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Immunology

Thymic tumorigenesis induced by overexpression of p56^{lck}

(tyrosine kinases/oncogenesis/transgenic mice)

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ABSTRACT **The *lck* gene encodes a membrane-associated protein tyrosine kinase (p56^{lck}) that is believed to participate in lymphocyte-specific signal transduction pathways. To investigate the function of this molecule, transgenic mice were generated carrying the wild-type *lck* gene or a mutated *lck* gene encoding a constitutively activated form of p56^{lck} (p56^{lckF505}). Transgene expression in thymocytes was achieved in each case using the *lck* proximal promoter element. Mice expressing high levels of either p56^{lckF505} or p56^{lckY505} reproducibly developed thymic tumors. The sensitivity of thymocytes to p56^{lck}-induced transformation suggests that disturbances in *lck* expression may contribute to the pathogenesis of some human neoplastic diseases.**

Protein tyrosine kinases are important signal transduction elements that have been implicated in the control of cell growth (1). Broadly speaking, the protein tyrosine kinases may be subdivided into two groups: a set of membrane-spanning receptor structures for which ligands are in many cases known (e.g., the receptors for platelet-derived and epidermal growth factors) and a second group of membrane-associated protein tyrosine kinases whose functions are less well established. Many membrane-associated protein tyrosine kinases were first identified as products of retrovirally transduced protooncogenes; hence it is widely believed that these molecules, like their growth factor receptor counterparts, assist in controlling proliferative responses to external stimuli.

Among the best-studied nonreceptor protein tyrosine kinases are those that structurally resemble p60^{src}, the product of the c-*src* protooncogene (2). Each of the eight src-family kinases contains a set of characteristic sequence motifs, including an amino-terminal myristoylation sequence, a central "homology" domain believed to assist in substrate recognition (3), and two tyrosine phosphorylation sites within the carboxyl-terminal kinase domain. The activity of src-family kinases appears to be regulated, in part, by phosphorylation of these two tyrosine residues (2).

Members of the src family frequently exhibit stereotyped and quite restricted expression patterns, particularly in hematopoietic cell lineages (4). Thus the *hck* and *fgr* gene products accumulate almost exclusively in myeloid cells (5), whereas the product encoded by the *blk* gene is found only in B lymphocytes (6). We have focused attention on a fourth src-like protein kinase, the product of the *lck* gene p56^{lck}, which is expressed exclusively in lymphocytes, particularly T cells (4). Previous studies (7–10) have revealed that p56^{lck} physically associates with the CD4 and CD8 coreceptors of the T-cell antigen recognition complex. Antibody-mediated cross-linking of CD4 molecules stimulates p56^{lck} activity (11), suggesting that p56^{lck} may participate in relaying signals derived from ligand occupancy of the T-cell receptor. Although the nature of the signal provided by p56^{lck} activation is enigmatic, the *lck* gene is rearranged and overexpressed in some murine lymphomas (12–14), suggesting that p56^{lck}-mediated phosphorylation may stimulate lymphocyte proliferation.

Like other src-family protein tyrosine kinases, p56^{lck} is phosphorylated *in vivo* at a tyrosine residue near its carboxyl terminus (Tyr-505). Elimination of this phosphorylation site by site-directed mutagenesis unmasks the transforming activity of p56^{lck} in fibroblast transformation assays (15, 16). Hence by this criterion the *lck* gene is a protooncogene.

To study the function of p56^{lck} in its normal cellular context, we have generated transgenic animals in which wild-type p56^{lck} and mutant p56^{lckF505} are overexpressed under the control of the normal *lck* transcriptional machinery. Mice expressing high levels of either protein develop thymic tumors by 6 weeks of age. These results indicate that increased phosphorylation of p56^{lck} substrates in normal lymphocytes can serve as an important predisposing factor for lymphoma development and focus attention on the potential contribution of p56^{lck} to the pathogenesis of human lymphoid malignancy.

MATERIALS AND METHODS

Transgene Construction. The 3′ coding region of the *lck* gene was reconstructed using an *Xmn* I fragment of the lckF505 cDNA in the pNUT vector (15) that contained 625 base pairs of the human growth hormone (hGH) gene 3′ untranslated region sequence within which is a consensus polyadenylylation site. This fragment was ligated to a 1.8-kilobase (kb) *Hin*dIII fragment containing exons 9–11 of the murine *lck* gene, and the remainder of the gene was reconstructed using characterized genomic clones (12). Transgenic animals were produced by microinjection of DBA/2 × C57BL6/J F_2 zygotes as described (17). Integration of transgenic constructs into the mouse germ line was assessed by the presence of hGH sequences in mouse tail DNA (18).

Quantitation of Transgene RNA in Mouse Tissues. RNA was recovered from homogenized tissue or single-cell suspensions using the guanidine isothiocyanate method (19). Quantitation of transgene mRNA was performed by solution hybridization (20) using oligonucleotides labeled with ^{32}P (21) and by densitometric analysis of RNA blots. RNA blotting was performed using 10 μg of total RNA as described (14). Quantities of RNA loaded for densitometry were verified by hybridization of blots with probes specific for the 3′ untranslated region of *lck*.

Antibody Staining and Flow Cytometric Analysis. Single-cell suspensions obtained from lymphoid organs were depleted of erythrocytes by ammonium chloride lysis (22). The

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Abbreviations: hGH, human growth hormone; SV40, simian virus 40.

resulting leukocytes were stained for surface expression of CD4, CD8, and CD3 molecules as described (17) using biotinylated GK1.5 (23), fluorescein isothiocyanate, labeled 53-6.71 (Becton Dickinson), and biotinylated 500AA2 (24), respectively. Detection of biotinylated antibodies was facilitated using phycoerythrin-conjugated streptavidin (Caltag, South San Francisco, CA) as a second step reagent. In some cases, cell lines were derived by cultivating dispersed thymocytes in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum as described (17). Multiparameter flow cytometric analysis was carried out on FACSCAN, FACSTAR, and FACSTAR-PLUS instruments (Becton Dickinson). Each analysis included 10,000 events collected in list mode files and analyzed using FACSTAR Consort 30 software.

Immunoblot Analysis. Whole-cell lysates of thymocytes or cell lines were boiled in SDS/PAGE loading buffer and analyzed by 10% SDS/PAGE. After electrophoresis, proteins were transferred to nitrocellulose, blotted, and developed as described (15) using rabbit anti-phosphotyrosine antiserum (the kind gift of Robert Abraham, Mayo Clinic, Rochester, MN) or rabbit anti-p56^{lck} (25) as primary reagents.

RESULTS

Construction of *lck*-Encoding Transgenes and Their Pattern of Expression *in Vivo*. The *lck* gene contains two widely spaced promoter elements (12, 13, 26). Sequences containing the proximal (or downstream) promoter element have been shown (18, 27, 28) to direct expression of heterologous genes in the thymocytes of transgenic mice. This promoter is active during the earliest stages of thymocyte development and in all thymocyte subsets defined by CD4 and CD8 expression (26,

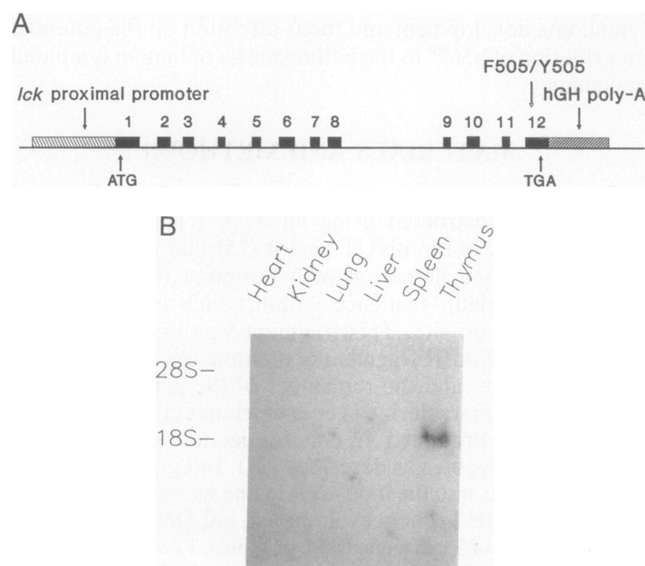

FIG. 1. Diagrammatic representation of the pLGF and pLGY constructs and expression of transgene derived mRNA. (*A*) pLGF and pLGY constructs contain 11.2 kb of murine genomic *lck* sequence including 1 kb 5′ to the proximal transcription start site (9) and exons 1–12 of the *lck* structural gene. A portion of exon 12 sequence was obtained from the murine lck cDNA encoding either the wild-type gene with tyrosine at position 505 in the pLGY construct or a point mutation replacing tyrosine with phenylalanine at position 505 in pLGF. The polyadenylylation signal for these constructs is provided by 625 base pairs of 3′ sequences from the hGH gene. (*B*) Total RNA (10 μg) was recovered from the indicated tissues, separated on formaldehyde/agarose gels, blotted, and subsequently analyzed for transgene expression by hybridization with an hGH probe. The migration of eukaryotic ribosomal RNAs, as markers, is indicated.

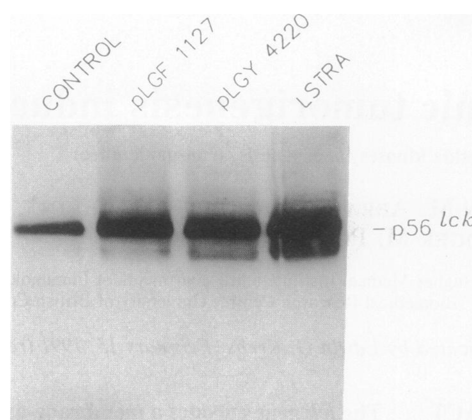

FIG. 2. Expression of pLGF and pLGY transgene-derived proteins in transgenic mice. An immunoblot analysis of 50 μg of whole-cell lysate protein obtained from dispersed thymic tumors of pLGF and pLGY transgenic mice, a normal littermate control, and the LSTRA cell line was done using p56^{lck}-specific antiserum. The migration position of p56^{lck} is noted.

29, 30). To manipulate levels of p56^{lck} in thymocytes *in vivo*, we assembled the constructs shown in Fig. 1. The pLGY and pLGF transgenes shown in Fig. 1*A* contain 1.0 kb of genomic sequence 5′ to the start of transcription and the entire coding region of the murine *lck* gene through exon 11. The remainder of the coding sequence was derived from murine cDNAs containing either tyrosine or phenylalanine codons at position 505 (9). These fragments were linked to a 3′ untranslated region segment from the hGH gene that contains a single polyadenylylation site (31). The presence of these hGH sequences permits easy detection of transgene integration in tail DNA and transgene expression in cellular RNA. By using these constructs, 14 pLGF founders and 5 pLGY founders were obtained after microinjection of C57BL/6 × DBA/2 F_2 embryos.

Fig. 1*B* demonstrates that pLGF-encoded transcripts accumulated in thymocytes, but not in other tissues. In particular, the transgene mRNA was not detectable in peripheral lymphoid tissues such as spleen (Fig. 1*B*) or lymph node (data not shown). This result is consistent with the low level of normal *lck* transcripts derived from the proximal *lck* promoter in mature circulating T lymphocytes (26, 30, 32). Hence the pLGF (and pLGY) transgenes are expressed in accord with the known pattern of endogenous proximal

Table 1. Characteristics of pLGF and pLGY transgenic mice

Founder	Transgene expression	Fold expression	Tumors at 8 weeks	Animals analyzed, no.
pLGF				
1127	33.8	4	+	23
2949	35.0	4	+	7
2943	23.4	3	+	1
2954	11.7	2	−	13
2964	10.0	2	−	8
3122	9.0	<2	−	1
3073	6.0	<2	−	11
701	1.4	<2	−	13
pLGY				
4220	70.0	6	+	10
1610	11.0	<2	−	1
1570	1.5	<2	−	1

Transgene mRNA expression is presented as pg/μg of total cellular RNA. Approximate total lck mRNA levels are expressed as the ratio of endogenous *lck* (12 pg/μg of total RNA) plus transgene-derived *lck* expression relative to endogenous *lck* levels. Tumor formation at 8 weeks was assessed by visual inspection and verified by forward scatter measurements. +, Tumor formed; −, no tumor.

promoter activity. In addition, thymocytes of animals expressing pLGF and pLGY mRNA also contain increased levels of $p56^{lck}$ protein when compared to normal littermate controls, as judged by immunoblot analysis using $p56^{lck}$-specific antisera (Fig. 2). The increase in $p56^{lck}$ expression correlated well with measurements of transgene-encoded mRNA abundance. Thus normal thymocytes contain ≈12 pg of *lck* mRNA per μg of total RNA (33), whereas the thymocytes that were used to generate data in Fig. 2 contain 3 to 6 times as much transgene-derived *lck* mRNA (Table 1).

The pLGF Transgene Functions as an Oncogene *in Vivo*. Mice expressing high levels of the pLGF transgene develop fatal thymic tumors. In line 1127, which has been propagated for 10 generations, all transgene-bearing mice developed large thymic lymphoid tumors that were grossly visible by about 6 weeks of age. Cells from pLGF tumors are larger than normal thymocytes (as measured by forward light scatter) and characteristically lack surface expression of the T-cell receptor/CD3 complex (Fig. 3). In addition, these thymocytes display a mixed $CD4^-8^-$, $CD4^-8^{lo}$ phenotype (Fig. 3). The pLGF 1127 tumors metastasize to extrathymic sites and can be readily adapted to *in vitro* culture in the absence of exogenous growth factors (see Fig. 5 and data not shown). Two other pLGF lines (2943 and 2949) expressing high levels of the transgene exhibited an identical phenotype with respect to thymoma development (Table 1). Expression of lckF505 transcripts at levels >20 pg/μg of total RNA appears to be required for tumorigenesis; animals expressing lower levels of the pLGF transgene fail to develop thymic tumors during at least the first year of life (Table 1).

Overexpression of Wild-Type *lck* Leads to Tumorigenesis *in Vivo*. Expression of $p56^{lckF505}$, but not wild-type $p56^{lck}$ protein, can confer a transformed phenotype on cultured fibroblasts (15). Surprisingly, overexpression of wild-type *lck* transcripts (using the pLGY construct) also stimulated thymoma development in transgenic mice. The target cells transformed under these circumstances were phenotypically similar to cells observed in pLGF tumors. However, all of the pLGY tumor-derived cells express low levels of surface CD8 protein, and a proportion also express CD4 molecules (Fig. 3). By these criteria, the transformed cells in pLGY-induced tumors are somewhat more mature T-lineage elements (34).

Phosphotyrosine Levels Are Increased in Thymocytes Transformed by *lck*. The signaling function of $p56^{lck}$ presumably

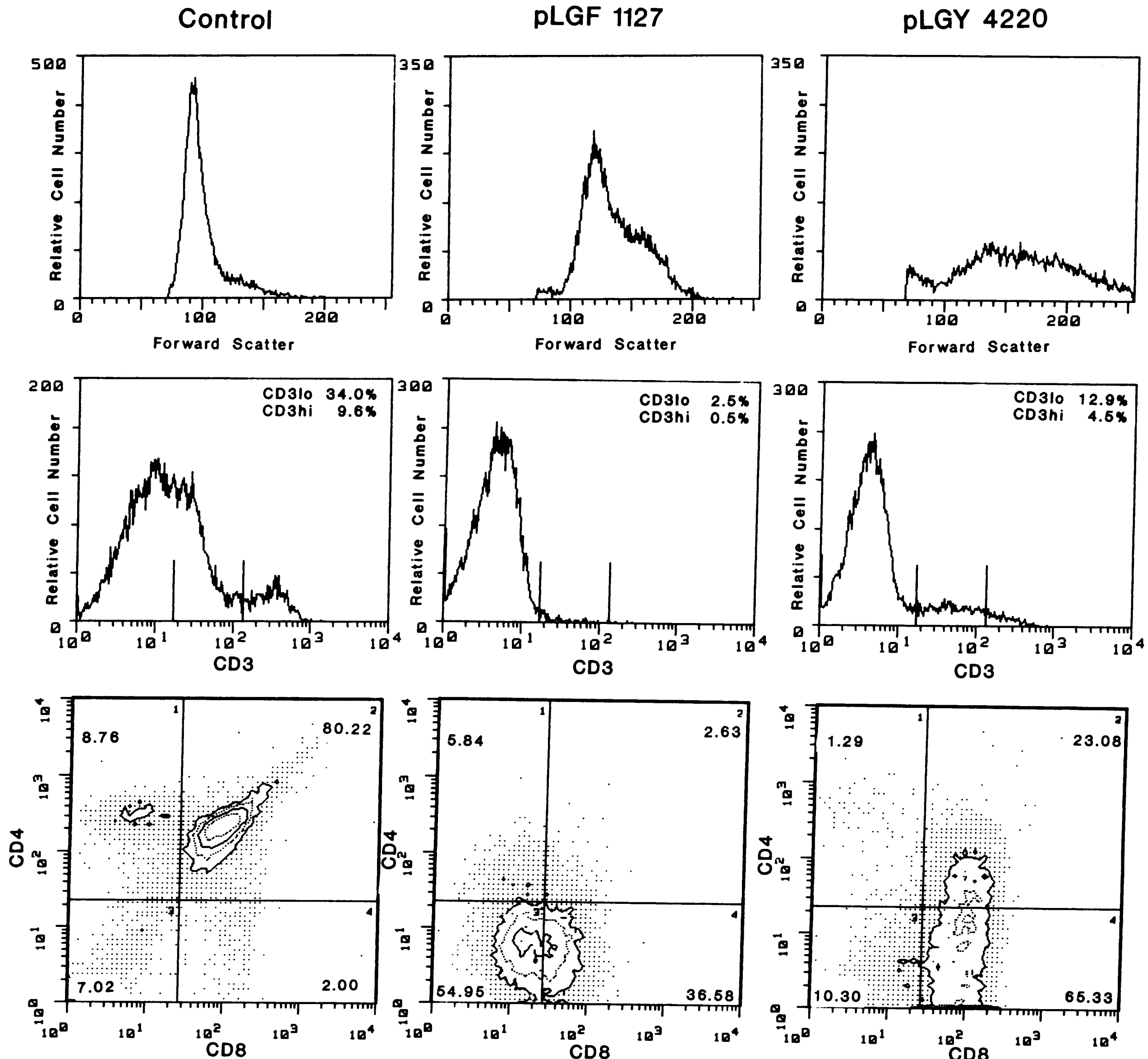

FIG. 3. Cell surface phenotype of thymomas originating in pLGF and pLGY transgenics. An analysis of cell size by forward light scatter and histograms of CD3, CD4, and CD8 levels obtained using dispersed cells from thymic tumors that arose in 1127 pLGF and 4220 pLGY animals or from a normal littermate control of the same age (8 weeks) is shown.

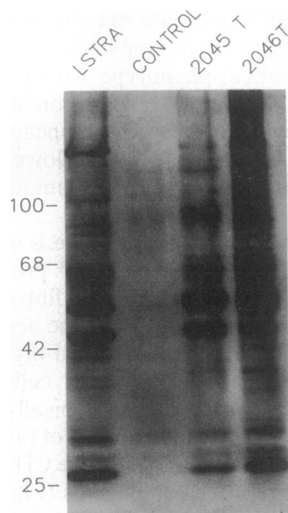

FIG. 4. Increased phosphotyrosine levels in pLGF thymic tumors. An immunoblot analysis of whole-cell lysates obtained from dispersed thymocytes of pLGF-tumor-bearing animals (2045 and 2046) and a normal littermate control was done using a phosphotyrosine-specific antiserum. The migration positions of protein standards in kDa are indicated to the left.

results from its ability to modify the phosphorylation state of tyrosine residues in other proteins. Examination of phosphotyrosine-containing proteins in pLGF and pLGY tumors might therefore permit identification of important regulatory molecules that assist in controlling lymphocyte growth. Immunoblot studies performed with phosphotyrosine-specific antisera reveal a very large number of targets for $p56^{lckF505}$ and $p56^{lckY505}$ in transgenic thymocytes. In fact, the array of proteins containing phosphotyrosine in these thymic tumors resembles the pattern observed in the LSTRA cell line, in which a retroviral insertion event results in 50-fold overexpression of *lck* (Fig. 4). Tumors obtained from pLGY animals exhibit similarly complex patterns of phosphotyrosine-containing proteins (data not shown). Hence with respect to tumorigenesis, it is very difficult to distinguish between the behavior of wild-type $p56^{lck}$, which is incapable of imposing transformed characteristics on NIH 3T3 fibroblasts (15, 16), and the mutant $p56^{lckF505}$, which is a potent transforming gene in cell culture.

Cell Lines Derived from pLGF and pLGY Thymomas. Thymomas from pLGF and pLGY animals provide a ready source of transformed cells with extremely immature phenotypes. Cell lines derived from these tumors were easily propagated *in vitro* in the presence of 10% fetal calf serum but without added growth factors. Such cell lines are uniformly $CD3^-$ (data not shown) and lack expression of CD4 and CD8 cell surface markers (Fig. 5). Hence, these cells may represent a transformed correlate of immature progenitor cells that ordinarily constitute a small fraction of normal thymocytes.

DISCUSSION

Three previous observations suggested that the *lck* gene is in fact a protooncogene. (*i*) It is structurally related to a set of oncogenes previously identified in acutely transforming retroviruses: v-*src*, v-*fgr*, and v-*yes* (4). (*ii*) It is specifically overexpressed in two murine lymphoma cell lines in which retroviral promoter insertion causes increased accumulation of an altered lck mRNA that is very efficiently translated (12–14, 35). (*iii*) Substitution of phenylalanine for tyrosine at position 505 unmasks the transforming potential of the *lck* gene in NIH 3T3 cell assays (15, 16). Tyr-505 of $p56^{lck}$ is normally phosphorylated *in vivo* (15, 36). Dephosphorylation of the analogous tyrosine (Tyr-527) of $p60^{c\text{-}src}$ substantially augments the kinase activity of this molecule (37). Thus there is reason to believe that $p56^{lck}$ activity is negatively regulated by Tyr-505 phosphorylation. Indeed, recent reports suggest that the leukocyte phosphotyrosine phosphatase CD45 can specifically dephosphorylate Tyr-505 of $p56^{lck}$, resulting in a modest increase in activity (38, 39).

Our studies demonstrate that $p56^{lckF505}$ and its wild-type counterpart $p56^{lckY505}$ behave as transforming elements *in vivo* in cells that normally express *lck*. Transformation occurred when transcription was appropriately directed by the normal *lck* proximal promoter. Moreover, the 5′ untranslated regions of the pLGF and pLGY transgene transcripts contain all of the sequence elements that have been shown (35) to reduce the efficiency of translation of $p56^{lck}$.

Efficiency of $p56^{lck}$-Induced Transformation. It is instructive to compare the efficacy of the *lck* oncogene with that of the simian virus 40 (SV40) large tumor antigen expressed using the same promoter element. SV40 large tumor antigen also induces maturational defects and thymic tumors in transgenic mice (17). However, SV40-derived tumors develop with a significantly longer latency (18–20 weeks as compared to 6–9 weeks for *lck*). Therefore, with reference to this highly efficient viral oncogene, *lck* is an extremely potent transforming agent in murine thymocytes. The *lck*-induced thymomas typically contained cells with less mature phenotypes (as judged by expression of cell surface CD3, CD4, and CD8 markers) than do those induced by SV40 large tumor antigen, and the cell lines derived from *lck*-induced thymomas retain their immature characteristics. This situation contrasts with SV40-induced cell lines that almost invariably express detectable CD8 protein and frequently bear near-normal levels of the T-cell antigen receptor complex (17). The sensitivity of thymocytes to transformation by $p56^{lck}$ presumably results from a unique substrate specificity of the kinase and not from the ability of $p56^{lck}$ to interact with CD4 and CD8 molecules, since thymocytes lacking both of these proteins are transformed. These observations suggest that

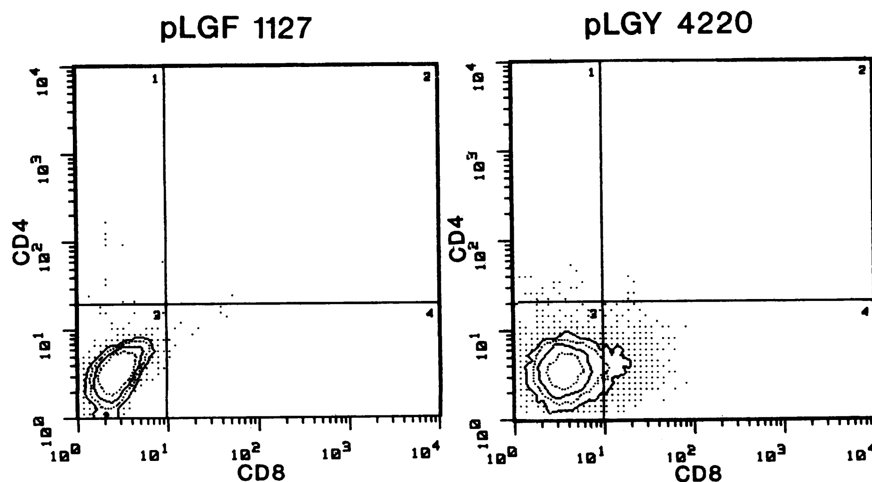

FIG. 5. Phenotypes of pLGF- and pLGY-derived thymoma cell lines. Cells derived from pLGF- and pLGY-induced thymomas were propagated *in vitro* for >4 months in RPMI medium containing 10% fetal calf serum but without additional growth factors. Representative flow histograms for CD4 and CD8 protein expression on the surfaces of these cells are shown.

mutations in the *lck* coding region or in regions involved in regulating *lck* expression levels might both potentially contribute to the pathogenesis of some human lymphoid malignancies. In fact, the human *lck* gene is positioned at a chromosomal site (1p32–35) that has been implicated in the pathogenesis of non-Hodgkin lymphomas and neuroblastomas (40). Of course since tumors in pLGF mice develop over a period of some weeks, additional somatically propagated genetic abnormalities presumably contribute to the establishment of the fully transformed phenotype of pLGF and pLGY thymomas.

Transformation by p56^{lck} Is Dose-Dependent. Interestingly, there appears to be a threshold mRNA level that must be exceeded to permit the development of thymic tumors in pLGF mice. Animals with steady-state accumulations of pLGF transcripts <12 pg/μg showed no evidence of tumor formation. In contrast, animal 2943, with just twice this level of pLGF transcripts, experienced the full malignant phenotype. Normal thymocytes contain ≈ 12 pg of lck mRNA per μg of total RNA (14) and we found no evidence for silencing of the endogenous *lck* gene in pLGF transgenic mice (data not shown). Although wild-type p56^{lck} has been shown to be incapable of mediating transformation of NIH 3T3 cells (15, 16), in the 4220 pLGY line a 6-fold increase in wild-type *lck* expression was sufficient to stimulate rapid tumorigenesis. Hence thymocytes appear to be especially susceptible to overexpression of p56^{lck} activity. Our data do not permit comparisons of the relative transforming efficiencies of lckF505 versus lckY505, except to say that the F505 mutation cannot increase the transforming behavior of the lck gene by more than 5-fold (Table 1). Conceivably, overexpression of p56^{lck} in 4220 pLGY animals may permit accumulation of some molecules lacking phosphate on Tyr-505, which would presumably have activated properties. This situation resembles that observed in the LSTRA cell line, where enhanced lck transcription and translation conspire to permit 50-fold overexpression of wild-type p56^{lck} molecules that are incompletely phosphorylated on Tyr-505 (15, 16). Whether p56^{lck} overexpression provokes phosphorylation of additional substrates or supraphysiologic phosphorylation of normal substrates is unclear; however, it is apparent that the level of p56^{lck} activity in developing thymocytes must be maintained under stringent control. When such control mechanisms are subverted, a profound predisposition to malignancy results.

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