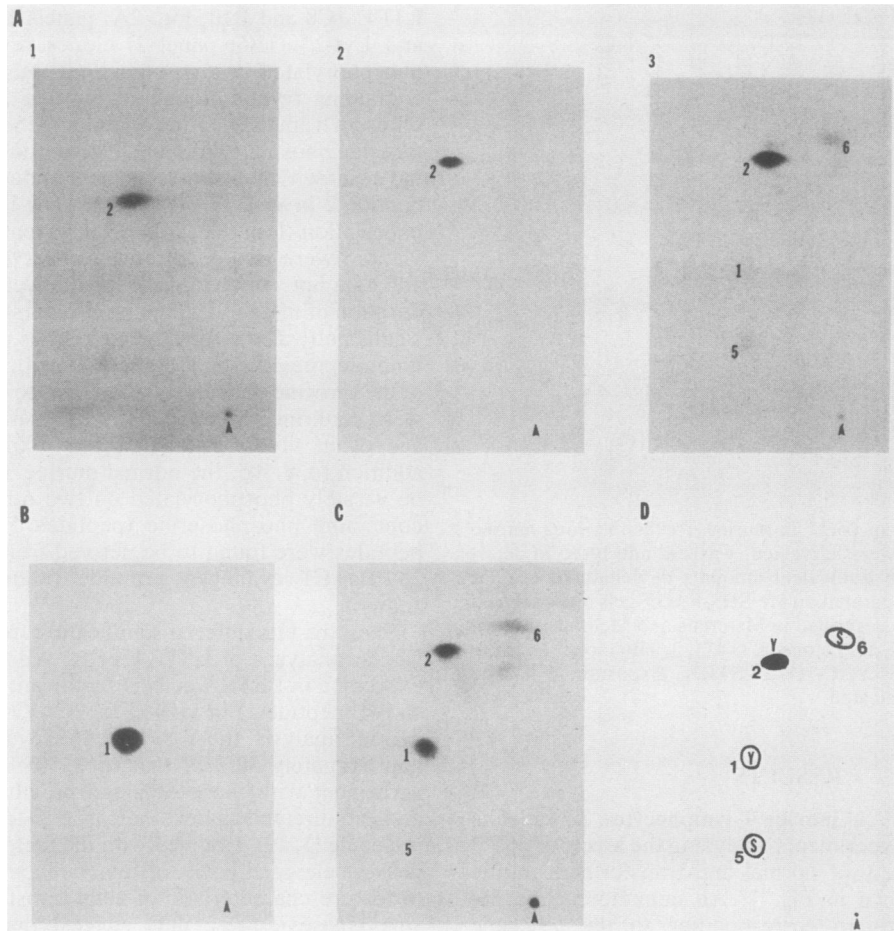


FIG. 2. Phosphorylation pattern of the p56^{lck} expressed in normal murine T-cell clones (tryptic peptide mapping). (A) In vivo phosphorylation. Approximately 5×10^7 resting cells were labeled for 4 h in medium containing 1.0 mCi of $^{32}P_i$ per ml. The cells were subsequently washed and lysed in a buffer containing 1% Nonidet P-40. p56^{lck} was immunoprecipitated as described in Materials and Methods and electroeluted from SDS-PAGE gels. The Lck proteins were oxidized and digested with trypsin according to protocol (13). The phospholabeled tryptic fragments were subsequently separated on thin-layer chromatography plates as described in the text. The arrows indicate the origin. Electrophoresis proceeded from right to left, and chromatography proceeded from bottom to top. Panels: 1, C8 cells; 2, B10 cells; 3, 1B6 cells. Exposure, 5 to 7 days. (B) In vitro-phosphorylated p56^{lck}. p56 was immunoprecipitated from LSTRA cells and reacted in immune-complex kinase assays. After electrophoresis in SDS-PAGE gels, the Lck proteins were processed as described for Fig. 2A for tryptic peptide mapping. Exposure, 12 h. (C) In vivo-labeled p56 from LSTRA cells. Exposure, 2 days. (D) Composite diagram of the major tryptic peptides detected in p56^{lck}. Phosphoamino acid analysis was performed as described elsewhere (13). Y, Tyrosine; S, serine.

TABLE 1. Characterization of L3T4$^+$ C8 T-cell clone

Treatment	Thymidine uptake[a]	Amt of gamma interferon mRNA[b]
Control	1,087 ± 10	0
Con A	45,061 ± 3,326	++
PMA	2,131 ± 213	0
Con A + PMA	43,801 ± 3,720	+++
A23187	784 ± 256	0
IL-2	58,618 ± 1,988	0

[a] Counts per minute (means plus or minus standard deviation) determined in triplicate as described in Materials and Methods. The highest levels of [^{3}H]thymidine incorporation obtained for each treatment modality are indicated here.

[b] The relative abundance of the reactive 1.3-kilobase mRNA species is indicated (0, absent; ++, moderate; +++, high).

induction of gamma interferon mRNA (Table 1). Within 30 min of con A treatment (Fig. 3, lane 2), we detected the appearance of a modified form of the Lck protein migrating at approximately 59 kDa (p59). Less prominent species of the Lck protein migrating at approximately 62 and 68 kDa could also be detected after a longer exposure of the autoradiogram (data not shown). Similar results were obtained after treatment with another activating mitogenic lectin, phytohemagglutinin (data not shown).

Interaction of specific antibodies with human T-cell receptor determinants does not usually result in significant activation of clonal or purified T-cell populations. However, cross-linking of the bound T-cell receptor antibodies by a second antibody can lead to a significant increase in the cytoplasmic calcium concentration, the activation of PKC, and, if sustained, lymphokine production and T-cell proliferation (18, 24, 33). To examine the correlation of these parameters of T-cell activation with posttranslational alterations in p56^{lck}, we evaluated the effect of an antibody

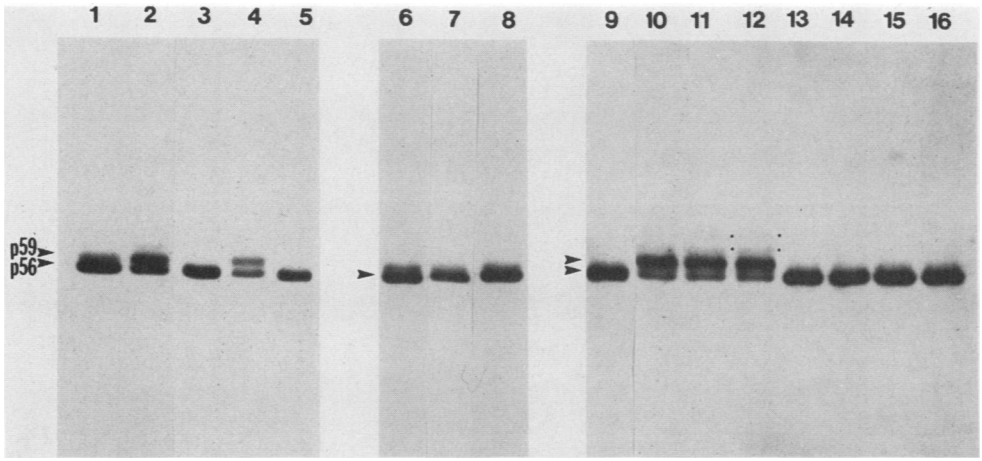


FIG. 3. Effect of various treatments on the Lck protein expressed in the C8 cells. C8 cells were treated as described in Materials and Methods, washed in phosphate-buffered saline, and processed as described for Fig. 1. Immunoblot was performed as described above. Lanes: 1, control; 2, con A; 3, 145-2C11 antibody alone; 4, 145-2C11 antibody plus cross-linking antibody; 5, cross-linking antibody alone; 6, GK1.5 antibody alone; 7, GK1.5 antibody plus cross-linking antibody; 8, cross-linking antibody alone; 9, control; 10, PMA; 11, diC8; 12, con A plus PMA; 13, A23187; 14, ionomycin; 15, IL-2; 16, dexamethasone. Exposure, 6 to 12 h. The positions of p56 and p59 are indicated by arrowheads. Dots denote positions of p62 and p68.

against the T-cell receptor (145-2C11) on the $p56^{lck}$ expressed in the C8 cells. Our results indicate that only treatment with 145-2C11 antibody cross-linked with a second antibody resulted in the appearance of p59 (Fig. 3, lane 4). Exposure of the C8 cells to antibody GK1.5 directed against another T-cell surface molecule (L3T4) either alone or with a cross-linking antibody did not induce the Lck protein mobility changes (Fig. 3, lanes 6 through 8). This is consistent with the lack of activation reported after exposure of T-cells to this antibody, even in the presence of a second-step antibody (16, 28, 34; our unpublished observations).

Exposure to phorbol esters is usually not sufficient to activate purified or cloned T-lymphocytes (12). However, these compounds are known to cause a rapid activation of PKC (for a review, see reference 25) and have been useful in evaluating the potential participation of this signal transduction pathway in various cellular responses. Indeed, PMA did not have any significant effect on the proliferative index or the levels of lymphokine mRNA in the C8 cells (Table 1). However, PMA was capable of inducing Lck protein alterations similar (Fig. 3, lane 10) to those described after treatment with activating lectins or antibodies to the T-cell receptor (Fig. 3, lanes 2 and 4). Similar results were also obtained after treatment with the synthetic analog of diacylglycerol, diC8 (Fig. 3, lane 11).

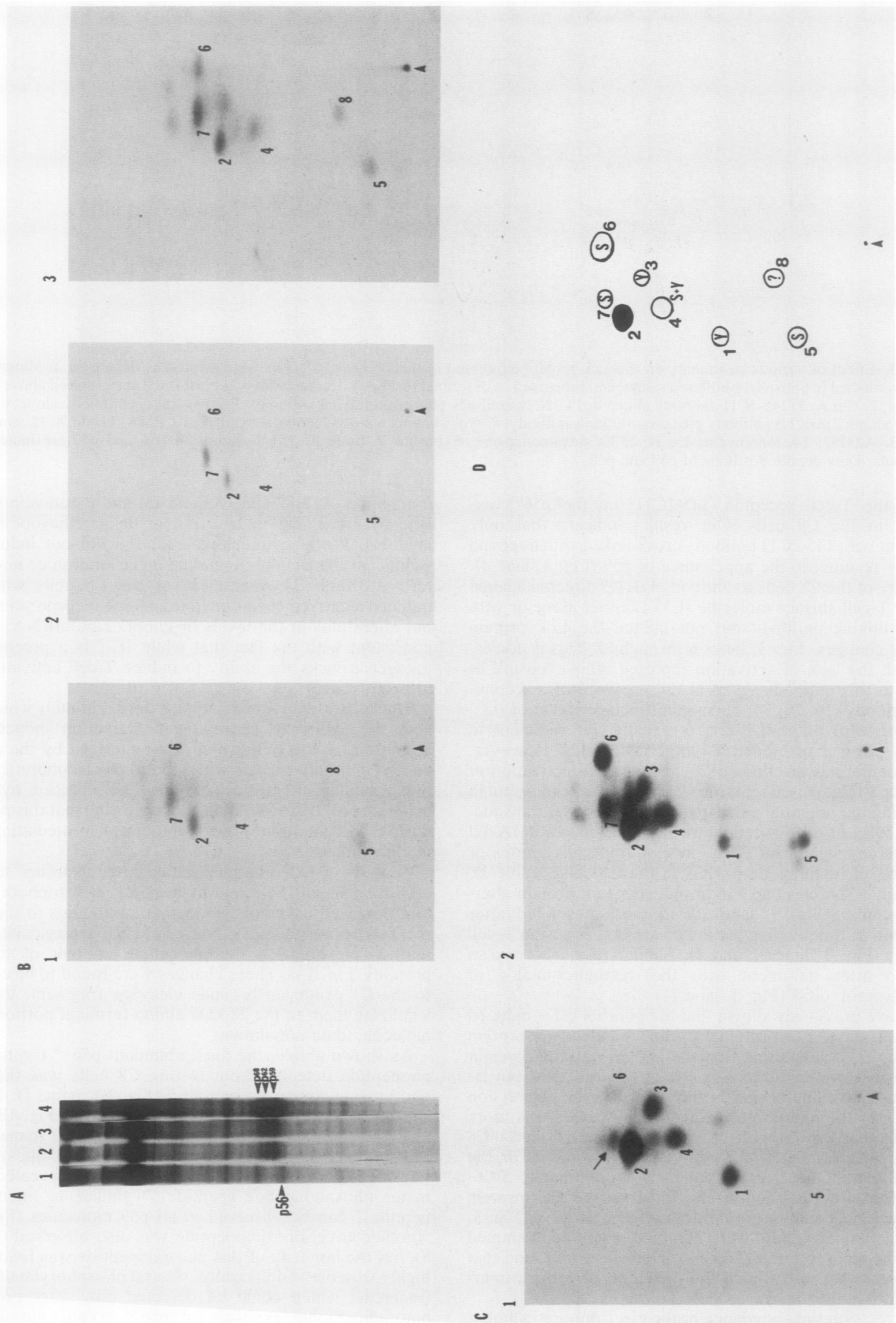
We have previously shown that the phorbol ester-induced alterations of the Lck protein occur independently of protein synthesis (31), suggesting that the higher-molecular-weight species observed in our assays are the result of posttranslational modifications of $p56^{lck}$. Interestingly, the conversion of $p56^{lck}$ to the higher-molecular-weight species was more efficient (nearly 80%) with PMA (Fig. 3, lane 10) and diC8 (Fig. 3, lane 11) than with con A (Fig. 3, lane 2) or the 145-2C11 antibodies (Fig. 3, lane 4) (approximately 50 to 60%). The effects of con A and PMA on the Lck protein expressed in C8 cells were not found to be additive (Fig. 3, lane 12). Similar results were obtained when we examined thymidine incorporation (Table 1). However, we found that PMA somewhat potentiated the induction of gamma interferon mRNA by con A.

No effects on the abundance or the electrophoretic mobility of $p56^{lck}$ were observed after the addition of the calcium ionophores A23187 (Fig. 3, lane 13) and ionomycin (Fig. 3, lane 14), IL-2 (Fig. 3, lane 15), or dexamethasone (Fig. 3, lane 16). We also found that A23187 did not induce any significant increase in thymidine incorporation or interferon mRNA (Table 1). Treatment of the C8 cells with IL-2 induced a marked elevation in thymidine incorporation without alterations in the levels of lymphokine mRNA. This is consistent with the fact that while IL-2 is a potent T-cell mitogen it lacks the ability to induce T-cell activation (12; also see above).

These data demonstrate that external stimuli which possess the ability to cause T-cell activation induce rapid alterations of the Lck protein characterized by the appearance of multiple species with altered electrophoretic mobilities. Similar alterations can also be induced by direct activators of PKC (PMA, diC8), suggesting that this pathway (and/or a related pathway) is important in mediating these modifications.

Analysis of Lck phosphorylation after exposure to T-cell activating signals. Alterations in $p56^{lck}$ electrophoretic mobility induced by mitogenic lectins, antibodies to the T-cell receptor complex, and activators of PKC are associated with marked elevation in the phosphate content of the Lck proteins (Fig. 4A). These changes were found to be confined to the C1 cyanogen bromide cleavage fragment, therefore localizing them to the 28-kDa amino-terminal portion of the molecule (data not shown).

As shown above, the most abundant $p56^{lck}$ tryptic phosphopeptide detected from resting C8 cells was the phosphotyrosine-containing peptide 2 (Fig. 2A, panel 1). The p59 species generated after treatment with con A (Fig. 4B, panel 1), cross-linked anti-T3 antibodies (145-2C11) (panel 2), or PMA (panel 3) contained several additional phosphopeptides. The results of these mapping studies revealed that a major phosphopeptide (peptide 7) similar in intensity to peptide 2 can be observed in all p59 molecules (Fig. 4B). Another novel phosphopeptide was also observed (peptide 8), but the intensity of this phosphopeptide was found to be highly variable. Additionally, several phosphorylated tryptic fragments which could be detected only after prolonged autoradiographic exposure of $p56^{lck}$ tryptic digests from untreated cells (Fig. 2A and data not shown) were found to


A
1 2 3 4
p56
B
1
2
3
C
1
2
D

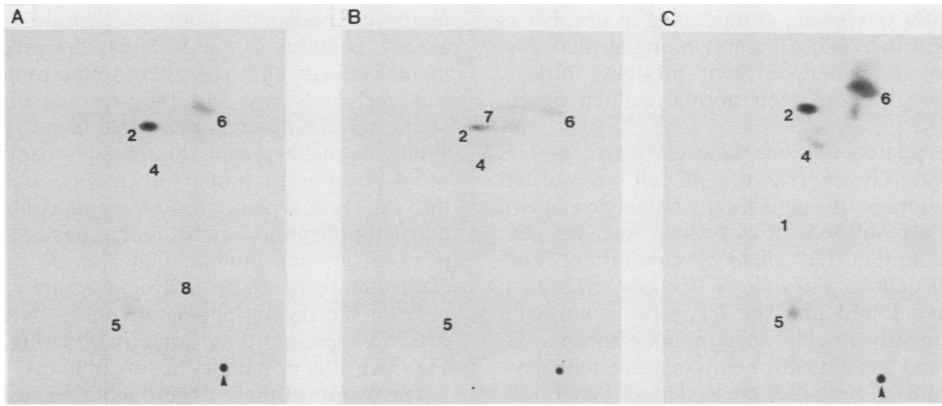


FIG. 5. Tryptic peptide mapping of $p56^{lck}$. Tryptic phosphopeptide analysis was performed as described for Fig. 4. (A) p56 remaining after treatment with PMA ($p56^{PMA}$); (B) p59 induced by PMA; (C) p56 after treatment with A23187. Exposure, 5 days.

be increased in relative intensity after these treatments (peptides 4, 5, and 6). Similar results were obtained after treatment of these cells with diC8 (data not shown).

Despite the fact that LSTRA cells cannot be activated, we and others have found that treatment of these cells with PMA (but not mitogenic lectins) can induce alterations in the electrophoretic mobility of $p56^{lck}$ similar to those described for normal T-cells (5; our unpublished data). Trypsin digestion of $^{32}P_i$-labeled Lck proteins from LSTRA cells revealed that the structure of the p59 generated in these cells (Fig. 4C, panel 2) is closely related to that of the p59 from C8 cells (Fig. 4B, panel 3). Noticeable differences were the presence of peptide 3 in the LSTRA Lck maps, the absence of phosphorylated peptide 8 in LSTRA Lck, and the apparent decrease in occupancy of peptide 1 (Y394) after treatment of LSTRA cells with PMA. Acid hydrolysis of the p59 tryptic phosphopeptides generated from LSTRA cells revealed that all the changes in phosphorylation detected were associated with increases in phosphoserine content (Fig. 4D and data not shown). We were unable to determine the phosphoamino acid content of peptide 8 from C8 cells. Tryptic peptide analysis of the two other altered forms of Lck protein (p62, p68) revealed that they possess phosphopeptides similar to those observed in p59 digests (data not shown). The reason for their slower electrophoretic mobility remains unclear.

Phosphoamino acid analysis of the p56 remaining after these treatments (named $p56^{PMA}$) revealed that this species also possesses increased amounts of phosphoserine compared with the p56 from untreated cells (data not shown). Exhaustive trypsin digestion of these phosphoproteins revealed that they share most of the changes present in p59 (Fig. 5A and B). Indeed, the increased phosphorylation of peptide 6 (and, to a lesser extent, peptides 4 and 5) detected in $p56^{PMA}$ (Fig. 5A) is approximately the same as that observed in p59 (Fig. 5B). It is of additional interest that calcium ionophores also caused an increase in amino-terminal serine phosphorylation of Lck even though they did not induce significant alterations in gel mobility (Fig. 3, lanes 10 and 11). Tryptic peptide mapping of the $p56^{lck}$ isolated after treatment of the C8 cells with A23187 (Fig. 5C) revealed that, like $p56^{PMA}$, these molecules possess increased phosphorylation of peptides 4, 5, and 6. The results of these mapping studies therefore demonstrate that peptide 7 phosphorylation was found exclusively in digests of Lck proteins demonstrating altered electrophoretic mobility. We were not able to detect any significant alterations in the occupancy of tyrosine residues 394 and 505 in C8 cells as a result of these treatments.

Effect of activating signals on $p56^{lck}$ tyrosine kinase activity. To determine whether the alterations in Lck phosphorylation had an effect on Lck tyrosine kinase activity, immune-complex kinase assays were performed after treatment of the C8 cells with the different regimens used previously. Although we found that the degree of autophosphorylation of the modified forms of Lck protein was somewhat less than that of phosphorylation of $p56^{lck}$, we could not detect any alteration in the ability of the various Lck species to phosphorylate exogenous substrates such as enolase or casein (data not shown).

DISCUSSION

There are currently eight genes that are considered members of the *src* family of tyrosine protein kinases: c-*src*, c-*yes1*, c-*fgr*, *fyn*, *lck*, *hck*, *lyn*, and *tkl* (for a review, see Cooper, in press). Three of these genes (c-*src*, c-*yes1*, and c-*fgr*) have been integrated and mutated within the genome of transforming retroviruses. In each case, the mutated cellular genes have been shown to be responsible for the transforming potential of these viruses. Introduction of mutations in the carboxy-terminal regulatory domain of two additional members of the gene family (*fyn* and *lck*) has also

FIG. 4. Effects of T-cell activating signals on the pattern of phosphorylation of the *lck* gene product. (A) C8 cells were metabolically labeled with $^{32}P_i$ and subsequently treated with the agents indicated below. The Lck proteins were immunoprecipitated from 100 μg of cell lysate and resolved by electrophoresis in SDS-PAGE gel. Lanes: 1, control; 2, con A; 3, 145-2C11 antibodies plus second antibody; 4, PMA. Exposure, 12 h. The positions of p56, p59, p62, and p68 are indicated. (B) Tryptic peptide mapping of the p59 generated after treatment with con A (panel 1), 145-2C11 antibodies plus second antibody (panel 2), and PMA (panel 3). Exposure, 7 days. (C) Effect of PMA on the Lck protein expressed in LSTRA cells (tryptic peptide mapping). Panel 1, Control p56; panel 2, p59 generated after treatment with PMA. Exposure, 12 h. The arrow in panel 1 indicates a peptide fragment different from peptide 7 that is derived from the same cyanogen bromide fragment (C3) as peptide 2 (containing Y-505). It is likely to result from partial oxidation or partial digest of peptide 2 (our unpublished data). (D) Composite diagram of the tryptic phosphopeptides detected in p59. Y, Tyrosine; S, serine.

been shown to confer oncogenic competence in fibroblasts (13a, 19). Despite our increasing understanding of the transforming potential of these mutated gene products, little is known about the functions of their normal cellular homologs.

Three of the *src*-related tyrosine kinases (c-*src*, c-*yes1*, *fyn*) have been found to be expressed in all cell types tested to date. Other members of this gene family have been shown to be expressed in a restricted set of cellular lineages (*lck*, *hck*, c-*fgr*), suggesting that they might play important functions in specialized cellular physiology (Cooper, in press). We and others have found that the *lck* gene is normally expressed predominantly in cells of lymphoid lineage (22, 30). Studies of normal and transformed lymphoid cells have demonstrated that the *lck* gene is expressed in all T-cells (20, 30) and is equally abundant in both helper-inducer ($L3T4^+$) and cytotoxic-suppressor ($Lyt\text{-}2^+$) cell lines (Fig. 1). Interestingly, the results of our analysis show that the level of $p56^{lck}$ detected in normal T-lymphocytes is generally higher than that observed in transformed T-cells (Fig. 1). These observations are consistent with the idea that the Lck protein plays a fundamental role in normal T-cell functions.

Our evaluation of the *lck* gene product expressed in several normal murine T-cell clones revealed that $p56^{lck}$ is predominantly phosphorylated in vivo on Y-505 (Fig. 2). This residue is the equivalent of Y-527 of avian $pp60^{c\text{-}src}$, which has also been shown to be extensively phosphorylated in vivo in a variety of cell types (7; for a review, see Cooper, in press) and is felt to negatively regulate the enzymatic activity of $pp60^{c\text{-}src}$. Mutation of $pp60^{c\text{-}src}$ Y-527 to phenylalanine has been shown to generate c-Src molecules with increased tyrosine kinase activity and oncogenic potential. Similar data have been obtained by mutating Y-505 of $p56^{lck}$ to phenylalanine (19). On the basis of these observations, it is likely that the enzymatic activity of $p56^{lck}$ can also be regulated by the degree of phosphate occupancy of this carboxy-terminal tyrosine residue. However, the actual stoichiometry of Y-505 phosphorylation in normal T-cells remains to be established.

After treatment of resting helper-inducer T-cells with the mitogenic lectin con A or antibodies directed against the invariant portion of the T-cell receptor complex (T3), approximately 50% of the Lck protein was converted into a product migrating at 59 kDa on SDS-PAGE gels (Fig. 3). This shift in mobility could also be induced by agents known to activate PKC and related phospholipid-dependent protein kinases (26) (Fig. 3), suggesting that one or more of these signal transduction pathways is involved in the Lck alterations. This possibility is further supported by the fact that con A and cross-linked anti-T3 antibodies have been shown to increase phosphatidyl inositol turnover and stimulation of PKC in T-lymphocytes (18, 24, 29, 33). This alteration in Lck electrophoretic mobility appears to be specific for activating signals, since it was not observed after treatment with single agents incapable of activating (as measured by increased thymidine incorporation and induction of lymphokine mRNA) these cells (e.g., calcium ionophores or antibodies directed against L3T4/CD4).

Peptide mapping and phosphoamino-acid analysis revealed that the p59 form of Lck possesses two major tryptic phosphopeptides: peptide 2, which contains Y-505, and the phosphoserine-containing peptide 7 (Fig. 4; data not shown). Several other peptides of lesser intensity were also observed after T-cell activation in both the residual p56 ($p56^{PMA}$) and p59 (peptides 4, 5, 6, and 8). Interestingly, treatment with the calcium ionophore A23187 (Fig. 5C) also resulted in an increased phosphorylation of peptide 6 and, to a lesser extent, peptides 4 and 5. Thus, the results of our experiments indicate that the altered electrophoretic mobility of Lck correlates best with the presence of a single phosphoserine-containing amino-terminal tryptic peptide, peptide 7. While we believe that this phosphopeptide results from a novel phosphorylation event, it is possible that tryptic peptide 7 in fact corresponds to a region containing a previously existent phosphorylation site that has acquired an additional posttranslational modification altering its migration during electrophoresis and/or chromatography. However, since none of the tryptic phosphopeptides detected in digests of $p56^{PMA}$ appeared diminished in p59 (Fig. 4B, panel 3, and Fig. 5A), this possibility seems unlikely.

The results of these experiments suggest that during T-cell activation at least two serine protein kinase systems are involved in the alterations of the *lck* gene product. The first pathway would involve a calcium-dependent process which induces predominantly the phosphorylation of peptide 6 (but not peptide 7). The second pathway presumably involves the activation of phospholipid-dependent protein kinases, results in the phosphorylation of peptide 7, and correlates with the appearance of p59 on SDS-PAGE gels. The ability of PMA and diC8 to induce phosphorylation of both peptide 6 and peptide 7 indicates that the two pathways suggested above do not represent mutually exclusive reaction series. This is supported by the fact that phorbol esters can directly activate both calcium-dependent and calcium-independent phospholipid-dependent kinases (26). Interestingly, T-cell activating signals (mitogenic lectins, anti-T3 antibodies) appear to stimulate both putative pathways, an observation consistent with the fact that phosphatidyl inositol turnover is stimulated by these agents (29, 33).

The alterations of $p56^{lck}$ during T-cell activation do not appear to result in any significant modification of its enzymatic activity as measured in in vitro assays, although these assays are not necessarily predictive of changes occurring in vivo. It is clearly possible that such alterations lead to changes in Lck substrate interactions. Indeed, the amino-terminal portion of the Src-related tyrosine kinases, particularly the nonconserved region of these proteins (approximately the first 70 amino acids), is considered critical for such interactions.

In summary, our data are consistent with the concept that $p56^{lck}$ or a posttranslationally modified form of this protein may play a critical role in the early signal transduction events during T-cell activation. The determination of the exact role of $p56^{lck}$ in this process and the nature of the cellular kinases responsible for its modification necessitates further evaluation.

ACKNOWLEDGMENTS

A.V. is supported by a fellowship grant from the National Cancer Institute of Canada.

We thank E. Gelmann for synthesis of oligonucleotide.

LITERATURE CITED

1. **Bolen, J. B., A. Veillette, A. M. Schwartz, V. DeSeau, and N. Rosen.** 1987. Analysis of $pp60^{c\text{-}src}$ in human colon carcinoma and normal human colon mucosal cells. Oncogene Res. **1**:149–168.
2. **Bookman, M. A., R. Swerdlow, and L. A. Matis.** 1987. Adoptive chemoimmunotherapy of murine leukemia with helper T-lymphocyte clones. J. Immunol. **139**:3166–3170.
3. **Casnellie, J. E.** 1987. Sites of *in vivo* phosphorylation of the T

cell tyrosine protein kinase in LSTRA cells and their alteration by tumor-promoting phorbol esters. J. Biol. Chem. **262**:9858–9864.

4. **Casnellie, J. E., M. L. Harrison, K. E. Hellstrom, and E. G. Krebs.** 1983. A lymphoma cell line expressing elevated levels of tyrosine protein kinase activity. J. Biol. Chem. **258**:10738–10742.
5. **Casnellie, J. E., and R. J. Lamberts.** 1986. Tumor promoters cause changes of the state of phosphorylation and apparent molecular weight of a tyrosine protein kinase in T lymphocytes. J. Biol. Chem. **261**:4921–4925.
6. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonucleases. Biochemistry **18**:5294–5299.
7. **Cooper, J. A., K. L. Gould, C. A. Carthwright, and T. Hunter.** 1986. Tyr^{527} is phosphorylated in $pp60^{c\text{-}src}$: implications for regulation. Science **231**:1431–1433.
8. **Davis, L. G., M. D. Dibner, and J. M. Battey.** 1986. Basic methods in molecular biology. Elsevier Science Publishing, Inc., New York.
9. **DeGraaf, P. W., E. Horak, and M. A. Bookman.** 1988. Adoptive immunotherapy of syngeneic murine leukemia is enhanced by the combination of recombinant IFN-γ and a tumor-specific cytotoxic T-cell clone. J. Immunol. **140**:2853–2857.
10. **Gould, K. L., J. R. Woodgett, J. A. Cooper, J. E. Buss, D. Shalloway, and T. Hunter.** 1985. Protein kinase C phosphorylates $pp60^{c\text{-}src}$ at a novel site. Cell **42**:849–857.
11. **Gray, P. W., and D. V. Goeddel.** 1983. Cloning and expression of murine interferon cDNA. Proc. Natl. Acad. Sci. USA **80**:5842–5846.
12. **Heckford, S. E., E. P. Gelmann, C. L. Agnor, S. Jacobson, S. Zinn, and L. A. Matis.** 1986. Distinct signals are required for proliferation and lymphokine gene expression in murine T cell clones. J. Immunol. **137**:3652–3663.
13. **Hunter, T., and B. M. Sefton.** 1980. Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc. Natl. Acad. Sci. USA **77**:1311–1315.

13a. **Kawakami, T., Y. Kawakami, S. A. Aaronson, and K. C. Robbins.** 1988. Acquisition of transforming properties by *fyn*, a *src*-related human gene. Proc. Natl. Acad. Sci. USA **85**:3870–3874.

14. **Koga, Y., N. Kimura, J. Minowada, and T. W. Mak.** 1988. Expression of the human T-cell-specific tyrosine kinase YT16 (*lck*) message in leukemic T-cell lines. Cancer Res. **48**:856–859.
15. **Ledbetter, J. A., L. E. Gentry, C. H. June, P. S. Rabinovitch, and A. F. Purchio.** 1987. Stimulation of T-cells through the CD3/T-cell receptor complex: role of cytoplasmic calcium, protein kinase C translocation, and phosphorylation of $pp60^{c\text{-}src}$ in the activation pathway. Mol. Cell. Biol. **7**:650–656.
16. **Ledbetter, J. A., C. H. June, L. S. Grosmaire, and P. S. Rabinovitch.** 1987. Cross-linking of surface antigens causes mobilization of intracellular ionized calcium in T-lymphocytes. Proc. Natl. Acad. Sci. USA **84**:1384–1388.
17. **Leo, O., M. Foo, D. H. Sachs, L. E. Samelson, and J. A. Bluestone.** 1987. Identification of a monoclonal antibody specific for the murine T3 polypeptide. Proc. Natl. Acad. Sci. USA **84**:1374–1378.
18. **Manger, B., A. Weiss, J. Imboden, T. Laing, and J. D. Stobo.** 1987. The role of protein kinase C in transmembrane signaling by the T cell antigen receptor complex. J. Immunol. **139**:2755–2760.
19. **Marth, J. D., J. A. Cooper, C. S. King, S. F. Ziegler, D. A. Tinker, R. W. Overell, E. G. Krebs, and R. M. Perlmutter.** 1988. Neoplastic transformation induced by an activated lymphocyte-specific protein tyrosine kinase ($pp56^{lck}$). Mol. Cell. Biol. **8**:540–550.
20. **Marth, J. D., D. B. Lewis, C. B. Wilson, M. E. Gearn, E. G. Krebs, and R. M. Perlmutter.** 1987. Regulation of $pp56^{lck}$ during T cell activation: functional implications for the src-like tyrosine kinases. EMBO J. **9**:2727–2734.
21. **Marth, J. D., R. W. Overell, K. E. Meier, E. G. Krebs, and R. M. Perlmutter.** 1988. Translational activation of the *lck* proto-oncogene. Nature (London) **332**:171–173.
22. **Marth, J. D., R. Peet, E. G. Krebs, and R. M. Perlmutter.** 1985. A lymphocyte-specific protein-tyrosine kinase is rearranged and overexpressed in the murine T-cell thymoma LSTRA. Cell **43**:393–404.
23. **Matis, L. A., S. K. Ruscetti, D. L. Longo, S. Jacobson, E. J. Brown, S. Zinn, and A. M. Kruisbeek.** Distinct proliferative T cell clonotypes are generated in response to a murine retrovirus-induced syngeneic T cell leukemia: viral gp70 antigen-specific $MT4^+$ clones and Lyt-2^+ cytolytic clones which recognize a tumor-specific cell surface antigen. J. Immunol. **135**:703–713.
24. **Meuer, S. C., J. C. Hodgdon, R. E. Hussey, J. P. Protentis, S. F. Schlossman, and E. L. Reinherz.** 1983. Antigen-like effects of monoclonal antibodies directed at receptors of human T-cell clones. J. Exp. Med. **158**:988–993.
25. **Nishizuka, Y.** 1984. The role of protein kinase C in cell surface signal transduction and tumor promotion. Nature (London) **308**:693–698.
26. **Ohno, S., Y. Akita, Y. Konno, S. Imajoh, and K. Suzuki.** 1988. A novel phorbol ester receptor/protein kinase, nPKC, distantly related to the protein kinase C family. Cell **53**:731–741.
27. **Ralston, R., and J. M. Bishop.** 1985. The product of the protooncogene *c-src* is modified during the cellular response to platelet-derived growth factor. Proc. Natl. Acad. Sci. USA **82**:7845–7849.
28. **Rosoff, P. M., S. J. Burakoff, and J. L. Greenstein.** 1987. The role of L3T4 molecule in mitogen and antigen-activated signal transduction. Cell **49**:845–853.
29. **Taylor, M. V., J. C. Metcalfe, R. B. Hesketh, G. A. Smith, and J. P. Moore.** 1984. Mitogens increase phosphorylation of phosphoinositides in thymocytes. Nature (London) **312**:462–465.
30. **Veillette, A., F. M. Foss, E. A. Sausville, J. B. Bolen, and N. Rosen.** 1987. Expression of the *lck* tyrosine kinase gene in human colon carcinoma and other non-lymphoid human tumor cell lines. Oncogene Res. **1**:357–374.
31. **Veillette, A., I. D. Horak, and J. B. Bolen.** 1988. Post-translational alterations of the tyrosine kinase $p56^{lck}$ in response to activators of protein kinase C. Oncogene Res. **2**:385–401.
32. **Voronova, A. F., and B. M. Sefton.** 1986. Expression of a new tyrosine protein kinase is stimulated by retrovirus promoter insertion. Nature (London) **319**:682–685.
33. **Weiss, A., J. Imboden, K. Hardy, B. Manger, C. Terhorst, and J. Stobo.** 1986. The role of the T3/antigen receptor complex in T-cell activation. Annu. Rev. Immunol. **4**:593–620.
34. **Wilde, D. B., P. Marrack, J. Kappler, D. P. Dialynas, and F. W. Fitch.** 1983. Evidence implicating L3T4 in class II MHC antigen reactivity. J. Immunol. **131**:2178–2183.