Phosphorylation of Serine 59 of p56Lck in Activated T Cells*

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p56Lck, a member of the src family of non-receptor protein tyrosine kinases, is expressed almost exclusively in cells of lymphoid origin. Recent evidence has implicated p56Lck in a critical role both in T cell development and activation. A variety of T cell stimuli induce a shift in the electrophoretic mobility of p56Lck from an apparent molecular mass of 56 kDa (p56Lck) to 60 kDa (p60Lck). This shift in electrophoretic mobility correlates with an increase in the phosphoserine content of the p60Lck. We have shown that both 4α-phorbol 12β-myristate 13α-acetate and OKT3 treatment of Jurkat cells, as well as 4α-phorbol 12β-myristate 13α-acetate treatment of 171.CD4 and LSTRA cells, induced phosphorylation of serine-59 on p56Lck in vivo, which correlated with the shift to p60Lck. We also demonstrated that the same serine residue could be phosphorylated in vitro with mitogen-activated protein kinases and that this event was capable of reducing p56Lck activity in vitro. Combined, these data suggest a novel pathway for the in vivo regulation of p56Lck activity.

p56Lck is a member of the src family of non-receptor protein tyrosine kinases and is expressed almost exclusively in lymphoid cells and tissues, primarily in T cells (Casnellie et al., 1984; Marth et al., 1985; Koga et al., 1986; Trevillyan et al., 1986). In T cells, p56Lck binds noncovalently to the cytoplasmic tails of the cell surface glycoproteins CD4 and CD8 (Veillette et al., 1988a; Turner et al., 1990; Chalupny et al., 1991), which bind nonpolymorphic regions of the class II and class I proteins of the major histocompatibility complex, respectively, during activation mediated by the T cell receptor-CD3 complex (TCR) (Doyle and Strominger, 1987; Norment et al., 1988; Ratnofsky et al., 1987; Rosenzweig et al., 1989). Recently, p56Lck has also been shown to interact with the β subunit of the interleukin-2 (IL-2) receptor in a manner distinct from its interaction with CD4 and CD8 (Hatakeyama et al., 1991).

Recent studies have revealed that p56Lck plays a vital role in both T cell activation through the TCR and in thymocyte development. Activation of CD4 positive T cells by antigen presenting cells has been found to be dependent on the association of p56Lck with CD4 (Glachienhaus et al., 1991; Collins et al., 1992). Signaling via the TCR following treatment of cells with soluble antibodies against a variety of TCR epitopes is strongly enhanced by the physical association of CD4 with the TCR (Dianzani et al., 1992). In fact, p56Lck modulates the association of CD4 with the TCR during this process (Collins et al., 1992). Furthermore, expression of a constitutively active p56Lck enhances T cell responsiveness (Abraham et al., 1991) and has been shown to stimulate antigen-independent IL-2 production in certain cell lines (Luo and Setton, 1992). Cell lines lacking p56Lck expression are profoundly impaired in TCR-mediated signal transduction (Straus and Weiss, 1992; Karnitz et al., 1992) and transgenic mice lacking p56Lck expression show pronounced thymic atrophy along with undetectable levels of mature CD4 or CD8 single positive thymocytes (Molina et al., 1992).

While the importance of p56Lck in T cell signaling is now clear, its in vivo regulation and substrates are not. When T cells are activated in a TCR-dependent manner, a rapid increase in the tyrosine phosphorylation of numerous proteins occurs (within 1–2 min) that appears to require the presence of functional p56Lck (Abraham et al., 1991; Caron et al., 1992; Straus and Weiss, 1992). Similarly, IL-2 stimulation of T cells causes a rapid and transient increase in p56Lck phosphorylase activity (Horak et al., 1991). This regulation may be due to dephosphorylation of p56Lck at Tyr-505, since the kinase activity of all src family protein tyrosine kinases can be reversibly regulated via phosphorylation of a conserved C-terminal tyrosine phosphorylation site (reviewed in Veillette and Davidson (1992)). The up-regulation of p56Lck at this site may be carried out by the protein tyrosine phosphatase CD45 (Ostergaard et al., 1989; Ostergaard and Trowbridge, 1990), known to be essential for T cell activation via the TCR (Koretzky et al., 1991) and down-regulation by the protein tyrosine kinase csk (Bergman et al., 1992).

While the initial activation of p56Lck has been studied extensively, subsequent signaling events and p56Lck deactivation have not. Following the initial burst in tyrosine kinase activity following cell activation, p56Lck becomes modified, resulting in a shift in electrophoretic mobility from an estimated 56- to 60-kDa form. This effect has been demonstrated by cross-linking the TCR with soluble antibodies (Veillette et al., 1988b) by treatment of cells with IL-2 (Horak et al., 1991), or with activators of protein kinase C such as 4α-phorbol 12β-myristate 13α-acetate (PMA) (Casnellie and Lamberts, 1986; Veillette et al., 1988a, 1988b; Marth et al., 1983; Hurley et al., 1989; Luo and Setton, 1990). This shift in electrophoretic mobility correlates with increased serine phosphorylation of 23275
p60^ik (Casnellie and Lamberts, 1986; Veillette et al., 1988a, 1988b; Luo and Sefton, 1990). In the case of IL-2 stimulation, phosphatase treatment of the induced p60^ik causes a reversion to a 56-kDa form (Horak et al., 1991). However, the kinase(s) responsible for serine phosphorylation of p56^ik, the site(s) of modification, and their functional consequences have remained elusive.

We have employed a number of lymphocyte-derived cell lines to study the serine phosphorylation of p56^ik following cell activation. We demonstrate the efficient conversion of p56^ck to p60^ik in the human T cell line Jurkat following activation, either by cross-linking the surface TCR or by treatment with PMA. Peptide mapping of the p60^ik from the activated cells revealed the induction of a major site of serine phosphorylation. Similar results were obtained following PMA treatment of the mouse cell lines 171.CD4 and LSTRA. Using purified p60^ik and purified mitogen-activated protein kinases (MAPKs), we demonstrated that MAPKs will phosphorylate p60^ik in vitro at a single serine residue, and have gone on to show that this is the same site as the one induced in vivo. This phosphorylation has been identified as being at Ser-59 via two-dimensional phosphopeptide mapping in conjunction with mass spectrometric analyses and solid-phase peptide sequencing of synthetic peptides derived from p56^ik. We also found that this modification results in a partial reduction in the in vitro catalytic activity of p56^ik, implying a regulatory pathway for p60^ik involving both Tyr-505 and Ser-59 phosphorylation-dephosphorylation events.

EXPERIMENTAL PROCEDURES

Antibody Preparations—Polyclonal rabbit antiserum raised against a p56^ik-3pe fusion protein was kindly provided by Dr. P. Johnson (University of British Columbia). Supernatant from the mouse hybridoma cell line OKT3 (ATCC), which secretes antibodies specific for the external domain of the ε subunit of the human CD3-TCR complex, was purified on a protein-A Sepharose column (Pharmacia LKB Biotechnology Inc.). Antibodies were eluted with 3.5 M MgCl2 and dialyzed extensively at 4 °C against phosphate-buffered saline. Antibody concentration was determined via UV absorbance at 280 nm and adjusted to 500 μg/ml in phosphate-buffered saline. All antibodies and antiserum were sterile-filtered and stored at −80 °C in small aliquots until required.

Cell Stimulations and Labeling—The human T cell line Jurkat and mouse lymphocyte cell line LSTRA were cultured in RPMI 1640 medium (Terry Fox Laboratories (TFL), University of British Columbia). The mouse T cell line 171.CD4 (Gischagen et al., 1991) was cultured in Dulbecco’s modified Eagle’s medium (TFL). All culture media were supplemented to 10% fetal calf serum (Life Technologies Inc.) and 50 μM 2-mercaptoethanol. Prior to biosynthetic labeling, all cells were transferred to phosphate-free RPMI 1640 medium supplemented with dialyzed fetal calf serum to 10% at 37 °C. Jurkat cells were labeled for 2 h at 37 °C at 2.5 × 106 cells/ml with H3[32P]O4 (ICN) added to 1 μCi/ml. LSTRA cells were labeled for 2 h at 37 °C at 5 × 106 cells/ml with H3[32P]O4 added to 0.5 μCi/ml. 171.CD4 cells were labeled for 2 h at 37 °C at 1 × 106 cells/ml with H3[32P]O4 added to 1 μCi/ml. Cells were stimulated with PMA (Sigma) at a final concentration of 50 ng/ml for 30 min at 37 °C or with a dimethyl sulfoxide (the PMA solvent) control. Jurkat cells were additionally stimulated with the anti-TCR monoclonal antibody (OKT3) at a final concentration of 10 μg/ml, or with total rabbit protein A-purified IgG (control) at 10 μg/ml for 30 min at 37 °C.

Immunoprecipitations and Immunoblots—Cells were pelleted and lysed at 4 °C in 50 mM Tris, pH 8, 150 mM NaCl, 5 mM NaF, 2 mM EDTA, 1% Nonidet P-40, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, 10 μM β-mercaptoethanol, 10 μg/ml soybean trypsin inhibitor (Sigma). Nuclei and insoluble matter were removed by centrifugation. SDS gel sample buffer (containing either 1% 2-mercaptoethanol or 50 mM dithiothreitol) was added directly to samples to be analyzed by immunoblotting. Immunoprecipitations were prepared with 1 μl of anti-p56^ik antiserum at 4 °C for 2 h, followed by 20 μl of protein-A Sepharose for 1 h at 4 °C.

Sepharose beads were washed 3 times in lysis buffer with LiCl added to 500 mM, then twice more in lysis buffer. All samples were boiled for 5 min in SDS gel sample buffer prior to analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels.

Gels to be analyzed by immunoblots were transferred to nitrocellulose membranes (Schleicher & Schuell) at 120 V overnight at 4 °C. Membranes were stained with Coomassie Blue (ICN) and phosphoproteins were visualized by autoradiography of the (wet) destained gels. Individual species observed by autoradiography were excised from the gel, and the gel slices washed extensively in deionized water and partially dried under vacuum. Proteolytic digestion was overnight at 37 °C in 150–200 μl of 1% ammonium bicarbonate, pH 7.8, with 20 μg of V3 protease (Boehringer Mannheim) added to each sample. Eluted peptides were recovered in the supernatant, Cerenkov counted in a Packard 2200CA Tri-Carb liquid scintillation counter to quantitate peptide recovery, and dried under vacuum. Samples were then resuspended in a minimal volume of 1% SDS, spotted onto (20 × 20 cm) cellulose thin-layer chromatography (TLC) plates (Kodak). These were electrophoresed in the first dimension (20 cm) in 10% acetic acid, 1% pyridine at 120 volts for 110 min, dried in air, and developed in the second dimension (10 cm) in 34% water, 30% pyridine, 30% 1-butanol, 6% acetic acid. Phosphopeptides were visualized by autoradiography.

Phosphoamino Acid Analysis—Phosphoamino acid analysis was performed as previously described (Watts et al., 1992) directly on phosphoproteins transferred onto Immobilon-P polyvinylidene difluoride membrane (Millipore) or on individual phosphopeptides eluted from a cellulose TLC plate in 20% acetonitrile and dried under vacuum.

In Vitro Kinase Phosphorylations—Mouse p56^ik was purified from Sf cells by a baculovirus expression system as described (Watts et al., 1992) and the MAPK p44^ik was purified from oocytes of the sea star Pisaster ochraceus as described (Sanghera et al., 1990). p56^ik was phosphorylated in the presence or absence of p44^ik in 20 mM Hepes, pH 7.2, 10 mM MgCl2, 0.1% Triton X-100, 1 mM ATP with [γ-32P]ATP (ICN) added to 2200 dpm/pmol. Reactions were performed for 45 min at 30 °C and were terminated by boiling in SDS gel sample buffer. Phosphorylated proteins were separated by SDS-PAGE in 10% gels and visualized by autoradiography.

Peptide and protein samples were digested with 50 μg of V8 protease of both mouse and human p56^ik (p56^ik 50–75) were kindly provided by Dr. I. Clark-McKie (Biomedical Research Centre, University of B.C.). Peptides were phosphorylated with p44^ik in 20 mM Hepes, pH 7.2, 10 mM MgCl2, 1 mM ATP with [γ-32P]ATP added to 500 dpm/pmol and repurified by high performance liquid chromatography (HPLC) on a Waters 990 Peptide Analyzer and a Vydac C18 column. Phosphorylated peptides were digested with 50 μg of V8 protease in 1% ammonium bicarbonate, pH 7.8, and analyzed either directly by two-dimensional phosphopeptide mapping, or by a second round of HPLC followed by two-dimensional phosphopeptide mapping of radiolabeled peptides detected by UV absorbance at 215 nm and Cerenkov counting.

Mass Spectroscopic and Protein Sequence Analyses—Mass spectrometric analyses were performed on a Sciex API III triple quadrupole mass spectrometer. Exact mass determination was performed either on individual HPLC peaks, or on phosphopeptides eluted in 20% acetonitrile from TLC plates following two-dimensional phosphopeptide mapping. Peptides eluted from the TLC plates were repurified over a 0.5-mm microcrome C8 HPLC column (Keystone Scientific) using a Microm Ultraltra Microprotein Analyzer HPLC connected on-line to the mass spectrometer for detection and mass determination (LC/MS). Full details of phosphopeptide mapping by LC/MS will be published elsewhere.2

Determination of serine phosphorylation sites induced in the p56^ik 50–75 peptides by p44^ik following V8 digestion and repurification by mass spectrometry was performed with a Microm MassLynx mass spectrometer (Microm). Two-dimensional phosphopeptide mapping was performed with a Microm Micromass ProteinChip Analyzer (Microm). Peptide mapping by LC/MS was performed on a Microm Ultima LC/MS High-Resolution Mass Spectrometer (Microm). The LC/MS instrument was interfaced on-line to the mass spectrometer for detection and mass determination (LC/MS). Full details of phosphopeptide mapping by LC/MS will be published elsewhere.2

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HPLC was performed by multiple Edman degradation cycles in a Milligen 6600 Protein Sequencer, as described elsewhere (Wettenhall et al., 1991; Aebersold et al., 1991).

**p56** Activity Assays—Purified p56 (final concentration of 40 μg/ml) was preincubated in 20 mM Hepes, pH 7.2, 10 mM MgCl₂, 100 μM ATP, 0.1% Nonidet P-40 at 30 °C for 15 min. An equal volume of p44 (final concentration of 2 μg/ml) was preincubated in 20 mM Hepes, pH 7.2, 12.5 mM MgCl₂, 17.5 mM MgCl₂, 5 mM EGTA, 0.5 mM NaF, 0.1% Nonidet P-40 or a buffer blank was added, followed by [γ-32P]ATP to 8800 dpm/pmol, and the mixture incubated for a further 40 min at 30 °C.

p56 autophosphorylation was analyzed by boiling samples in SDS gel sample buffer and loading on 7.5%-polyacrylamide gels. Phosphorylated p56 was visualized in situ by autoradiography, and the level of autophosphorylation determined by V8 phosphopeptide mapping as described above.

p56 activity towards the TCR-ζ (52-164) peptide was determined by adding 5-μl aliquots of the p44-phosphorylated and nonphosphorylated p56 to 20 μl of 20 mM Hepes, pH 7.2, 10 mM MgCl₂, 100 μM ATP, 0.1% Nonidet P-40 with [γ-32P]ATP to either 1100 or 8800 dpm/pmol, and TCR-ζ (52-164) added to either 6 or 30 μM (Kₘ = 6.5 μM (Watts et al., 1992)). Assays were performed for 3 min at 30 °C and terminated by boiling in SDS gel sample buffer (with EDTA added to 100 mM). Phosphoproteins were visualized by autoradiography following electrophoresis on 15% SDS-polyacrylamide gels and transfer to polyvinyldene difluoride membrane. TCR-ζ (52-164) phosphorylation was quantified by excising bands and scintillation counting.

**RESULTS**

The human T cell line Jurkat was treated, following biosynthetic 32P labeling of the ATP pool for 30 min, with either PMA, or the anti-CD3/TCR antibody OKT3. In both cases, immunoprecipitated Lck protein shifted in electrophoretic mobility from a 56-kDa species (p56) to one of 60 kDa (p60) following stimulation (Fig. 1A). Immunoblot analyses indicated that p56 levels remained essentially unchanged following stimulation (Fig. 1B). Furthermore, in Jurkat cells, conversion to p60 was highly efficient with both OKT3 and PMA. The autoradiogram shown in Fig. 1A revealed enhanced phosphorylation of p56 during its conversion to p60. Cerekova counting of the excised Lck protein bands confirmed that p60 contained about 2 to 3 times the amount of radiolabel than did p56 prior to stimulation. In agreement with the published literature, phosphoamino acid analyses of these bands showed a substantial increase in the phosphoserine content of p60 compared with p56 (data not shown).

Two-dimensional phosphopeptide maps of both the p56 and p60 species observed in Fig. 1A were obtained by digestion of visualized phosphoproteins with V8 protease in the gel matrix. Phosphoamino acid analyses were performed on the phosphopeptides eluted from TLC plates, whenever sufficient signal was available, and these results have been incorporated as (S) for phosphoserine and (Y) for phosphotyrosine into all the figures relating to phosphopeptide maps. p56 from unstimulated Jurkat cells (Fig. 2A) yielded one major phosphotyrosine-containing peptide (spot A) confirmed as containing Tyr-505 by its co-migration with a synthetic peptide corresponding to the expected V8 protease-derived tyrosine-phosphorylated C-terminal fragment of p56 (data not shown). Analysis of p60 observed following PMA treatment (Fig. 2B) revealed the presence of a new phosphopeptide (spot B) in addition to spot A. An identical two-dimensional pattern was obtained for p60 following OKT3 treatments of Jurkat cells (data not shown). Phosphoamino acid analyses of spots A and B before and after stimulation confirmed this induced phosphorylation as being at serine residues (Fig. 2C).

Although phosphoamino acid analysis had shown a substantial increase in the phosphoserine content of p60 observed following PMA treatment, there was a basal level of phosphoserine observed in the 56-kDa region of the gel prior to PMA treatment (data not shown). A number of earlier studies, all using PMA for cell activation had suggested multiple sites of serine phosphorylation on p56. However, these studies used peptide-mapping systems susceptible to artifacts, since chymotrypsin (Luo and Sefton, 1990) is not very specific and trypsin (Veillette et al., 1988b; Hurley and Sefton, 1989)

**Fig. 1. Conversion of p56 to p60 in Jurkat cells.** Analytical Lck protein species present in Jurkat cells before and after stimulation. Lanes 1 and 3, control (unstimulated) cells; lane 2, 30-min treatment with PMA (50 ng/ml); lane 4, 30-min treatment with OKT3 (10 μg/ml). A, in vivo phosphorylated p56 (p56) and p60 (p60) immunoprecipitated from 2 × 10⁶ cells per lane following biosynthetic 32P labeling of the cellular ATP pool. Visualization of the phosphoproteins was by 10% SDS-PAGE and autoradiography. The migration of molecular mass standard proteins are also indicated (kDa). B, anti-p56 immunoblot analyses of the p56 (p56) and p60 (p60) content in total cell lysates (2 × 10⁶ cells per lane) following 10% SDS-PAGE. Visualization was by enhanced chemiluminescence (Amersham). The migration of molecular mass standard proteins are also indicated (kDa).

**FIG. 2. Two-dimensional phosphopeptide mapping of p56 and p60 from Jurkat cells.** Lck protein was immunoprecipitated from 2 × 10⁶ Jurkat cells following biosynthetic 32P labeling of the ATP pool. p56 was separated from p60 by 10% SDS-PAGE, and two-dimensional V8 protease phosphopeptide maps were obtained for A, p56 precipitated from unstimulated cells (from Fig. 1A, lane 1) and B, p60 precipitated from PMA-treated (30 min, 50 ng/ml) cells (from Fig. 1A, lane 2). The origin of sample application is also indicated (O) along with the orientation of the positive (+) and negative (−) electrodes. C. phosphoamino acid analysis was performed on phosphopeptides eluted from the two-dimensional maps. Lane A, spot A eluted from A; lane B, spot B eluted from B. The migration of standard ninhydrin visualized phosphoserine (PS), phosphothreonine (PT), and phosphotyrosine (PY) are indicated, along with the origin of sample application (O). The phosphoamino acid detected is indicated in parentheses in the two-dimensional maps when determined (single letter code). Exposure time, 20 h for panels A and B.
frequently produces nonspecific proteolytic artifacts. We thus employed a more specific protease, V8, for these studies. Cerenkov counting of the gel pieces prior to and following V8 phosphopeptide mapping was performed to show efficient recovery into solution (consistently around 75–80%) of the radiolabel for both p56\textsuperscript{ck} prior to and p60\textsuperscript{ck} following treatment of cells with either PMA or OKT3. This suggests that the V8 enzyme employed in these experiments successfully recovers into solution the major phosphopeptides present in both species. The possibility remains, however, that other minor phosphorylation sites may be present in both p56\textsuperscript{ck} and p60\textsuperscript{ck).

To test whether this induced serine phosphorylation of p56\textsuperscript{ck} was peculiar to Jurkat cells, PMA treatment and two-dimensional phosphopeptide mapping was performed in a similar fashion on the mouse T cell line 171.CD4 (Glaichenhaus et al., 1991) and the mouse cell line LSTRA, which overexpresses the Lck protein about 50-fold (Marth et al., 1988a). Fig. 3, A–D, show that in both of these cell lines, Tyr-505 was again constitutively phosphorylated (spot A). In LSTRA cells (Fig. 3, C and D), a second phosphotyrosine containing peptide (spot C) was apparent in both p56\textsuperscript{ck} and p60\textsuperscript{ck}. As was seen in Jurkat cells, PMA treatment of both cell lines induced a single new phosphoserine-containing peptide (spot B') in p60\textsuperscript{ck} (Fig. 3, B and D). Since both mouse and human cells were used in these studies, the resulting differences in the amino acid sequence of p56\textsuperscript{ck} could account for the difference seen in the mobility of the phosphoserine-containing peptides observed in stimulated mouse cells (Fig. 3, spot B') and in stimulated human cells (Fig. 2, spot B). Since there are no differences in the mouse and human p56\textsuperscript{ck} sequences at the C terminus, the mobility of the Tyr-505 containing phosphopeptide remained unchanged (spot A).

Our previous work had shown that p56\textsuperscript{ck} could directly phosphorylate and activate p44\textsuperscript{mpk} (Ettehadieh et al., 1992), a sea star homologue of MAPKs (Sanghera et al., 1990; Rossmann et al., 1991; Posada et al., 1991) also known as extracellular signal-regulated kinases (ERKs). Furthermore, the p42\textsuperscript{mpk} isozyme is also tyrosine-phosphorylated and activated in TCR-stimulated 171.CD4 cells in a CD4-dependent fashion (Ettehadieh et al., 1992), thus implying a role for p56\textsuperscript{ck}. Since MAPKs may also be activated by treatment of T cells with PMA (Nel et al., 1990; Whitehurst et al., 1992), we focused on the MAPK family to test whether they were able to phosphorylate p56\textsuperscript{ck} in vitro. We found that when purified baclovirus-expressed murine p56\textsuperscript{ck} (Watts et al., 1992) was incubated in the presence of purified p44\textsuperscript{mpk} (Sanghera et al., 1990), p56\textsuperscript{ck} became serine-phosphorylated (data not shown). We also tested the human p44\textsuperscript{er} MAPK isofrom, known to be expressed and active in T cells (Nel et al., 1990; Ettehadieh et al., 1992, 1992) in the form of an Escherichia coli-expressed glutathione S-transferase/p44\textsuperscript{er} (GST-erk1) fusion protein, and again observed serine phosphorylation of p56\textsuperscript{ck} (data not shown).

Two-dimensional phosphopeptide mapping of p56\textsuperscript{ck} prepared from PMA-treated cells (30 min, 50 ng/ml). The origin of sample application is indicated (O) along with the orientation of the positive (+) and negative (−) electrodes. Phosphoamino acid analysis was performed on individual spots (not shown), and the phosphoamino acid detected is indicated in parentheses (single letter code). Exposure times: 90 h, panel A; 340 h, panel B; 16 h, panel C; and 20 h, panel D.

**Fig. 3.** Two-dimensional phosphopeptide mapping of p56\textsuperscript{ck} and p60\textsuperscript{ck} from 171.CD4 and LSTRA cells. Lck protein immunoprecipitates were made from A and B, 1 × 10\textsuperscript{6} 171.CD4 cells, or C and D, 5 × 10\textsuperscript{5} LSTRA cells following biosynthetic \textsuperscript{32}P labeling of the ATP pool. p56\textsuperscript{ck} was separated from p60\textsuperscript{ck} by 10% SDS-PAGE. Two-dimensional phosphopeptide mapping of p56\textsuperscript{ck} in vitro: B, p56\textsuperscript{ck} phosphorylated in vitro with p44\textsuperscript{mpk}, and C, a 50:50 mixture (in cpm) of p56\textsuperscript{ck} phosphorylated by p44\textsuperscript{mpk} from B, and p60\textsuperscript{ck} immunoprecipitated from \textsuperscript{32}P-labeled PMA-treated LSTRA cells from Fig. 3D. The origin of sample application is also indicated (O) along with the orientation of the positive (+) and negative (−) electrodes. Phosphoamino acid analysis was performed on individual spots (not shown), and the phosphoamino acid detected in each case is indicated in parentheses (single letter code). Exposure time, 20 h for panels A, B, and C.

**Fig. 4.** Two-dimensional phosphopeptide mapping of p56\textsuperscript{ck} phosphorylated in vitro with MAPK. Two-dimensional V8 protease phosphopeptide maps of A, purified p56\textsuperscript{ck} autophosphorylated in vitro; B, p56\textsuperscript{ck} phosphorylated in vitro with p44\textsuperscript{mpk}, and C, a 50:50 mixture (in cpm) of p56\textsuperscript{ck} phosphorylated by p44\textsuperscript{mpk} from B, and p60\textsuperscript{ck} immunoprecipitated from \textsuperscript{32}P-labeled PMA-treated LSTRA cells from Fig. 3D. The origin of sample application is also indicated (O) along with the orientation of the positive (+) and negative (−) electrodes. Phosphoamino acid analysis was performed on individual spots (not shown), and the phosphoamino acid detected in each case is indicated in parentheses (single letter code). Exposure time, 20 h for panels A, B, and C.
toposphorylated p56\textsuperscript{\textalpha\textkappa} yielded two phosphopeptides (Fig. 4A). The major site was identical to the minor phosphotyrosine-containing peptide (spot C) observed in p56\textsuperscript{\textalpha\textkappa} and p60\textsuperscript{\textalpha\textkappa} immunoprecipitated from LSTRA cells (Fig. 4C) and presumably contains Tyr-394, the expected p56\textsuperscript{\textalpha\textkappa} autophosphorylation site (Amrein and Sefton, 1988; Marth et al., 1988b). A second phosphotyrosine-containing peptide was observed (spot D) that may be either a second in vitro autophosphorylation site of p56\textsuperscript{\textalpha\textkappa}, or an artifact due to incomplete proteolysis around Tyr-394. When p56\textsuperscript{\textalpha\textkappa} was phosphorylated in vitro with p44\textsuperscript{\textalpha\textkappa} (Fig. 4B) a third phosphopeptide, containing phosphoserine, was observed (spot B'). This peptide exhibited an identical mobility to the PMA-induced phosphopeptide (spot B') observed in both murine cell lines, 171. CD4 and LSTRA (Fig. 4C), as measured by co-migration.

The preferred target sequence for MAPK action is Pro-X-Ser/Thr-Pro (where X is a variable residue) (Clark-Lewis et al., 1991). One such sequence, centered around Ser-59, occurs in the N-terminal region of p56\textsuperscript{\textalpha\textkappa}. While human and mouse p56\textsuperscript{\textalpha\textkappa} are highly related, the expected V8 fragments derived from the two species would contain Ser-59 and either Leu (mouse) or Asn (human) at position 55 in a single peptide. Since both mouse and human cell lines were studied, we synthesized peptides corresponding to residues 50–75 of human and mouse p56\textsuperscript{\textalpha\textkappa}. These peptides were phosphorylated in vitro with p44\textsuperscript{\textalpha\textkappa}, digested with V8 protease, fragments purified by HPLC, and then analyzed by 2-D phosphopeptide mapping.

Fig. 5A shows the co-migration of the synthetic phosphorylated human peptide (following V8 digestion and HPLC repurification) with the serine-phosphorylated peptide produced in vivo following PMA (or OKT3) treatment of Jurkat cells (spot B). The synthetic phosphorylated peptide was eluted from a duplicate two-dimensional map and analyzed by microbore HPLC and ion-spray mass spectroscopy (LC/MS) (Fig. 5, B and C). The observed mass (M+H\textsuperscript{+} = 1162.6) corresponded to the calculated mass (M+H\textsuperscript{+} = 1162.51) for residues 53–63 of the murine p56\textsuperscript{\textalpha\textkappa} sequence plus 80 mass units due to a single phosphate-ester group. Since this peptide contained 2 serine residues, it was subjected to peptide sequence analysis, collecting and scintillation counting of column eluate at each cycle in order to detect the loss of radiolabel (Wettenhall et al., 1991; Aebersold et al., 1991) (Fig. 5D). This confirmed the phosphorylated residue as being Ser-59. These analyses were repeated with the synthetic murine p56\textsuperscript{\textalpha\textkappa} 50–75 peptide, again confirming phosphorylation at Ser-59, with the phosphorylated V8 fragment having an identical mobility to the induced phosphoserine peptide (spot B') observed in 171.CD4 and LSTRA cells (data not shown).

The co-migration of the singly phosphorylated synthetic p56\textsuperscript{\textalpha\textkappa} 53–63 peptides, as well as the V8-derived phosphoserine-containing peptides produced in vitro with purified p56\textsuperscript{\textalpha\textkappa} and p44\textsuperscript{\textalpha\textkappa} with the induced phosphoserine-containing peptides observed in vivo (Figs. 5A and 4C, and data not shown), thus demonstrate the presence of a single induced phosphoserine residue in this region of p56\textsuperscript{\textalpha\textkappa} in vivo. Consistent with

**Fig. 5. Identification of Ser-59 as an in vitro and in vivo site of serine phosphorylation on p56\textsuperscript{\textalpha\textkappa}.** A synthetic peptide corresponding to residues 50–75 of human p56\textsuperscript{\textalpha\textkappa} was phosphorylated in vitro with p44\textsuperscript{\textalpha\textkappa}, digested with V8 protease, and the phosphorylated fragment, now corresponding to residues 53–63 of p56\textsuperscript{\textalpha\textkappa} repurified by HPLC. A, two-dimensional phosphopeptide map of the synthetic, phosphorylated human p56\textsuperscript{\textalpha\textkappa} 53–63 fragment mixed with V8 digested p56\textsuperscript{\textalpha\textkappa} immunooprecipitated from 32P-labeled PMA-treated Jurkat cells from Fig. 2B. B and C, around 15 pmol of the synthetic, phosphorylated human p56\textsuperscript{\textalpha\textkappa} 53–63 peptide was run alone on a duplicate phosphopeptide map, eluted from the cellulose TLC plate, and repurified by HPLC, with the column eluate being injected on-line into an ion-spray mass spectrometer. B, the total ion current, measured as a function of time. C, the mass/charge (m/z) spectrum within the indicated time window. The singly charged (1162.6) and doubly charged (581.8) peptide ions are labeled. The observed mass corresponds to the actual mass (M) plus an additional mass unit due to the addition of a proton (H\textsuperscript{+}) during the ionization process. D, V8 digested and HPLC-purified phosphorylated human p56\textsuperscript{\textalpha\textkappa} 53–63 from A was analyzed by peptide sequencing. Radiolabel released from the sequenator following each Edman cycle was collected and scintillation counted. The radioactive counts recovered following each sequencing cycle are shown, along with the sequence of the human p56\textsuperscript{\textalpha\textkappa} 53–63 peptide.
Serine Phosphorylation of p56\textsuperscript{ck} in T Cells

The in vitro phosphorylation of p56\textsuperscript{ck} at Ser-59 is the in vitro phosphorylation of this residue by p44\textsuperscript{mapk} and GST-erkl, the activation of the p42\textsuperscript{mapk} isozyme following TCR antibody treatment of T cells (Ettehadieh et al., 1992) and the serine phosphorylation of p56\textsuperscript{ck} at the same site following cross-linking of the surface IgM on B cells\textsuperscript{4} and IL-2 stimulation of IL-2 dependent splenic T cells,\textsuperscript{5} both of which also induce activation of the p42\textsuperscript{mapk} isozyme (Gold et al., 1992).\textsuperscript{6}

While we have demonstrated that Ser-59 phosphorylation of p56\textsuperscript{ck} is occurring in vivo and that MAPK can effect this modification in vitro, we addressed the possibility that other protein kinases could also phosphorylate p56\textsuperscript{ck} at that site, at least in vitro. Since Ser-59 phosphorylation is inducible with a protein kinase C-activating compound (PMA), we tested a purified protein kinase C α/β/γ isoform mixture for its ability to phosphorylate the synthetic p56\textsuperscript{ck} 50–75 peptide compared with a positive myelin basic protein control. Scintillation counting and MS analyses confirmed the phosphorylation of myelin basic protein by protein kinase C, but we were not able to detect significant levels of phospho-p56\textsuperscript{ck} 50–75 in the mass spectrometer (data not shown). Thus it is unlikely that the in vivo induction of Ser-59 phosphorylation induced by PMA is due to the direct phosphorylation of p56\textsuperscript{ck} by protein kinase C. We also tested purified protein kinase C, as well as cdc2 kinase, casein kinase II, and the catalytic subunit of cyclic AMP-dependent kinase for their ability to phosphorylate intact p56\textsuperscript{ck} in vitro, compared with appropriate positive controls for each kinase activity. We found p56\textsuperscript{ck} to be a poor in vitro substrate for cdc2 kinase and protein kinase C, and was not phosphorylated by the other two (data not shown).

To test whether phosphorylation of p56\textsuperscript{ck} at Ser-59 could affect the enzymatic activity of p56\textsuperscript{ck}, aliquots of purified p56\textsuperscript{ck} were incubated with [γ-\textsuperscript{32P}]ATP in the presence or absence of p44\textsuperscript{mapk}. p56\textsuperscript{ck} activity was then measured either by visualizing p56\textsuperscript{ck} autophosphorylation in SDS-polyacrylamide gels and two-dimensional phosphopeptide maps or by measuring the tyrosine phosphorylation of a model substrate (Fig. 7) in this case a synthetic peptide corresponding to the TCR-ζ subunit cytoplasmic domain (residues 52–164, Watts et al. (1992)).

Since p56\textsuperscript{ck} was being used as a substrate for p44\textsuperscript{mapk} and was thus in excess (~25-fold), p56\textsuperscript{ck} was incubated with (unlabeled) ATP for a short time in order to reduce the substantial tyrosine autophosphorylation signal, prior to the addition of [γ-\textsuperscript{32P}]ATP and either p44\textsuperscript{mapk} or a buffer control. Fig. 6A demonstrates that p56\textsuperscript{ck} phosphorylation was reduced in the presence of p44\textsuperscript{mapk}. Phosphopeptide mapping of the MAPK-treated and control p56\textsuperscript{ck} showed a significant reduction in the p56\textsuperscript{ck} autophosphorylation-derived peptides (spots C and D) and appearance of the Ser-59-containing phosphopeptide (spot B’) upon the addition of p44\textsuperscript{mapk} (Fig. 6, B and C). This same effect was also observed with the addition of the GST-erkl fusion protein in place of p44\textsuperscript{mapk} (data not shown). In order to allow direct comparison of spot intensities, the phosphopeptides derived from the two phosphorylated p56\textsuperscript{ck} species seen in Fig. 6A were applied to the TLC plates in entirety. The resultant phosphopeptide maps (Fig. 6, B and C) were then exposed to film for identical periods of time.

We also looked at the phosphorylation by p56\textsuperscript{ck} of an external substrate, the synthetic TCR-ζ (52–164) peptide, in response to MAPK phosphorylation. Again, p56\textsuperscript{ck} was auto-phosphorylated for a short time prior to the addition of

\textsuperscript{4} M. Gold, R. Chiu, R. Ingham, T. Saxton, I. van Oostveen, J. Watts, and R. Aebersold, manuscript in preparation.

\textsuperscript{5} J. D. Watts, M. Welham, L. Kalt, J. Schrader, and R. Aebersold, manuscript in preparation.

\textsuperscript{6} J. D. Watts, M. Welham, L. Kalt, J. Schrader, R. Aebersold, manuscript in preparation.
Serine Phosphorylation of p56^ck in T Cells

[\gamma-32P]ATP (to allow confirmation of serine phosphorylation of p56^ck by p44^ak] and either p44^ak or a buffer control. Aliquots of these preparations were then used to phosphorylate the TCR-\zeta (52-164) peptide. Fig. 7A shows the reduced TCR-\zeta (52-164) phosphorylation by p56^ck (and p56^ak auto-phosphorylation) when the p56^ck was initially phosphorylated with p44^ak. Scintillation counting of the TCR-\zeta (52-164) bands from several experiments (n = 5) gave an average inhibition of p56^ak activity towards this substrate of \~50% relative to the control (Fig. 7B). Quantitative 125I-immuno-blotting of soluble proteins following phosphorylation reactions of p56^ck showed that the reduction in p56^ak activity was not due to enzyme depletion (data not shown). Additional control experiments were performed demonstrating that the TCR-\zeta (52-164) is not a substrate for p44^ak, that the effect was not dependent on the presence of p44^ak protein alone by heat-inactivating the p44^ak prior to the experiment, and is not dependent on the TCR-\zeta (52-164) concentration (data not shown). Once again, the same effect was observed with the addition of the GST-erk1 fusion protein in place of p44^ak (data not shown).

**DISCUSSION**

While the serine phosphorylation of p56^ak and its shift in apparent molecular mass to p60^ak have been known about for a number of years, the site(s) of modification, their physiological significance, and the protein kinase(s) responsible for them have remained elusive. The phorbol ester PMA has been well documented as capable of inducing this type of modification in p56^ck (Cassellie and Lamberts, 1986; Veillette et al., 1988b, 1986c; Luo and Sefton, 1990). We have identified, for the first time, a major site of serine phosphorylation (Ser-59) induced on p56^ck by treatment of T cells with either PMA or anti-CD3/TCR antibodies and have shown, at least in vitro, that a MAPK can introduce the same modification on p56^ck.

*In vitro* kinase assays have also shown that phosphorylation of this residue can down-regulate the *in vitro* catalytic activity of p56^ak.

We have previously documented the specific tyrosine phosphorylation and activation within T cells of the p42^mek MAPK family member in a CD4-dependent (thus presumably a p56^ck-dependent) manner (Ettehadieh et al., 1992). Since it has also been shown that MAPKs can be activated in T cells via protein kinase C (Nel et al., 1990; Whitehurst et al., 1992), it seems plausible that a MAPK may also be responsible for this modification of p56^ck *in vivo*. Further evidence in support of such a model comes from our work on B cells, where the same serine residue of p56^ck becomes phosphorylated *in vivo* in response to cross-linking of the surface IgM, known to also result in activation of MAPK (Gold et al., 1992). The same effects (Ser-59 phosphorylation of p56^ak and MAPK activation) have also been observed following IL-2 stimulation of splenic T cells. Thus in conjunction with these previous data, the results presented here suggest a possible regulatory loop for p56^ck, whereby dephosphorylation of Tyr-505, possibly by CD45, causes the initial activation of p56^ck activity. Subsequent down-regulation of the pathway would then be achieved by phosphorylation of Ser-59, possibly by a MAPK, and perhaps involving additional mechanisms, such as re-phosphorylation of Tyr-505. However, while we have clearly demonstrated that Ser-59 of p56^ck is phosphorylated *in vivo* and that a MAPK can effect this modification *in vitro*, it has yet to be determined whether a MAPK performs this modification *in vivo*.

Several nonreceptor (e.g. p60^k) and receptor protein tyrosine kinases (e.g. the epidermal growth factor and insulin receptors) are known to become serine-phosphorylated under various conditions (reviewed in Pelech et al. (1990)), although little is known about what the *in vivo* roles of such modifications are. While the regulation of Ser/Thr kinases by tyrosine phosphorylation is well documented (see Pelech et al. (1990)), the possible regulation of tyrosine kinases by Ser/Thr phosphorylation is unclear. Using purified kinases, we have now been able to demonstrate the *in vitro* down-regulation of a tyrosine kinase activity (p56^ck) due to serine phosphorylation, in this case by MAPKs. However, it still remains to be determined what the *in vivo* consequences of this event are. There are several *in vivo* roles one could envisage phosphorylation of Ser-59 as mediating apart from the direct inhibition of p56^ak activity. These would include the internalization of the CD4-p56^ck complex, the interaction of p56^ck with other signaling pathway components or receptor-type molecules, or the proteolytic degradation of such pathway components.

Following T cell activation, CD4 becomes serine-phosphorylated, possibly by protein kinase C (Acres et al., 1986; Hoxie et al., 1986), the CD4-p56^ck complex dissociates (Hurley et al., 1989), and CD4 becomes internalized (Acres et al., 1986; Blue et al., 1987; Maddon et al., 1988). However, this process does not seem to be dependent on the serine phosphorylation and concurrent shift in apparent molecular mass of p56^ck (Hurley et al., 1989). Rather, the mutation of several serine residues in CD4 abolishes its internalization following treatment of cells with PMA (Shin et al., 1990; Glaienhauss et al., 1991). It has also been shown that not all the p56^ck is targeted for proteolysis following PMA stimulation, since metabolic labeling and pulse-chase experiments have indicated that at least some of the p56^ck is recycled to p56^ak following conversion (Veillette et al., 1988c).

The data presented here show that the induced Ser-59 phosphorylation of p56^ck correlates well with the shift in electrophoretic mobility to p60^ak. Nevertheless, the precise biochemical differences between p56^ck and p60^ak in *in vivo* still remain unclear. While Ser-59 phosphorylation represents one such difference, other post-translational modifications may also be required. Indeed, reversible *in vivo* palmitoylation of p56^ck has recently been demonstrated (Paige et al., 1993), although this modification does not appear to alter the electrophoretic mobility of p56^ck by itself. Thus the shift to p60^k is probably dependent on a more complex state of post-translational modification, including both tyrosine and serine phosphorylation.

There are several other possible functions that Ser-59 of p56^ck could be mediating in *in vivo* other than direct regulation of p56^ck activity. Ser-59 could mediate the interaction of other enzymes with the ability to modulate p56^ck activity via Tyr-505, such as the skb protein tyrosine kinase, or the protein tyrosine phosphatase CD45. On the other hand, Ser-59 phosphorylation may determine whether interactions between p56^ck and potential substrates such as the TCR-\zeta subunit (Barber et al., 1989) and phospholipase C-\gamma1 (Weiss et al., 1991; Weber et al., 1992), or between p56^ck and receptor-type molecules such as CD4 and CD8. Alternatively, as discussed above, it may play a role in the induction of CD4/p56^ck internalization and/or recycling within the cell. It is worth noting here that while Ser-59 occurs within the N-terminal domain unique to p56^ck among src-family protein tyrosine kinases (reviewed in Veillette and Davidson (1992)), it lies just prior to the start of the SH3 domain, a conserved region common to all src-family protein tyrosine kinases, which may mediate their interaction with components of the cytoskeleton (Kanner et al., 1991). It also remains to be determined whether sites of phosphorylation other than Ser-59 can be induced on p56^ck.
following other means of T cell activation.

The generation and expression of mutant p56\textsuperscript{tk} molecules in relevant cell lines and transgenic animal systems will permit all of these possibilities to be addressed, and such approaches are now underway. Expression of Ser-59 mutants of p56\textsuperscript{tk} will allow studies on CD4/p56\textsuperscript{tk} association with the TCR and potential substrates, as well as the internalization and recycling of this complex. Mutation of Ser-59 will also demonstrate whether this residue can indeed regulate p56\textsuperscript{tk} activity in vivo, as it appears to in vitro. If this turns out to be the case, the expression of double mutants (at Tyr-505 and Ser-59, for example) may provide valuable insight into the early events of T cell activation.

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