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Effects of p56^{lck} Deficiency on the Growth and Cytolytic Effector Function of an Interleukin-2-Dependent Cytotoxic T-Cell Line

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The growth, differentiation, and functional activities of antigen-stimulated T lymphocytes are regulated by the interaction of the T-cell-derived cytokine, interleukin-2 (IL-2), with the high-affinity IL-2 receptor (IL-2R). IL-2R occupancy initiates a rapid increase in intracellular protein tyrosine phosphorylation, suggesting that a receptor-coupled protein tyrosine kinase (PTK) serves as a proximal signaling element for the IL-2R. Previous studies implicated the *src*-family kinase, p56^{lck}, as a potential IL-2R-linked signal transducer. In this study, we have characterized a spontaneous variant of the IL-2-dependent cytotoxic T-cell line, CTLL-2, which contains no detectable *lck*-derived mRNA transcripts, protein, or PTK activity. The p56^{lck}-deficient CTLL-2 cells retained strict dependence on IL-2 for both viability and growth, indicating that p56^{lck} activity was not required for the transduction of IL-2-mediated mitogenic signals. However, the p56^{lck}-deficient cells exhibited a moderate decrease in their rate of IL-2-dependent proliferation. In contrast to this relatively modest proliferative defect, the p56^{lck}-deficient cell line displayed a profound reduction in T-cell antigen receptor-dependent cytolytic effector functions. Both the proliferative and the cytolytic defects observed in the p56^{lck}-deficient cells were completely reversed by transfection of these cells with a wild-type *lck* expression vector. These results indicate that p56^{lck} expression is not obligatory for IL-2-mediated T-cell growth stimulation; however, this PTK plays a central role in the generation T-cell-mediated cytotoxic responses.

Antigenic or mitogenic stimulation of quiescent (G_0-phase) T lymphocytes triggers cell-cycle entry (G_0- to G_1-phase transition) and cell surface expression of high-affinity interleukin-2 (IL-2) receptors (IL-2R). The progression of these cells through the remainder of the cell cycle is subsequently dependent on the interaction of the T-cell-derived growth factor, IL-2, with the IL-2R. In addition to its T-cell growth-promoting activities, IL-2 stimulates the differentiation of cytotoxic T-cell (CTL) precursors into mature effector cells (20, 49). Furthermore, exposure of mature CTL to IL-2 augments the capacity of these cells to mediate target cell killing (11, 16, 34, 44). The mechanism by which IL-2 exposure enhances or "primes" cytotoxic function is unknown; however, earlier data suggest that CTL priming can occur independently of IL-2-induced cell-cycle progression (28).

In contrast to the wealth of information regarding the biological actions of IL-2, the molecular mechanisms involved in the transmission of mitogenic and other regulatory signals from the ligand-stimulated IL-2R remain unclear. The high-affinity IL-2R is a multi-subunit complex composed of two ligand-binding subunits, designated p55 and p75, which independently bind IL-2 with low and intermediate affinities, respectively (19, 42, 43). The p75 subunit apparently represents the signal-transducing component of the receptor complex (18, 41); however, the deduced amino acid sequence of the p75 subunit provides no definitive insights into the mechanism of transmembrane signal generation by this receptor. In particular, the IL-2R p75 subunit exhibits no intracytoplasmic protein tyrosine kinase (PTK) domain, which clearly differentiates it from a number of other growth factor receptors, including the platelet-derived growth factor and colony-stimulating factor-1 receptors. Nonetheless, stimulation of activated T cells with IL-2 induces the phosphorylation of multiple intracellular proteins on tyrosyl residues (4, 13, 14, 22, 33, 38), suggesting that IL-2R occupancy induces an increase in PTK activity in these cells.

The *src* family kinase, p56^{lck}, has attracted particular attention as a potential signal-transducing element for the IL-2R. p56^{lck} is expressed at relatively high levels in IL-2-responsive lymphoid cells, including T cells and natural killer cells (36). Hatakeyama et al. have recently demonstrated that p56^{lck} is capable of associating noncovalently with an acidic region of the intracytoplasmic domain of the IL-2R p75 subunit (17). Finally, stimulation of activated T cells with IL-2 leads to a rapid, transient increase in the catalytic activity of p56^{lck}, accompanied by the phosphorylation of this PTK on multiple N-terminal serine residues (12, 21). Although these findings have provoked speculation that p56^{lck} serves as an IL-2R-coupled PTK, a compelling body of evidence indicates that the signal-transducing function of p56^{lck} in T cells is not restricted to the IL-2R. The coreceptor molecules, CD4 and CD8, which subserve both adhesion-promoting and transmembrane signaling functions during antigen-specific T-cell activation, are physically associated with p56^{lck} (6, 39, 40, 45, 47). Antibody-mediated cross-linkage of CD4 molecules results in an increase in the catalytic activity of the associated p56^{lck} (30, 46, 48). Furthermore, Abraham et al. have shown that expression of a

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constitutively activated version of $p56^{lck}$ in an antigen-specific T-cell hybridoma line enhances the responsiveness of these cells to T-cell antigen receptor (TCR)-mediated stimuli (1). Although these studies suggest that $p56^{lck}$ participates in the transduction of both antigen- and IL-2-mediated regulatory signals, the actual roles of endogenous $p56^{lck}$ activity in the regulation of T-cell activation and/or growth responses remain unclear.

In the present study, we have characterized a spontaneous variant of the murine IL-2-dependent, cytotoxic T-cell line, CTLL-2, which fails to express detectable levels of *lck*-derived mRNA or protein. The $p56^{lck}$-deficient CTLL-2 cells remained fully dependent on exogenous IL-2 for viability maintenance and proliferation in culture, indicating that the $p56^{lck}$ expression defect did not prohibit the transmission of growth-promoting signals from the IL-2R. However, the loss of $p56^{lck}$ severely impaired the TCR-dependent cytolytic responses of both factor-deprived and IL-2-stimulated CTLL-2 cells. The defective cytotoxic functions of the variant cell line were completely reversed by transfection of these cells with an *lck* expression vector. These studies indicate that although $p56^{lck}$ is not essential for IL-2-dependent T-cell proliferation, this PTK plays an important role in the generation of cytotoxic responses by CTL.

MATERIALS AND METHODS

Cells and cell culture. Parental CTLL-2 cells (15) were obtained from the American Type Culture Collection (Rockville, Md.). CTLL-2 sublines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 10% conditioned medium from concanavalin A-stimulated rat splenocytes (2), 5 U of human recombinant IL-2 (a gift of Hoffman-La Roche, Inc., Nutley, N.J.) per ml, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.2). Cell lines transfected with an *lck* expression plasmid (see below) were maintained in the same medium supplemented with 400 μg of G418 (GIBCO Laboratories, Grand Island, N.Y.) per ml. Prior to experimental manipulations, exponentially growing cells were harvested from standard growth medium by centrifugation (250 × *g*, 5 min) and stripped of bound IL-2 by incubation for 30 s in low-pH buffer (10 mM sodium citrate, 0.14 M NaCl [pH 4.0]). The cell suspension was diluted with 10 volumes of basal medium (RPMI 1640 medium containing 10% fetal bovine serum, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol, and buffered to pH 7.2 with 10 mM HEPES-NaOH), and the cells were harvested by centrifugation. The cells were then resuspended in basal medium and cultured for the indicated times prior to restimulation with IL 2.

Transfections. CTLL.lck$^-$ cells were transfected by electroporation as previously described (5) with the *lck* expression plasmid, pMNC-LCK, which was kindly provided by R. Perlmutter (University of Washington, Seattle). pMNC-LCK contains the neomycin resistance marker and a full-length murine *lck* cDNA under the transcriptional control of a cytomegalovirus promoter. Transfectants were selected and cloned by limiting dilution in G418-containing growth medium. Transfected clones were screened for expression of $p56^{lck}$ by immunoblot analysis (see below). Three *lck*-expressing clones, designated CTLL.LCK10, CTLL.LCK15, and CTLL.LCK16, were selected for further analysis.

RNA analysis. Cells were stripped of bound IL-2 and cultured for 4 h in basal medium. The IL-2-deprived cells were stimulated for 1 h with either basal medium only or IL-2 (100 U/ml), and total cellular RNA was prepared by the guanidine isothiocyanate-cesium chloride technique (8). Purified RNA (30 μg per sample lane) was electrophoresed through formaldehyde-containing agarose gels and subsequently transferred to Hybond N paper (Amersham, Arlington Heights, Ill.). The blots were successively hybridized with ^{32}P-labeled cDNA probes for *lck*, c-*myc*, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) by using previously described techniques (37). Hybridizing species were detected by autoradiography at −70°C with Quanta III screens (Dupont, Wilmington, Del.).

DNA probes. Probes were prepared by restriction enzyme digestion of the plasmid DNAs described below. After gel purification, plasmid inserts were labeled with [α-^{32}P]dCTP (specific activity, 3,000 Ci/mmol) by random oligomer priming to a minimum specific activity of 5×10^8 cpm/μg of DNA. The murine *lck* probe was a 1.75-kb *Eco*RI fragment from plasmid NT18 (32), which was provided by R. Perlmutter (University of Washington, Seattle). The c-*myc* probe was a 0.98-kb *Xba*I-*Sac*I fragment from plasmid pSVC-MYC-1 (27) obtained from the American Type Culture Collection. GAPDH was a 1.85-kb *Pst*I fragment from plasmid pIBI 30-GAPDH, kindly provided by S. Kang (Mount Sinai Medical Center, New York, N.Y.).

Immunoblot analyses. Levels of $p56^{lck}$ were determined by immunoblot analysis of total cellular proteins with rabbit polyclonal antibodies prepared against a bacterially expressed *trpE* fusion protein containing amino acid residues 5 to 148 of murine $p56^{lck}$. CTLL-2 cells were washed with phosphate-buffered saline (PBS; pH 7.2) containing 0.5 mM EDTA, (PBS-EDTA) and were lysed by heating for 10 min at 100°C in 1% sodium dodecyl sulfate (SDS). Lysates were sonicated to shear cellular DNA, and proteins were precipitated at −20°C with 3 volumes of acetone-ammonium hydroxide (30:1). Insoluble material was collected by centrifugation, washed once with cold acetone, and dissolved in reducing sample buffer (26) prior to separation by SDS-polyacrylamide gel electrophoresis (PAGE) in 10% gels. After electrophoretic transfer to Immobilon-P (Millipore, Bedford, Mass.), the blot was blocked overnight at 4°C in PBS containing 10% nonfat dry milk and 0.05% Tween 20. The blot was then incubated for 2.5 h at 23°C with a 1:1,000 dilution of $p56^{lck}$-specific antiserum in blocking buffer, and then washed three times (10 min each) with PBS containing 0.05% Tween 20. Immunoreactive proteins were detected by incubating the blot for 2.5 h with 0.5 μCi of ^{125}I-protein A (Amersham) per ml in blocking buffer. After three washes with PBS containing 0.3% Tween 20, the membrane was autoradiographed at −70°C with an intensifying screen.

For detection of phosphotyrosyl-containing proteins, cells were cultured for 4 h in IL-2-free basal medium at a cell density of 10^6 cells per ml. Samples (5×10^6 cells) were stimulated with medium alone or IL-2 (30 U/ml), and, at the indicated times, reactions were stopped by diluting the cell suspensions in ice-cold PBS-EDTA. The cells were pelleted by centrifugation (250 × *g*, 5 min), washed once with PBS-EDTA, and solubilized in lysis buffer (20 mM Tris-HCl, 30 mM $Na_4P_2O_7$, 50 mM NaF, 40 mM NaCl, 5 mM EDTA [pH 7.6], containing 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 40 μg of leupeptin per ml, 5 μg of aprotinin per ml, and 1 mM Na_3VO_4). Detergent-insoluble material was removed by centrifugation (2,000 × *g*, 5 min), and one-third volume of 4× reducing sample buffer was added to the resulting supernatant. After being heated at 100°C for 10 min, the denatured proteins were fractionated by SDS-PAGE and transferred to an Immobilon-P membrane. The

membrane was incubated overnight in blocking buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl [TBS] containing 2% bovine serum albumin [BSA] and 0.5% Tween 20). The blots were incubated for 2 h in TBS containing 0.5% Tween 20 and 0.1 μg of the monoclonal anti-phosphotyrosine antibody, 4G10 (Upstate Biotechnology Inc., Lake Placid, N.Y.), per ml. The membrane was washed three times with TBS containing 0.2% Tween 20 and then incubated for 1 h with 1 μg of rabbit polyclonal anti-mouse antibody (Pierce, Rockford, Ill.) per ml in blocking buffer. After three washes with TBS containing 0.2% Tween 20, the membrane was incubated for 1 h with a 1:8,000 dilution of protein A-horseradish peroxidase conjugate (Amersham) in blocking buffer. The membrane was washed as described above, treated with the chemiluminescent detection reagent, ECL (Amersham), and exposed to photographic film.

Cellular proliferation. Growth factor-dependent DNA synthesis was determined by measurement of [^{3}H]thymidine incorporation into cellular DNA as previously described (2). Briefly, cells were deprived of IL-2 for 4 h, and triplicate samples (10^4 cells per sample) were plated in 0.2 ml of basal medium containing the indicated concentrations of IL-2. After 18 h in culture, cells were pulsed with [^{3}H]thymidine (1 μCi per well) for 6 h, and incorporation of radioactivity into DNA was measured by liquid scintillation counting.

IL-2-dependent cellular proliferation in exponentially growing cultures was also assessed by cell counting. Cells were harvested from standard growth medium and cultured at an initial density of 5×10^3 cells per ml for 3 days in basal RPMI containing recombinant human IL-2 (100 U/ml). After this preculture, the cells were diluted into the same medium at an initial cell density of 5×10^3 cells per ml. At 24-h intervals, cell densities in duplicate cultures were assayed with a Coulter counter. Each culture was assayed in duplicate. When the cells reached an approximate density of 1.5×10^5 cells per ml, the cultures were diluted to 1×10^4 cells per ml with IL-2-containing medium to maintain an exponential growth rate. Population doubling times were determined by fitting first-order exponential curves to each set of data.

Immune-complex kinase assays. Immunoprecipitations were performed by using polyclonal antisera prepared or obtained as follows. The anti-p56^{lck} and anti-p59^{fyn} antisera were prepared by immunizing rabbits with keyhole limpet hemocyanin-coupled peptides corresponding to amino acid (aa) residues 39 to 64 and 26 to 74 of the p56^{lck} and p59^{fyn} sequences, respectively. Antisera raised against synthetic peptides derived from polymorphic N-terminal regions of p56^{lyn} (aa 18 to 62), p59^{hck} (aa 7 to 58), p62$^{c\text{-}yes}$ (aa 5 to 71), p58$^{c\text{-}fgr}$ (aa 16 to 58), p55^{blk} (aa 2 to 50) were provided by J. Bolen (Bristol-Meyers, Squibb Pharmaceuticals, Princeton, N.J.) (7).

Samples were prepared for immune-complex kinase assays by harvesting cells from standard growth medium. The cells were deprived of IL-2 for 4 h, and samples (10^7 cells) were stimulated with medium alone or IL-2. Cell lysates were prepared in Triton X-100-containing lysis buffer supplemented with 0.1% BSA. Detergent-soluble proteins were immunoprecipitated for 1 h at 4°C with the appropriate antiserum and protein A-coupled Sepharose beads. Protein A-Sepharose beads were preblocked by incubation for 2 h in lysis buffer containing 1% nonfat dry milk. The immunoprecipitates were washed once with lysis buffer (without BSA), twice with 0.1 M Tris-HCl (pH 7.4) containing 0.5 M LiCl, and finally, once with kinase buffer (50 mM PIPES (piperazine-*N*,*N*′-bis(2-ethanesulfonic acid; pH 7.0), 10 mM NaCl, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM Na_3VO_4). Phosphorylation reactions were initiated by addition of 20 μl of kinase buffer containing 10 μCi of [γ-^{32}P]ATP (6,000 Ci/mmol) and 3 μg of acid-denatured rabbit muscle enolase. Denatured enolase was prepared by incubating the native protein in 50 mM acetic acid for 5 min at 30°C. The phosphorylation reaction mixtures were incubated at 30°C for 2 min and were terminated by addition of 4× SDS-PAGE sample buffer. After being heated at 100°C for 5 min, the solubilized proteins were separated by SDS-PAGE in a 10% gel. Proteins were transferred electrophoretically to Immobilon-P, and the membrane was incubated in 1 M KOH for 1 h at 55°C to enrich phosphoproteins for phosphotyrosine (10, 23). ^{32}P-labeled phosphoproteins were detected by autoradiography at −70°C.

Cell-mediated cytotoxicity assays. The hamster B-cell hybridoma line, 145-2C11 (kindly provided by J. Bluestone, University of Chicago), which expresses on its cell surface an immunoglobulin G antibody directed against the CD3-ε subunit of the T-cell antigen receptor complex (29), was the target cell for assays of cell-mediated cytotoxicity. Hybridoma cells (3×10^6 cells) were labeled with 150 μCi of $Na_2{}^{51}CrO_4$ in 0.4 ml of basal medium for 90 min. Effector cells (CTLL sublines) were deprived of IL-2 for 12 h in basal medium and then incubated with either 0 or 10 U of IL-2 per ml for 4 h prior to cytotoxicity assays. A fixed number of labeled target cells (10,000 cells per well) was mixed with various numbers of effector cells at the indicated ratios, and the mixture was incubated for 4 h at 37°C. Cells were pelleted by centrifugation, and the ^{51}Cr released into the supernatant was quantitated by gamma counting. Maximal ^{51}Cr release was obtained by target cell lysis in 2.5% Triton X-100. Specific ^{51}Cr release was calculated as previously described (35).

RESULTS

Identification of a p56^{lck}-deficient CTLL-2 subclone. The murine CTLL-2 cell line was originally established as a tumor-specific CTL line which required both periodic antigenic stimulation and continuous exposure to IL-2 for maintenance of viability and proliferation in culture (15). These cells were subsequently converted to an antigen-independent growth state by long-term culture in the presence of IL-2 only. In our laboratory, we have maintained the CTLL-2 line in culture by using concanavalin A-stimulated rat splenocyte-conditioned medium as the principal source of IL-2 and, possibly, other T-cell growth factors. During a routine series of p56^{lck} immunoblot analyses, we unexpectedly observed that a stock culture of CTLL-2 cells appeared negative for the p56^{lck} polypeptide. Because p56^{lck} was normally expressed at readily detectable levels in both freshly isolated T cells and T-cell lines, we reasoned that this p56^{lck}-deficient CTLL-2 cell line provided a novel opportunity to examine the roles of p56^{lck} in signal transduction through the IL-2R and, possibly, other T-cell surface receptors. Initially, we recovered the parental CTLL-2 cell line from a cryopreserved stock and determined whether these cells, in fact, contained immunoreactive, *lck*-derived protein. The immunoblot analyses in Fig. 1A show that detergent extracts from wild-type CTLL-2 cells (designated CTLL.WT) contained readily detectable levels of p56^{lck}, whereas extracts from the variant subline (designated CTLL.lck$^-$) reproducibly yielded no p56^{lck}-specific immunoreactivity. Subsequently, CTLL.lck$^-$ cells were transfected with an *lck* expression vector, and the transfected

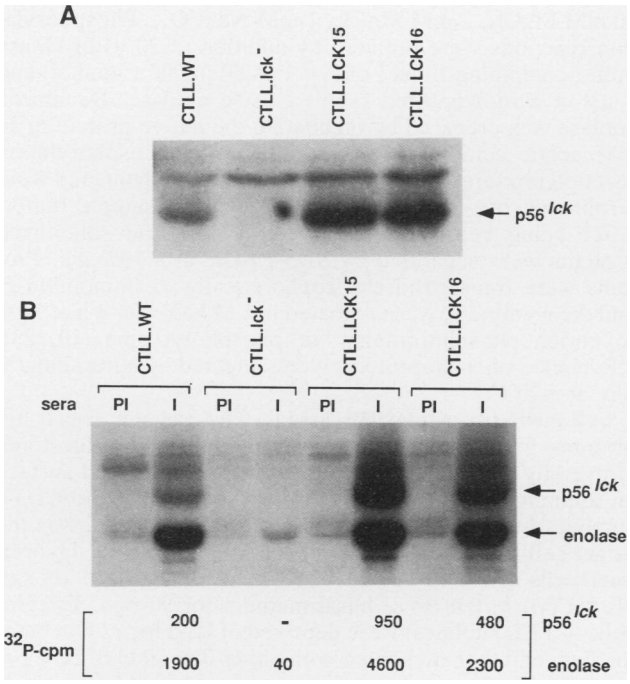

FIG. 1. Expression of p56^{lck} in CTLL-2 sublines. (A) Detergent-soluble proteins from CTLL.WT, CTLL.lck$^-$, CTLL.LCK15, and CTLL.LCK16 cells were fractionated by SDS-PAGE in 10% gels, transferred to Immobilon-P, and probed with a p56^{lck}-specific antiserum. The p56^{lck}-specific immunoreactive band is indicated by the arrow. (B) Detergent-soluble proteins from the indicated cell lines were immunoprecipitated with either preimmune (PI) or p56^{lck}-specific immune (I) serum. Immune-complex kinase assays were performed in the presence of [γ-^{32}P]ATP and enolase. Reaction products were separated by SDS-PAGE in 10% gels. After transfer to Immobilon-P, the membrane was treated with 1 M KOH for 1 h at 55°C to enrich for phosphotyrosine-containing proteins. Alkali-stable $^{32}P_i$ incorporated into p56^{lck} and enolase was quantitated by excising the appropriate sections of the membrane and then by liquid scintillation counting. Total ^{32}P counts per minute measured in the p56^{lck} and enolase-containing bands were corrected for background radioactivity present in the corresponding area of the preimmune serum lane. −, not above background.

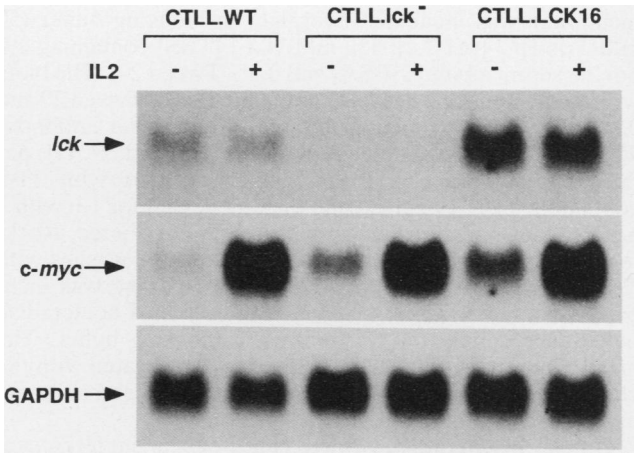

FIG. 2. Northern blot analysis of CTLL-2 sublines. CTLL.WT, CTLL.lck$^-$, and CTLL.LCK16 cells were deprived of IL-2 for 4 h. Cells were stimulated for 1 h at 37°C with either medium only (−) or IL-2 (+). Total cellular RNA was prepared and electrophoresed through formaldehyde-agarose gels. The RNA was transferred to nitrocellulose and hybridized with an *lck*-specific probe. The membrane was stripped, hybridized with a c-*myc*-specific probe, stripped again, and hybridized with a GAPDH-specific probe.

cells were cloned at a limiting dilution. Three *lck*-expressing subclones (designated CTLL.LCK10, CTLL.LCK15, and CTLL.LCK16) were selected for further study. Immunoblot analyses demonstrated that both the CTLL.LCK15 and CTLL.LCK16 subclones (Fig. 1A), as well as the CTLL.LCK10 subclone (not shown), expressed p56^{lck} at significantly higher levels than that observed in the CTLL.WT cell line.

To further establish the apparent p56^{lck} expression defect in CTLL.lck$^-$ cells, anti-p56^{lck} antibody immunoprecipitates from each subline were assayed for PTK activity with denatured enolase added as an exogenous substrate. As shown in Fig. 1B, immune complex kinase reactions derived from CTLL.WT, CTLL.LCK15, and CTLL.LCK16 cells each generated a radioactive band corresponding to autophosphorylated p56^{lck}. Quantitation of $^{32}P_i$ incorporation into the p56^{lck} and enolase bands indicated that the CTLL.LCK15 and CTLL.LCK16 transfectants expressed 2.5- and 5-fold-higher levels of p56^{lck} catalytic activity, respectively, than did the CTLL.WT cells. In contrast, anti-p56^{lck} immunoprecipitates from CTLL.lck$^-$ cells contained no autophosphorylated p56^{lck} (Fig. 1B). Similar results were obtained after prolonged autoradiographic exposure of this membrane (results not shown). The anti-peptide antiserum used to prepare the p56^{lck} immunoprecipitates also precipitated a low level of enolase-phosphorylating activity from the CTLL.lck$^-$ cells. However, this activity was not reduced by addition of excess competing *lck*-derived peptide to the immunoprecipitation reactions, indicating that the substrate phosphorylation was catalyzed by a nonspecifically precipitated PTK, rather than p56^{lck} (24). Taken together, these studies demonstrated that CTLL.lck$^-$ cells exhibited a profound to absolute deficiency in p56^{lck} expression when compared with that of the parental CTLL-2 cell line.

In order to more fully characterize the abnormality in p56^{lck} expression in CTLL.lck$^-$ cells, we next determined whether the cells expressed *lck* mRNA transcripts. Total cellular RNA was isolated from factor-deprived and IL-2-stimulated cells, and *lck* mRNA transcript levels were determined by Northern (RNA) blot analysis with a murine *lck*-specific probe (Fig. 2). As a control for variations in sample loading, the blot was stripped and rehybridized with a GAPDH-specific probe. CTLL.WT and CTLL.LCK16 cells expressed readily detectable levels of *lck* mRNA, whereas CTLL.lck$^-$ cells contained no detectable *lck* transcripts. Southern blot analysis of DNA isolated from the CTLL.lck$^-$ and CTLL.WT cell lines demonstrated no loss or detectable rearrangement of the *lck* gene in CTLL.lck$^-$ cells (24). Thus, these results indicate that the p56^{lck} deficiency in CTLL.lck$^-$ cells is due either to a defect in *lck* gene transcription or to an abnormally high rate of *lck* mRNA transcript degradation.

***src* family PTK expression in CTLL-2 cell sublines.** The p56^{lck} expression defect in CTLL.lck$^-$ cells might be compensated for by the anomalous expression of other *src* family kinases. CTLL-2 cells, like other T cells, express a cell lineage-restricted isoform of p59^{fyn} (9). To determine whether the p56^{lck} deficiency in CTLL.lck$^-$ cells was countered by a reciprocal alteration in the expression and/or

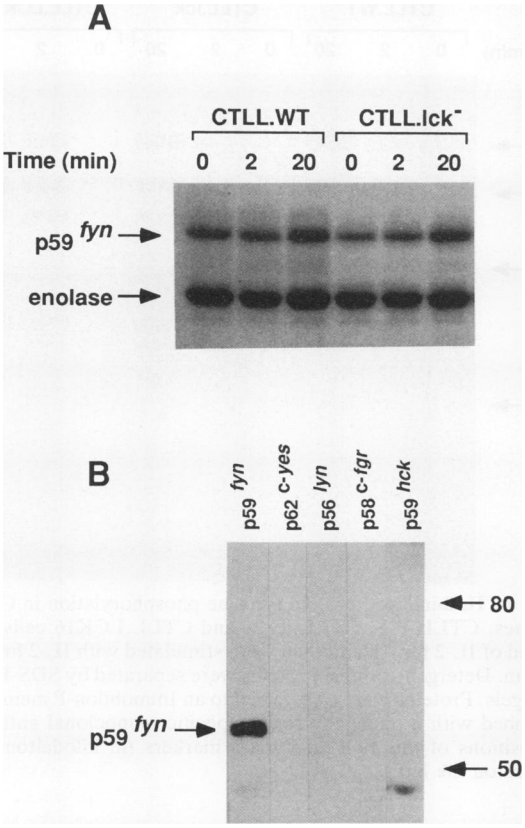

FIG. 3. *src* family PTK expression in CTLL-2 sublines. (A) CTLL.WT and CTLL.lck⁻ cells were deprived of IL-2 for 4 h and then stimulated for 0, 2, or 20 min with 30 U of IL-2 per ml. Detergent-soluble proteins were immunoprecipitated with anti-p59^{fyn} antiserum, and immune complex kinase assays were performed in the presence of [γ-^{32}P]ATP and denatured enolase. Proteins were separated by SDS-PAGE in a 10% gel and then transferred to an Immobilon-P membrane. Alkali-stable radioactivity was detected by autoradiography at −70°C. (B) Detergent-soluble proteins from IL-2-stimulated CTLL.lck⁻ cells were immunoprecipitated with the indicated *src* family kinase-specific antisera. Immune-complex kinase assays were performed in the presence of [γ-^{32}P]ATP. Reaction products were fractionated by SDS-PAGE through 8% gels. After treatment with KOH, the gels were dried and autoradiographed at −70°C.

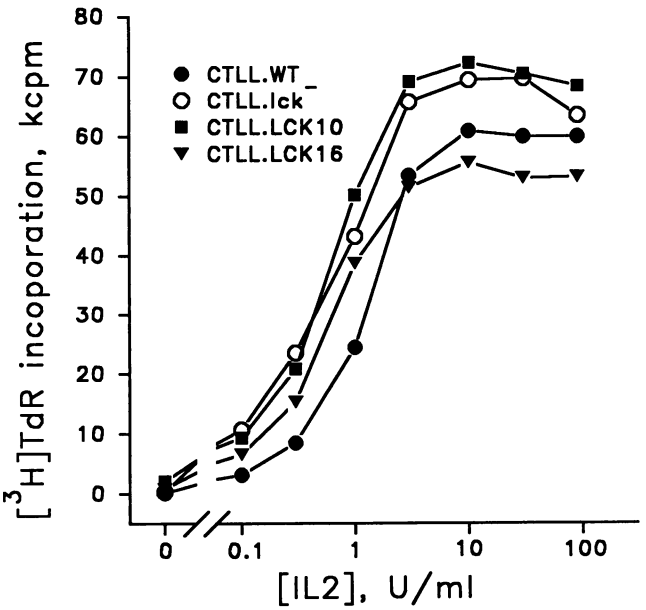

FIG. 4. IL-2-stimulated DNA synthesis in CTLL-2 sublines. CTLL.WT, CTLL.lck⁻, and CTLL.LCK16 cells were cultured for 4 h in IL-2-free medium. The factor-deprived cells were stimulated for 24 h with various concentrations of IL-2. [^{3}H]thymidine was added during the final 6 h of culture, and incorporation of radioactivity into DNA was quantitated by liquid scintillation counting. Datum points represent mean [^{3}H]thymidine incorporation from triplicate samples. Coefficients of variation for each mean value were less than 10%.

activity of p59^{fyn}, PTK activities in anti-p59^{fyn} antibody immunoprecipitates prepared from CTLL.WT and CTLL.lck⁻ cells were examined (Fig. 3A). These assays demonstrated that IL-2-deprived CTLL.WT and CTLL.lck⁻ cells contained comparable levels of p59^{fyn} catalytic activity and that this activity was not detectably altered by cellular stimulation with IL-2. In addition, detergent-soluble proteins from CTLL.lck⁻ cells were immunoprecipitated with antibodies specific for p62$^{c\text{-}yes}$, p53/56^{lyn}, p55$^{c\text{-}fgr}$, and p59^{hck} (Fig. 3B), as well as p55^{blk} and p60$^{c\text{-}src}$ (data not shown). Immune-complex kinase assays revealed that, like the parental CTLL.WT cell line, CTLL.lck⁻ cells expressed only p59^{fyn}-derived catalytic activity.

Effect of p56^{lck} deficiency on IL-2-dependent cell growth. Reports that p56^{lck} is physically associated with and perhaps functionally regulated by the IL-2R have provoked speculation that this PTK participates in the transmission of IL-2-dependent mitogenic signals in T cells (17, 21). Therefore, we examined the impact of the p56^{lck} deficiency on the growth-promoting activity of IL-2 in CTLL.lck⁻ cells. The CTLL.lck⁻ cells, as well as the parental CTLL.WT cell line and the CTLL.LCK transfectants, were strictly dependent on exogenous IL-2 for continued survival in culture. Growth factor deprivation resulted in a complete loss of cellular viability within 48 h (data not shown). The IL-2-induced mitogenic responses of the CTLL-2 sublines were subsequently compared by measuring growth factor-dependent [^{3}H]thymidine incorporation into cellular DNA. As shown in Fig. 4, stimulation of factor-deprived cells with IL-2 induced comparable levels of DNA synthesis in both p56^{lck}-positive and -negative CTLL-2 cell lines.

To more directly examine the effect of a p56^{lck} deficiency on IL-2-dependent cellular proliferation, the growth rates of CTLL.WT, CTLL.LCK16, and CTLL.lck⁻ cells in serum-containing culture medium supplemented with saturating concentrations of recombinant IL-2 were determined (Fig. 5). Under exponential growth conditions, the population doubling time of the CTLL.lck⁻ cell line was 18.3 ± 0.4 h (mean ± standard deviation; n = 4), whereas the CTLL.WT cells doubled every 15.7 ± 0.2 h. The IL-2-dependent growth rate of the CTLL.lck⁻ cell line was, therefore, moderately but significantly ($P < 0.05$, unpaired t test) slower than that of the CTLL.WT cell line. In contrast, the CTLL.LCK16 cell population doubled every 15.7 ± 0.4 h, indicating that reexpression of p56^{lck} in the CTLL.lck⁻ cells completely reversed the partial proliferative defect in these cells. Similar growth kinetics were observed with the CTLL.LCK15 subline (24), which argues against the possibility that the moderate increase in growth rate was simply an artifact of the

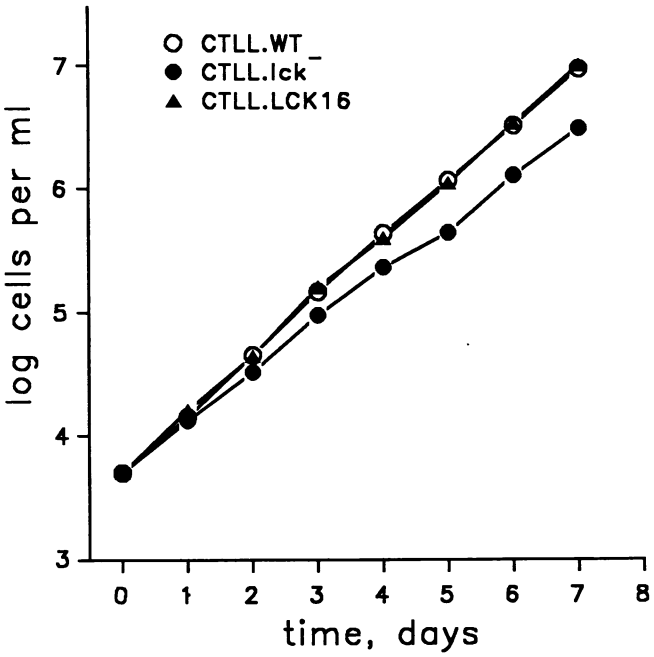

FIG. 5. IL-2-dependent growth of CTLL-2 sublines. CTLL.WT, CTLL.lck$^-$, and CTLL.LCK16 cells were precultured in basal RPMI containing 100 U of recombinant IL-2 per ml for 3 days. Duplicate cultures from each cell population were prepared at initial cell concentrations of 5,000 cells per ml. At 24-h intervals, cell densities in each culture were determined from duplicate samples. Datum points represent mean cell numbers per ml. Standard deviation bars were smaller than the symbol size. The population doubling times for the CTLL.WT, CTLL.LCK16, and CTLL.lck$^-$ sublines were 15.7 ± 0.2 h (mean ± standard deviation; n = 4), 15.4 ± 0.3 h, and 18.3 ± 0.4 h, respectively. The doubling time of the CTLL.lck$^-$ cells was significantly different from that of the CTLL.WT subline ($P < 0.05$, unpaired t test).

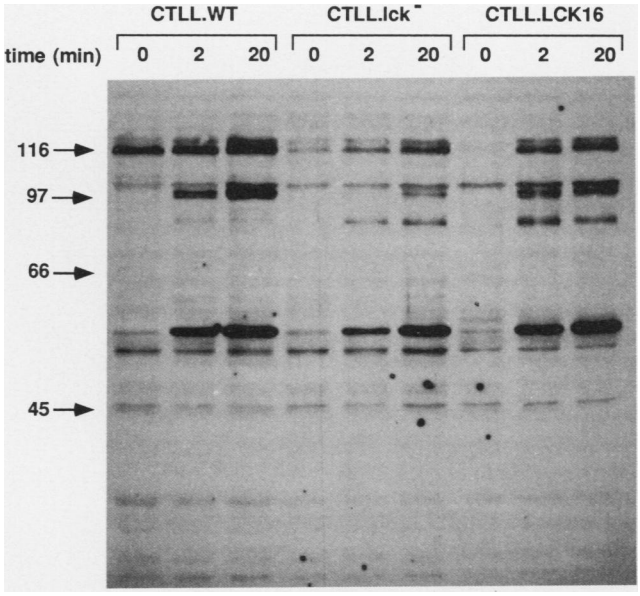

FIG. 6. IL-2-induced protein tyrosine phosphorylation in CTLL-2 sublines. CTLL.WT, CTLL.lck$^-$, and CTLL.LCK16 cells were deprived of IL-2 for 4 h and then were stimulated with IL-2 for 0, 2, or 20 min. Detergent-soluble proteins were separated by SDS-PAGE in 10% gels. Proteins were transferred to an Immobilon-P membrane and probed with a phosphotyrosine-specific monoclonal antibody. The positions of the molecular mass markers (in kilodaltons) are indicated on the left.

cloning procedure used to generate these *lck*-transfected sublines.

Previous studies have shown that stimulation of activated T cells with IL-2 induces a rapid increase in c-*myc* gene transcription (37). To determine whether IL-2-stimulated c-*myc* gene expression was dependent on p56^{lck} activity, the p56^{lck}-negative and -positive CTLL-2 cell sublines were stimulated with IL-2 for 1 h, and c-*myc* mRNA levels were measured by Northern blot analysis (Fig. 2). These studies clearly demonstrated that c-*myc* mRNA transcript accumulation was strongly induced by IL-2 addition to p56^{lck}-deficient CTLL-2 cells. Thus, p56^{lck} apparently does not participate in the postreceptor signaling pathway leading to IL-2-induced c-*myc* gene expression. Although the basal levels of c-*myc* mRNA in the CTLL-2 cell sublines appeared variable, additional studies have shown that these differences probably reflect intersample variability in the degree of IL-2 starvation, rather than intrinsic differences in the growth factor-independent c-*myc* mRNA expression levels in these sublines (24).

IL-2-dependent protein tyrosine phosphorylation. Stimulation of responsive T cells with IL-2 induces a rapid increase in intracellular protein tyrosine phosphorylation (4, 13, 14, 22, 33, 38). If p56^{lck} functions as a proximal, IL-2R-coupled PTK, then p56^{lck}-deficient CTLL-2 cells should exhibit an altered pattern of IL-2-inducible protein tyrosine phosphorylation. To test this possibility, CTLL.WT, CTLL.lck$^-$ and CTLL.LCK16 cells were deprived of growth factor for 4 h and then stimulated with recombinant IL-2 for 0, 2, or 20 min. Detergent-soluble proteins were separated by SDS-PAGE and immunoblotted with a monoclonal anti-phosphotyrosine antibody. IL-2 stimulation induced the tyrosine phosphorylation of proteins with molecular masses of 116, 97, 85, and 56 kDa in both p56^{lck}-expressing and -deficient CTLL-2 cells (Fig. 6). Thus, these phosphorylation events were not contingent upon the presence of p56^{lck}. Although the phosphorylation intensities of a number of protein bands varied quantitatively among the CTLL-2 cell sublines (cf. the 116- or 85-kDa proteins), these differences were, with a single exception, not reproducible in repeat experiments. The 97-kDa substrate was consistently phosphorylated more slowly and less intensively in the CTLL.lck$^-$ cell line relative to the *lck*-positive cell lines. Thus, variations in p56^{lck} expression lead to quantitative rather than qualitative alterations in IL-2-inducible protein tyrosine phosphorylation in CTLL-2 cells.

Effect of p56^{lck} on TCR-mediated cytolytic activity. Triggering of the TCR expressed by antigen-specific CTL initiates a series of biochemical events that culminate in the lysis of sensitive target cells (25). In addition, the efficiency with which cytotoxic lymphocytes mediate target cell killing is significantly enhanced by acute exposure of these cells to IL-2 (11, 16, 28, 34, 44). Experiments were therefore performed to determine whether the loss of p56^{lck} altered the cytolytic activity of CTLL.lck$^-$ cells. In preliminary studies, we demonstrated that CTLL-2 cells exhibited significant lytic activity toward the hamster B-cell hybridoma cell line, 145-2C11, which expresses surface immunoglobulin specific for the CD3-ε subunit of the TCR. Control experiments with surface immunoglobulin-negative 145-2C11 cells demonstrated that the target cell killing measured in this assay was

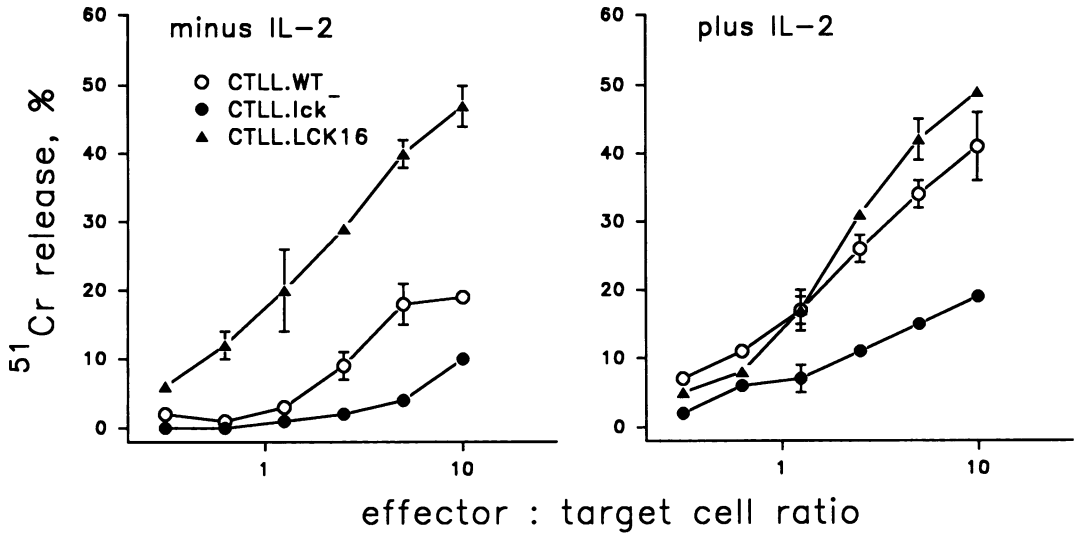

FIG. 7. Cytotoxic effector functions of CTLL-2 cell sublines. CTLL.WT, CTLL.lck$^-$, and CTLL.LCK16 cells (effector cells) were deprived of IL-2 for 12 h and then incubated with either no IL-2 (left panel) or 10 U of IL-2 per ml (right panel) for an additional 4 h. The effector cells were cultured for 4 h with ^{51}Cr-labeled 145-2C11 B-hybridoma target cells at the indicated effector-to-target cell ratios. Specific ^{51}Cr release was measured as described in Materials and Methods. Datum points represent mean ^{51}Cr release from triplicate samples, and standard deviations are indicated by error bars. The results shown are representative of those obtained in four independent experiments.

strictly dependent on cell surface expression of CD3-ε-specific immunoglobulin by the hybridoma cells (24). This redirected target cell lysis assay was subsequently used to determine the impact of a p56^{lck} deficiency on both the constitutive and IL-2-inducible cytolytic activities of CTLL-2 cells. The CTLL sublines were deprived of IL-2 for 12 h and then incubated with either vehicle only or a maximal stimulatory concentration of IL-2 (10 U/ml) for 4 h. The cytolytic activities of both factor-deprived (Fig. 7A) and IL-2-stimulated (Fig. 7B) cells were then measured in a 4-h cytotoxicity assay by using ^{51}Cr-labeled 145-2C11 hybridoma cells as targets. Factor-deprived CTLL.lck$^-$ cells mediated a relatively low level of target cell killing, which was slightly enhanced by pretreatment of the cells with IL-2. In contrast, CTLL.WT cells displayed a strong cytolytic response in the absence of IL-2, and IL-2 stimulation further increased the cytolytic function of these cells. The CTLL.LCK16 subline, which expresses an approximately fivefold-higher level of p56^{lck} than CTLL.WT cells (Fig. 1B), demonstrated a high level of cytolytic activity in both the absence and the presence of IL-2. Similar results were obtained with the CTLL.LCK15 subline (data not shown). The specificity of the p56^{lck} requirement was further substantiated by the finding that CTLL.lck$^-$ cell transfectants expressing either p60$^{c\text{-}src}$ or the polyoma virus middle-size tumor antigen (which induces constitutive activation of endogenous p59^{fyn} [5]) retained the defective cytolytic phenotype of the parental CTLL.lck$^-$ cell line (24).

DISCUSSION

The cytotoxic T-cell line, CTLL-2, provides a useful model system for studies of T-cell growth regulation by IL-2. In serum-containing medium, CTLL-2 cells exhibit strict dependence upon a single growth factor, IL-2, for both proliferation and maintenance of viability. In addition, these cells have retained the capacity to direct TCR-dependent cytolytic activity toward appropriate target cells. The spontaneous emergence of a p56^{lck}-deficient CTLL-2 variant therefore provided a unique opportunity to address the potential roles of p56^{lck} in both IL-2-mediated mitogenesis and CTL effector function.

The initial finding that IL-2 stimulation induced a rapid increase in protein tyrosine phosphorylation in T cells suggested that an undefined PTK served as a proximal signal-transducing element for this receptor. Furthermore, these results provoked speculation that, by analogy with members of the growth factor receptor tyrosine kinase superfamily, IL-2R mitogenicity was dependent on the ligand-dependent activation of a cytoplasmic PTK domain. Several lines of evidence render p56^{lck} an attractive candidate for the putative IL-2R-coupled PTK. Stimulation of activated human T cells with IL-2 provokes a rapid increase in the catalytic activity of p56^{lck}, and the subsequent phosphorylation of this PTK on multiple N-terminal serine residues (21). Furthermore, the intracytoplasmic domain of the IL-2R p75 subunit contains an acidic region which provides a specific binding site for p56^{lck} (17). Finally, the demonstrated cellular transforming potential of a constitutively activated version of p56^{lck} (Y505F) suggests that this PTK is capable of interacting with normal growth-regulatory pathways (3, 31). In the present study, the availability of p56^{lck}-deficient CTLL-2 cells allowed us to directly examine the potential role of p56^{lck} in mitogenic signal transmission from the IL-2R. These studies demonstrated that IL-2 triggered both cell cycle progression and proliferative responses in p56^{lck}-deficient T cells. Interestingly, the finding that IL-2R-mediated mitogenic signaling occurred in the absence of detectable p56^{lck} corroborated results obtained in an earlier study of a series of cytoplasmic domain deletion mutants of the IL-2R p75 subunit (18). This study demonstrated that removal of the acidic region of the receptor cytoplasmic domain, i.e., the putative p56^{lck} binding site, did not impair IL-2-dependent mitogenic signal transduction in pre-B cells transfected with the mutated p75 subunit. Although data obtained with established T-cell lines should be extrapolated to normal T lymphocytes with caution, the available data

strongly suggest that p56^{lck} does not play an essential role in mitogenic signal delivery from the IL-2R.

The failure of p56^{lck}-deficient CTLL-2 cells to exhibit a major defect in mitogenic responsiveness to IL-2 prompted a search for other phenotypic responses that might be influenced by the expression of p56^{lck}. The TCR complex plays direct roles in target cell recognition and lytic activation of antigen-specific CTL. The effector functions of cytotoxic lymphocytes are also up-regulated or primed by prior exposure of these cells to IL-2 (28, 49). On the basis of the accumulating evidence that p56^{lck} participates in downstream signal propagation from the TCR, as well as the IL-2R, we postulated that the cytolytic response of CTLL-2 cells might be sensitive to alterations in p56^{lck} expression. In contrast to the relatively minor growth defect of CTLL.lck$^-$ cells, the cytolytic function of this cell line was markedly impaired compared with those of the parental CTLL.WT cells or the lck-transfected subclones. Interestingly, the CTLL.LCK transfectants, which overexpressed p56^{lck} by 2.5- to 5-fold relative to the CTLL.WT cells, displayed similarly high levels of cytolytic activity in both the presence and absence of IL-2. The divergent effector phenotypes of the CTLL.lck$^-$ cells and the CTLL.LCK transfectants suggest that variations in p56^{lck} expression may have a major influence on the lytic activities of normal CTL populations.

The results discussed above indicate that both the TCR and the IL-2R deliver intracellular signals that activate the cytolytic potential of CTLL-2 cells. Clearly, p56^{lck} activity could be involved in the signal transduction pathways linked to either one or both of these receptor systems. The impaired cytolytic function of factor-deprived CTLL.lck$^-$ cells strongly suggests that a p56^{lck} deficiency results in an intrinsic defect in the coupling mechanism between TCR ligation and the cytolytic response of these cells. Indirect evidence supporting an important role for p56^{lck} in TCR-dependent signaling is provided by a previous report which indicated that expression of an activated p56^{lck} mutant (Y505F) in a CD4$^-$ murine helper T-cell hybridoma resulted in increased levels of antigen-dependent lymphokine production (1). However, the observation that p56^{lck}-deficient CTLL-2 cells retain a basal level of cytolytic activity is consistent with the notion that p56^{lck} is not indispensable for signal transduction through the TCR.

The postulated role of p56^{lck} as a TCR-linked signal amplifier is reminiscent of the current paradigm for the function of CD4- and CD8-associated p56^{lck} in helper- and cytotoxic-lineage T cells, respectively (46). According to this model, the extracellular domains of CD4 and CD8 serve as coreceptors for nonpolymorphic determinants expressed by major histocompatibility complex (MHC)-encoded class I and class II molecules. During antigen-specific, MHC-restricted T-cell activation, CD4 or CD8 molecules colocalize with the TCR and thereby deliver cytoplasmic p56^{lck} into close proximity with the TCR complex. Earlier studies indicated that the binding of p56^{lck} to the intracytoplasmic domain of CD8 was required for optimal cytokine secretion by MHC class I-restricted hybridoma cells (50), which was consistent with an enhancing role of p56^{lck} in the propagation of TCR-dependent activating signals. However, this mechanism does not explain the effects of altered p56^{lck} expression on the cytolytic functions of CTLL-2 cells, as our CTLL-2 sublines lack detectable surface expression of CD4 or CD8 (24). Although the association of p56^{lck} with CD8 may be necessary for maximal CTL activation by antigen-bearing target cells, results obtained with the redirected lysis assay used in the present study indicate that p56^{lck} can enhance TCR-dependent cellular responses in the absence of CD8 or CD4.

The failure of IL-2 pretreatment to reverse the cytolytic defect of CTLL.lck$^-$ cells demonstrates that the ability of IL-2 to prime these cells for cytolytic activation is directly or indirectly dependent on p56^{lck}. Nonetheless, IL-2 stimulation leads to a moderate elevation of cytolytic activity in the p56^{lck}-deficient cells, which again suggests that p56^{lck} activity provides an amplifying, rather than an obligatory, stimulatory signal for this TCR-mediated cellular response. The impaired cytolytic function of IL-2-stimulated CTLL.lck$^-$ cells might be explained by an intrinsic defect in the TCR-dependent stimulus-response coupling pathway which renders this mechanism insensitive to the priming signal generated as a consequence of IL-2R ligation. Alternatively, the loss of p56^{lck} might directly interfere with generation of the IL-2-mediated priming signal in these cells. The observation that the p56^{lck}-overexpressing CTLL.LCK transfectants displayed maximal target cell killing in the absence of IL-2 suggests that the priming effect of IL-2 on cytotoxic function can be replaced by increased levels of endogenous p56^{lck}. Clearly, further studies of the interplay among the TCR, the IL-2R, and intracellular p56^{lck} activity will provide important insights into the regulation of the cytolytic effector functions of antigen-specific CTL.

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