Regulation of pp56^{lck} during T-cell activation: functional implications for the *src*-like protein tyrosine kinases

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The lymphocyte-specific protein tyrosine kinase pp56^{lck}, encoded by a member of the *src* gene family, is implicated in the control of T-cell growth and differentiation. Purified resting human T lymphocytes contain appreciable levels of *lck* mRNA and of pp56^{lck}. Upon activation of these T cells, levels of *lck* mRNA and of pp56^{lck} promptly decline. These reductions in *lck* mRNA and protein expression are closely correlated with the induction of lymphokine production. Both require identical stimuli and follow a similar time course of response. Down-regulation of *lck* expression, however, is not correlated with proliferation. Our results provide an example of regulation of a *src*-like protein tyrosine kinase in a normal fully differentiated cell population and suggest that modulation of *lck* RNA and protein expression is an important feature of the lymphocyte activation sequence leading to lymphokine production.

Key words: tyrosine kinases/lymphocyte activation/lymphokine expression

Introduction

Among those molecules implicated in the control of cell growth and differentiation, the protein tyrosine kinases are particularly prominent. A subgroup of protein tyrosine kinases, exemplified by the product of the *c-src* proto-oncogene (pp60^{c-src}), includes molecules that typically associate with cellular membranes but which lack the membrane-spanning domains characteristic of the growth factor receptor subgroup (reviewed in Bishop, 1985; Hunter and Cooper, 1985). Of the seven known members of the *src*-family of protein tyrosine kinases (*fgr*, *hck*, *lck*, *lyn*, *src*, *syn/slk* and *yes*), three were originally identified as products of retroviral oncogenes (reviewed in Bishop, 1985; Hunter and Cooper, 1985). Thus although the normal functions of these enzymes are completely unknown, it has been attractive to view this class of protein tyrosine kinases, like the related growth factor receptor kinases, as components of signal transduction systems regulating cellular proliferation.

Analysis of the cell physiology of the *src*-family protein tyrosine kinases has been particularly difficult since it has not thus far been possible to associate alterations in *c-src* expression with defined metabolic events. The strong transforming activity of *v-src* notwithstanding, *c-src* is most abundantly expressed in fully differentiated cells with little replicative potential (Cotton and Brugge, 1983; Sorge *et al.*, 1984). Interestingly, at least some members of the *src* gene family are normally expressed in a lineage-specific fashion, suggesting that each may participate in

a cell-type specific regulatory pathway (Marth *et al.*, 1985; Ziegler *et al.*, 1987).

To investigate the processes whereby protein tyrosine phosphorylation mediated by the *src*-like kinases may govern cell behavior, we have focused attention on a lymphocyte-specific protein tyrosine kinase gene (*lck*) that is closely related to *c-src* (Marth *et al.*, 1985; Voronova and Sefton, 1986). Like pp60^{c-src}, pp56^{lck} is membrane associated, presumably by virtue of covalently linked myristate at its amino terminus (Marchildon *et al.*, 1984). The *lck* gene is rearranged and overexpressed in some murine lymphomas (Marth *et al.*, 1985; Voronova and Sefton, 1986), and is located at a site of frequent chromosomal abnormalities in human lymphomas and neuroblastomas (Marth *et al.*, 1986). Thus alterations in *lck* expression may contribute to the pathogenesis of some forms of neoplastic disease. At the same time, the specific accumulation of *lck* transcripts in lymphocytes, particularly T lymphocytes, suggests that pp56^{lck} normally participates in growth regulatory or differentiative pathways unique to these cells.

T lymphocytes occupy a critical role in immune function since activation of these cells by antigen results in the production of multiple lymphokines, including those that stimulate antibody production and the maturation of cytotoxic effector cells (Smith, 1984). The process of T-cell activation has been the subject of intense scrutiny in a number of laboratories, with the result that a biochemical dissection of the activation sequence has been partially achieved (reviewed in Weiss *et al.*, 1986). Here we report that activation of resting T lymphocytes provokes a rapid decline in *lck* mRNA abundance and in the level of pp56^{lck}. These alterations are not associated with proliferation *per se*, but are maximally induced by stimuli that also promote lymphokine production. Our results suggest that the *lck* gene product may function in the control of early events in lymphocyte activation, and encourage the view that *src*-family kinases participate in a signal transduction pathway only indirectly related to the control of cell growth.

Results

In resting T lymphocytes, binding of antigen or lectin mitogen (e.g. concanavalin A) is associated with a rapid increase in intracellular calcium concentration (Allwood *et al.*, 1971; Freedman and Raff, 1975; Nisbet-Brown *et al.*, 1985), and later with the expression of receptors for the T-cell growth factor interleukin-2 (IL-2; reviewed in Weiss *et al.*, 1986). Propagation of normal T lymphocytes is absolutely dependent on the presence of both the antigenic stimulus and sufficient amounts of IL-2, itself a T-cell product (reviewed in Smith, 1984). Although antigen or mitogen by itself can trigger some features of the activated phenotype, full activation requires a second stimulus (perhaps interleukin-1) that is normally provided by mononuclear accessory cells (reviewed in Palacios, 1982). The accessory cell stimulus can be replaced by phorbol myristic acid (PMA), indicating that this signal probably involves, at least in part, the

Fig. 1. Reduction in *lck* transcript levels following T lymphocyte activation. **Left panel.** Purified human T cells were stimulated with Con A (25 μ g/ml) and PMA (50 ng/ml) for the indicated intervals prior to isolation of total RNA. Shown is an RNA blot analysis performed using the human *lck* cDNA probe (see Materials and methods). IL-2 expression data was obtained after removal of the *lck* probe from the blot and rehybridizing with a human IL2 cDNA probe. **Right panel.** Shown are the results of RNA blotting analyses from an independent T-cell stimulation experiment in which both *lck* and interferon- γ (IFN) cDNA probes were used to identify transcripts on a single filter. The lines at the right of the figure denote the position of 18S ribosomal markers in both the left and right panels.

activation of protein kinase C (Rosenstreich and Mizel, 1979; Castagna *et al.*, 1982). Similarly, the expression and secretion of the lymphokines IL-2 and interferon- γ (IFN) also requires both an accessory cell signal, replaceable by PMA, and an antigenic signal, replaceable by agents that elevate intracellular Ca^{2+} concentrations (Wiskocil *et al.*, 1985; reviewed in Palacios, 1982). Early events in the physiologic activation of T lymphocytes can thus be mimicked by the synergistic effect of treatment with both a calcium ionophore and PMA (MacDonald and Nabholz, 1986; Truneh *et al.*, 1985).

To investigate the regulation of *lck* expression in lymphoid cells, we have isolated human peripheral blood T cells avoiding, in so far as possible, potentially activating stimuli that interact with T-cell surface receptors (see Materials and methods). These T-cell preparations maintain a two-stimulus requirement for induction of lymphokine mRNA, indicating that they are both unstimulated at the time of isolation and relatively free of accessory cells. Activation of these T lymphocytes *in vitro* provides an excellent model for the behavior of normal resting circulating T cells after antigen recognition *in vivo* (Cantrell and Smith, 1984).

Reduction in lck mRNA levels accompanies lymphocyte activation

Purified resting human T lymphocytes contain easily detectable levels of *lck* mRNA as visualized by RNA blot analysis. Stimulation of these cells with mitogenic lectin (Con A) and PMA in doses optimal for lymphokine production results in a profound decline in *lck* transcript abundance, reaching a nadir between 4 and 6 h after treatment, followed by reappearance of *lck* mRNA to levels that exceed those found in resting T cells (Figure 1). This biphasic expression pattern is the reciprocal of that obtained when levels of either IL-2 or IFN transcripts are analyzed. In accord with the published work of ourselves and others (Wiskocil *et al.*, 1985; Lewis *et al.*, 1986) stimulation of T cells with Con

Table I. Abundance of *lck* mRNA in lymphoid cells

Cell type	<i>lck</i> level (pg mRNA/ μ g total RNA)	Copies <i>lck</i> mRNA/cell
T cells ($t = 0$) ^a	2.3	3.6
T cells ($t = 6$ h) ^a	0.5	0.8
T cells ($t = 24$ h) ^a	4.4	10.2
B cells	1.3	2.0
Jurkat	12.2	38.2
KE37	2.3	7.2
HSB-2	12.7	40.8
CEM	9.0	28.2
Thymus (human)	12.3	19.8 ^b
Spleen (human)	1.0	1.5 ^b
LSTRA ^c	122.3	408.0
YAC-1 ^c	18.0	58.0

^aPurified human T cells were stimulated with Con A (25 μ g/ml) and PMA (50 ng/ml) for the indicated periods prior to analysis. The precision of these assays from repeated determinations is $\pm 5\%$.

^bThese tissues contain heterogeneous cell types that likely vary in RNA content. No independent measure of cell number was obtained, and hence these concentration data are approximate.

^cDeterminations in these murine cell lines were performed using a murine *lck* cDNA probe (see Materials and methods).

A and PMA induces both IL-2 and IFN mRNA, yielding maximal levels between 4 and 8 h after activation (Figure 1). Furthermore, the subsequent decline in IL-2 and IFN message abundance is associated with an increase in *lck* transcript abundance (Figure 1).

A quantitative assessment of *lck* mRNA levels in resting and activated T cells was achieved using solution hybridization methods (see Materials and methods). As shown in Table I, resting human T cells contain 2.3 pg of *lck* mRNA per μ g of

Fig. 2. Two signals are required to depress the level of *lck* transcripts in normal T cells. Shown are a series of RNA blot analyses probed with either the *lck* or the IFN cDNA probes. Purified T lymphocytes were stimulated for the indicated time intervals with either Con A (25 μ g/ml), PMA (50 ng/ml) or the combination of both at either high (50 ng/ml) or low (1 ng/ml) PMA concentrations. The figure was assembled from a single RNA blot which was first exposed using the *lck* probe and subsequently rehybridized with the IFN probe. The positions of 18S rRNA markers are noted at the left of the figure.

total cellular RNA, corresponding to approximately four molecules of *lck* mRNA per cell (see Materials and methods for details regarding these calculations). This value may be compared with levels observed in normal human thymus RNA (12.3 pg/ μ g or in human spleen (1.0 pg/ μ g). Six hours after stimulation *lck* mRNA abundance has decreased by >4-fold to 0.5 pg/ μ g of total RNA, or less than one molecule of *lck* mRNA per cell. Thus it seems likely that *lck* transcripts transiently disappear completely at some point in the activation sequence of individual T cells. By 24 h after activation, *lck* mRNA levels increase to 4.4 pg/ μ g of total RNA and are >10-fold increased over those observed 6 h after stimulation on a per cell basis. Basal levels of *lck* transcripts are again observed ~72 h post-activation (Figure 1).

Reduction of *lck* mRNA levels requires both Con A and PMA signals

As shown in Figure 2, neither Con A nor PMA independently induce reductions in *lck* expression at 6 h, while both administered together have a synergistic effect, even when a minimal dose of PMA (1 ng/ml) is employed. The doses of Con A (25 μ g/ml) and PMA (50 ng/ml) used here were chosen in preliminary titration experiments selecting for maximal reductions in *lck* mRNA levels. Interestingly, with this protocol T-cell proliferation, as judged by [3 H]thymidine incorporation, is inhibited by the combination of Con A and PMA as compared with either stimulus alone, and is especially low when compared with the combined Con A/low dose PMA regimen (Figure 2), most likely as a result of inhibition of cell cycle progression at high PMA doses. Thus the decline in *lck* mRNA does not simply reflect provision of a proliferative stimulus. At the 24-h time point, Con A alone, PMA alone and the combination of both agents all induced similar increases in the level of *lck* transcripts. As previously observed (Figure 1), there is an inverse correlation between *lck* mRNA levels and the levels of IL-2 or IFN

Fig. 3. Specific elimination of *lck* transcripts is a marker for lymphocyte activation. Whole mononuclear cells were treated with Con A (25 μ g/ml) and PMA (lane 2), OKT3 antibody (1:100 dilution of ascitic fluid) and PMA (lane 3), PHA (2 μ g/ml) and PMA (lane 4), ionomycin (2.5 μ M) and PMA (lane 5) or SEA (0.15 μ g/ml) and PMA (lane 6) for 6 h prior to harvesting total RNA. PMA concentrations were 50 ng/ml in all lanes. Shown is an RNA blot analysis of these samples as compared with unstimulated cells maintained in RPMI media for 6 h (lane 1) using either the *lck* or IFN probes. The positions of rRNA markers are noted at the left. Identical results were obtained when purified T cells were treated with OKT3/PMA or ionomycin/PMA (data not shown).

mRNAs (Figure 2). Thus lymphokine-specific mRNA is only detectable in those lanes where *lck* mRNA levels are decreased. Furthermore, reductions in *lck* mRNA levels were observed in murine splenocyte populations using Con A alone (data not shown), indicating that the PMA signal which contributes to down-regulation of *lck* in purified human T cells acts by replac-

Fig. 4. Immunoprecipitation of pp56^{lck} using rabbit antiserum lck-2102 directed against a carboxy-terminal peptide. Membranes derived from two murine cell lines, LSTRA (L) and YAC-1 (Y), were incubated in an *in vitro* phosphorylation reaction using [³²P]ATP and immunoprecipitated as previously described (Marth *et al.*, 1985) with protein-A Sepharose. The resulting immunoprecipitates were resolved by SDS-PAGE prior to autoradiography. The overexpression of pp56^{lck} in LSTRA, as compared with YAC-1, is clearly visible in the profiles of total phosphoprotein from the two cell lines. Immunoprecipitates obtained using LSTRA membranes and the lck-2102 antiserum in the presence of the indicated amounts of peptide are shown in the four right-hand lanes. A single phosphorylated protein, pp56^{lck}, is immunoprecipitated.

Fig. 5. Expression of pp56^{lck} in human T lymphoma cell lines visualized by immunoblotting. Total membranes were boiled in SDS sample buffer, separated by SDS-PAGE, electroblotted onto nitrocellulose, and visualized using affinity-purified lck-2102 antibodies and a standard alkaline phosphatase-coupled facilitating antiserum (see Materials and methods). The addition of lck peptide (5 μg/ml) during the immune incubation (plus peptide) effectively competes antibody binding to pp56^{lck} demonstrating antibody specificity.

ing the accessory cell stimulus (Weiss *et al.*, 1986). We therefore tested other combinations of activating stimuli in order to further investigate the reciprocal relationship between *lck* and lymphokine transcript levels.

Reduction in *lck* mRNA level is an activation marker

Stimulation of lymphocytes by antigen or mitogen can be mimicked using a monoclonal antibody (OKT3) directed against a component of the T-cell receptor complex (CD3) or by using a calcium ionophore (ionomycin). In each of these cases, an early increase in intracellular calcium concentrations is implicated as the proximal stimulatory signal (Metcalfe *et al.*, 1980; Oettingen *et al.*, 1985). As in the case of combined Con A and PMA stimulation, *lck* mRNA levels are profoundly reduced and IFN mRNA levels are elevated within 6 h after treatment of whole mononuclear

Fig. 6. Elimination of pp56^{lck} accompanies T-lymphocyte activation. Purified T cells were stimulated with Con A (25 μg/ml) and PMA (50 ng/ml) for the indicated intervals prior to the isolation of membrane proteins and analysis of pp56^{lck} levels by immunoblotting. Equivalent amounts of protein (by cell number and Bradford analysis) were loaded in all normal T-cell lanes. The position of pp56^{lck}, identified in the human T-cell lymphoma Jurkat (half of protein amount used in normal T-cell lanes) is indicated at the right of the figure.

Fig. 7. Two stimuli act synergistically to eliminate pp56^{lck} during lymphocyte activation. Shown are immunoblot analyses on SDS-soluble total membrane protein from 5×10^7 purified T cells stimulated with Con A (25 μg/ml), PMA (50 ng/ml) or both, or OKT3/PMA, for the indicated intervals.

cells with phytohemagglutinin/PMA, ionomycin/PMA, OKT3/PMA or staphylococcal enterotoxin A (SEA)/PMA (Wilson *et al.*, 1986) activating regimens (Figure 3). Similar results were obtained when purified T cells were treated with ionomycin/PMA or OKT3/PMA regimens; neither *lck* mRNA reduction nor IFN mRNA accumulation was observed when purified T cells were treated with ionomycin or OKT3 alone (data not shown). Thus marked reduction in *lck* mRNA levels is an invariable consequence of stimulation protocols that activate T lymphocytes to produce lymphokines.

Activation of T lymphocytes alters the expression of pp56^{lck}

To examine the significance of reduction in *lck* mRNA expression at the protein level, we generated polyclonal rabbit antisera directed against a synthetic peptide encompassing residues 476–505 of murine pp56^{lck}. Figure 4 demonstrates radio-immunoprecipitation of pp56^{lck} from a murine T-cell lymphoma in which the *lck* gene is overexpressed (LSTRA; Marth *et al.*, 1985; Voronova and Sefton, 1986) using our antisera. The immunoprecipitation reaction can be specifically inhibited with micromolar concentrations of free peptide (Figure 4). This representative antiserum, lck-2102, reacts strongly with pp56^{lck} but not with pp60^{c-src} (J.D.Marth, J.A.Cooper, E.G.Krebs and R.M.Perlmutter, unpublished data). For use in immunoblotting,

the reactive component of *lck*-2102 was affinity purified (see Materials and methods). This reagent permits the detection of pp56^{lck} on human lymphoid cell membranes as shown in Figure 5. Note that the immunoassay is completely inhibited by exogenous peptide, and that the amount of detectable pp56^{lck} correlates well with relative *lck* mRNA levels determined for these same cell lines (Table I). A 52-kd immunoreactive species is occasionally observed (Figure 5) and likely results from partial proteolysis of pp56^{lck}.

Using the affinity-purified *lck*-2102 reagent, pp56^{lck} is clearly identifiable in immunoblot analyses of SDS-PAGE-separated membrane proteins derived from resting human T lymphocytes. Figure 6 shows that within 6 h after combined Con A and PMA stimulation, pp56^{lck} has disappeared and does not reappear until >48 h later. Although it cannot be appreciated in this experiment, we have found that PMA treatment of resting T cells results in the rapid (within 30 min) appearance of a second immunoreactive species migrating at ~62 kd in SDS gels. This second species may result from phosphorylation of pp56^{lck} by protein kinase C, in accord with the observations of Casnellie and Lamberts (1986) who demonstrated higher mol. wt forms of the LSTRA kinase induced by PMA treatment of LSTRA cells, and showed that these larger forms contained increased levels of phosphoserine. Whether pp56^{lck} is completely degraded after lymphocyte stimulation or rather modified is currently being investigated. Regardless of the precise mechanism responsible, lymphocyte activation is clearly associated with the early loss of immunoreactive pp56^{lck}.

This phenomenon is presented in more detail in Figure 7, once again associating down-regulation of pp56^{lck} with combinations of signals that provoke lymphokine expression. Clearly although either Con A or PMA alone induced some diminution in the level of pp56^{lck}, complete elimination of pp56^{lck} is only achieved with the combination of PMA and either Con A or OKT3. Thus specific loss of membrane-associated pp56^{lck}, like the attenuation of *lck* transcripts, is a characteristic and early feature of the T-cell activation sequence.

Discussion

The mammalian *lck* gene encodes a myristylated membrane-associated 56-kd protein tyrosine kinase that is specifically expressed in lymphoid cells (Marchildon *et al.*, 1984; Marth *et al.*, 1985). Close homology between *lck* and *src* at the nucleotide level, and between pp56^{lck} and pp60^{src} at the protein level, strongly suggests that these genes (and other members of the *src* gene family) may mediate similar functions. The *lck* gene is rearranged and overexpressed in a subset of murine lymphoid tumors, in one case as a result of retroviral insertion (Marth *et al.*, 1985; Voronova and Sefton, 1986; A.M. Garvin, J.D. Marth and R.M. Perlmutter, unpublished data), and these malignant cells contain high levels of active pp56^{lck} (Casnellie *et al.*, 1982). In addition, the human *lck* gene is located at a site of frequent chromosomal abnormalities (1p32-35) in neuroblastomas and non-Hodgkin lymphomas (Marth *et al.*, 1986). Thus there is reason to believe that pp56^{lck} may normally regulate aspects of lymphocyte growth and/or differentiation.

Resting human T lymphocytes contain appreciable levels of *lck* mRNA. This observation is in accord with studies of other *src* family members and of *src* itself: all are expressed in fully differentiated cells. The *src* gene transcript and pp60^{c-src} are present at high levels in neurons, a population with little growth potential (Brugge *et al.*, 1985), and increases in *c-src* expression are associated with the acquisition of a mature post-mitotic

phenotype in the developing chick retina and cerebellum (Sorge *et al.*, 1984; Fults *et al.*, 1985). Similarly, the recently described *hck* gene, a member of the *src* gene family, is preferentially expressed in granulocytes (Ziegler *et al.*, 1987; Quintrell *et al.*, 1987), cells with an *in vivo* lifespan measured in hours (Boggs, 1967).

Lymphocytes provide an ideal proving ground for theories regarding the function of *src*-family kinases since they can be isolated in quantity as resting cells, and can be readily activated using physiologic or near-physiologic stimuli (reviewed in Palacios, 1982; Larsson *et al.*, 1980). We have demonstrated that stimuli which lead to lymphokine production in normal T lymphocytes specifically attenuate the expression of *lck* mRNA and of pp56^{lck}.

Control of *lck* mRNA levels

Quantitative analysis of *lck* transcripts in purified populations of stimulated human T lymphocytes yields an intriguing biphasic profile: early suppression (to less than one copy per cell) followed by relative induction (Figure 1, Table I). There are two particularly important features of this response. First, the early loss of *lck* transcripts in stimulated T cells is synergistically induced by combinations of agents that also synergistically stimulate lymphokine production, and second, the *lck* response is the inverse of that observed for IL-2 or IFN mRNA (Figures 1 and 2).

In T lymphocytes, as in other cell types, growth stimulation is associated with rapid accumulation of *c-fos* and subsequently *c-myc* transcripts, believed to be essential for progression through the cell cycle. These transcripts are induced, however, by either mitogen or phorbol esters singly as well as in combination (Granelli-Piperno *et al.*, 1986; Kern *et al.*, 1986; and unpublished data). Importantly, Con A and PMA alone stimulated proliferation in our experiments even though neither of these agents by themselves triggered reductions in *lck* mRNA levels (Figure 2 and data not shown). Stimulation of [³H]thymidine incorporation by Con A alone likely results from residual contamination of our T-cell preparations with accessory cells. Apparently this contamination, although adequate to promote progression into the S phase of the cell cycle, is not sufficient to permit significant lymphokine production or to induce reductions in *lck* mRNA levels (Figure 2). Thus the observed decline in *lck* mRNA levels does not correlate directly with increased replicative activity, a finding that is reinforced by the high levels of *lck* mRNA observed in actively proliferating T-lymphoma lines (Table I). Indeed these neoplastic lines frequently contain increased levels of *lck* mRNA, perhaps analogous to the increase in *fgr* transcript abundance induced by Epstein-Barr virus transformation of human B lymphocytes (Cheah *et al.*, 1986).

To our knowledge, the biphasic expression profile of *lck* mRNA in the activation of T lymphocytes is unique. No other transcripts, and certainly no proto-oncogene transcripts, are present in resting T cells and decline promptly after activation (Granelli-Piperno *et al.*, 1986; Reed *et al.*, 1986). Furthermore, *lck* mRNA levels are reciprocally related to levels of IL-2 and IFN mRNAs whether these are induced by Con A/PMA, PHA/PMA, OKT3/PMA, ionomycin/PMA or SEA/PMA regimens (Figure 3). These observations implicate the *lck* gene product in the process of lymphokine production by activated T lymphocytes.

Reductions in the levels of *lck* mRNA may reflect changes in mRNA stability or in the transcriptional activity of the *lck* gene. While we favor the latter view, a complete analysis awaits simultaneous measurements of *lck* mRNA turnover and of *lck* transcription. In order to understand the physiologic significance

of this alteration in mRNA levels, however, we have instead focused attention on changes in pp56^{lck} induced by lymphocyte activation.

Two signals are required for elimination of pp56^{lck} from T cells

Summarizing the experiments presented in Figures 6 and 7, it is clear that Con A plus PMA treatment provokes substantial elimination of pp56^{lck} from lymphocyte membranes within 2 h, in contrast to either PMA alone or Con A alone which both leave significant levels of pp56^{lck} protein. The Con A signal can be replaced by OKT3 (Figure 7), indicating again that elimination of pp56^{lck} is a correlate of lymphocyte activation. This rapid disappearance of pp56^{lck} documented using immunoblotting does not represent redistribution of the kinase within the cell since experiments using cytosolic proteins or whole cell preparations did not yield any additional pp56^{lck} immunoreactive materials (data not shown). It is widely believed that PMA by itself maximally activates protein kinase C at physiological calcium concentrations (Kikkawa and Nishizuka, 1986). For this reason, the combined effect of mitogens together with PMA on pp56^{lck} (Figure 7) cannot easily be explained by invoking additive activation of protein kinase C. Thus Con A and PMA are probably contributing two independent signals, both of which are required for pp56^{lck} elimination.

Although high-level expression of protein tyrosine kinases is in some circumstances incompatible with cell growth (an example is the lethal effect of *v-abl* on murine 3T3 fibroblasts; Ziegler *et al.*, 1981), suppression of pp56^{lck} expression is apparently not a prerequisite for lymphocyte proliferation since, again, our Con A-stimulated T lymphocytes undergo significant DNA synthesis as judged by [³H]thymidine incorporation without complete elimination of pp56^{lck} expression (Figures 3 and 7 and data not shown). In addition, actively proliferating T-lymphoma cell lines have readily detectable levels of pp56^{lck} protein (Figure 5).

Alterations in pp56^{lck} expression may mediate T-lymphocyte activation

Examination of the level of *lck* transcripts in stimulated lymphocytes produced a straightforward result: only combinations of stimuli capable of inducing lymphokine expression could successfully down-regulate *lck* expression. Surprisingly, the levels of pp56^{lck} do not correlate well with simultaneously determined levels of *lck* mRNA. Thus pp56^{lck} disappears after activation hours before mRNA levels reach their nadir, and pp56^{lck} protein does not reappear until at least 36 h after *lck* mRNA levels have already rebounded (Figures 1, 2, 6 and 7). These results sound a cautionary note regarding the use of mRNA abundance measurements as indices of gene function.

Since the extent of elimination of pp56^{lck}, like the degree of reduction in *lck* mRNA transcript levels, appears to correlate with the efficacy of the activating stimulus applied to resting T lymphocytes (Figures 6 and 7), it is possible that pp56^{lck} is somehow required for the maintenance of a resting phenotype. For example, pp56^{lck} may be constitutively active in resting cells, generating phosphorylated substrates that aid in maintaining quiescence.

The behavior of other members of the *src* gene family is somewhat consistent with this model. All of the seven known *src*-family members are expressed in fully differentiated cells. In cases where it has been possible to measure changes in gene expression during development, they are maximally expressed in post-mitotic cells (Sorge *et al.*, 1984; Fufts *et al.*, 1985; Simon *et al.*, 1985; Vardimon *et al.*, 1986; Quintrell *et al.*, 1987).

Similarly, introduction of *v-src* into PC12 pheochromocytoma cells induces neurite outgrowth, a feature of more mature neuronal cells (Alema *et al.*, 1985). By analogy pp56^{lck} may ordinarily promote the imposition of lymphocytoid features on stimulated lymphoblasts, and hence specific suppression of pp56^{lck} expression may be required to permit T-cell activation. With these facts in mind, deliberate overexpression of *lck* in activated T lymphocytes might inhibit or even reverse some aspects of the T-cell activation sequence.

Lymphocyte activation has been reported to both augment and attenuate protein tyrosine kinase activity (e.g. Fischer *et al.*, 1984; Piga *et al.*, 1984), probably because of technical limitations in measurements of tyrosine kinase activity *in vitro* (Hunter and Cooper, 1985). Furthermore, our studies of lymphocyte membranes reveal that pp56^{lck} does not comigrate with the dominant p58 or p64 phosphoproteins previously identified as protein tyrosine kinases (or tyrosine kinase substrates) in T cells (data not shown). Like Casnellie and Lamberts (1986), we have also observed the PMA-induced appearance of an *lck* immunoreactive species of higher apparent mol. wt (p62) in T lymphocytes (data not shown). While it is possible that this p62 protein is in fact an altered version of pp56^{lck}, a definitive identification of this molecule will require purification and structural analysis. Despite these complexities, it is attractive to view alterations in the activity of pp56^{lck} or in its substrate selection as an important feature of lymphocyte activation.

Two signal systems and src-like protein tyrosine kinases

Many of the cell types known to contain high levels of *src*-family tyrosine kinases exhibit secretory responses that are maximally induced by combinations of calcium ionophore and phorbol ester (reviewed in Kikkawa and Nishizuka, 1986). In platelets, which reportedly express pp60^{c-src} (Golden *et al.*, 1986), activation of protein kinase C acts synergistically with increases in intracellular calcium concentration to effect serotonin release (Kaibuchi *et al.*, 1983; Yamanishi *et al.*, 1983). Similarly, neutrophils, cells that are nearly unique in expressing high levels of the *src*-like protein tyrosine kinase *hck* (Ziegler *et al.*, 1987; Quintrell *et al.*, 1987), are maximally stimulated to degranulate by a synergism between calcium ionophore and diacylglycerol (Kajikawa *et al.*, 1983). In fact, this same two-stimulus requirement, which mimics appropriate hormonal stimulation and also leads to granule membrane fusion and exocytosis, has been observed in virtually all secretory cells including those of adrenal, myeloid, neuronal and pancreatic origin (Kojima *et al.*, 1983; de Pont and Fleuren-Jakobs, 1984; Katakami *et al.*, 1984; Tanaka *et al.*, 1984; Zawulich *et al.*, 1983). In some cotransformation systems expression of pp60^{v-src} appears to induce growth factor secretion (Adkins *et al.*, 1984). Furthermore, a significant proportion of cellular pp60^{c-src} in adrenal medullary cells reportedly resides in the membranes of secretory vesicles (Parsons and Cruetz, 1986).

We find the association between two-signal activation systems and *src*-family gene expression intriguing in light of our demonstration that pp56^{lck} is completely eliminated from T cells exposed to these same stimuli. Activation of T lymphocytes is associated with rapid changes in membrane structure, fluidity and ion permeability (reviewed in Ashman, 1984). Components of the submembranous cytoskeleton (e.g. lipocortin, talin and vinculin) are prominent among substrates for pp60^{v-src} (Erikson and Erikson, 1980; Sefton *et al.*, 1981; Saris *et al.*, 1986; DeClue and Martin, 1987) and alterations in these and other membrane surface modulating components were observed early in the study of *v-src* effects in cell culture (Edelman and Yahara, 1976). Our

observations suggest that an important function of the *src*-like protein tyrosine kinases may be to catalyze modifications in membrane structure of a type that in many cells facilitates the production and secretion of regulatory molecules, e.g. cytokines. Experiments designed to probe the relationship between T-cell activation, pp56^{lck} activity and lymphokine production may therefore prove especially revealing with regard to the cell physiology of the membrane-associated protein tyrosine kinases.

Materials and methods

Isolation and stimulation of human T lymphocytes

Peripheral blood mononuclear cells were obtained from normal adult donors by multiple Ficoll-Hypaque centrifugations, and T cells were isolated by treatment with a monoclonal antibody and complement reagent, T cell Lymphokwik (One Lambda, Los Angeles, CA) as previously described by Wilson *et al.* (1986). The reagent includes a monoclonal antibody directed against HLA-DR and therefore depletes activated T cells as well as monocytes, B cells and granulocytes. Resulting cell populations were >98% T cells as judged by immunofluorescence with monoclonal antibody 9.6 (anti-CD2, SRBC receptor) provided by Dr John Hansen and had negligible thymidine uptake values when incubated in basal medium consisting of RPMI 1640 with 5% human AB serum (Wilson *et al.*, 1986). Stimulation of purified T cells were performed using Con A (Pharmacia Fine Chemicals, Piscataway, NJ), PMA (Sigma Chemical Co., St Louis, MO), PHA (Sigma), ionomycin (Calbiochem, San Diego, CA), SEA (Dr Reginald Bennett, Food and Drug Administration, Washington, DC) and OKT3 (from ascitic fluid harvested in our laboratories) in described concentrations (see Figure legends). Thymidine uptake was determined as previously described by Wilson and Remington (1979).

Membrane fractionation

T cells (up to 7×10^7) were harvested by low-speed centrifugation to remove culture media. Cells were then washed once in phosphate-buffered saline (PBS, pH 7.4) and resuspended in 5 ml of hypotonic lysis buffer containing 20 mM Hepes (pH 7.4), 10 mM EGTA, 2 mM EDTA and 2 mM DTT. Homogenization was achieved with 20 strokes in a Dounce homogenizer and the homogenate was then centrifuged at 40 000 g at 4°C for 30 min to separate cellular membranes from cytosolic components. Membranes were resuspended in hypotonic lysis buffer prior to SDS-PAGE and immunoblotting analyses in experiments using total membrane components. Cytosolic components were obtained by acetone precipitation in the presence of sucrose (5% w/v) followed by centrifugation at 12 000 g at 4°C. In all experiments protein quantitation was done by the method of Bradford (1976) and supplemented prior quantitation by cell counting.

Antibody production and characterization

Antisera specific for residues 476–505 of murine pp56^{lck} (CWKERPEDRPT-FDYLRSLDDFFFTATEGQY) were obtained through immunization of New Zealand white rabbits with unconjugated peptide, or with peptide coupled to bovine serum albumin (BSA) using glutaraldehyde (Reichlin, 1980) at a mass ratio of 1:10 (peptide:protein). Immunizations using 100 µg of free peptide or 1 mg of peptide–BSA conjugate were initially performed in complete Freund's adjuvant (Gibco, Grand Island, NY), and thereafter twice in incomplete Freund's adjuvant allowing at least 7 days between immunizations. At 6 weeks, sera were obtained and assayed for their ability to immunoprecipitate pp56^{lck} from LSTRA cell membranes that had been labelled by *in vitro* autophosphorylation as described by Casnellie *et al.* (1982). Immunoprecipitations were performed as described previously (Marth *et al.*, 1985) except using protein A Sepharose as an immunoadsorbent. Although specific immunoprecipitation was observed with all antisera, in no case could we quantitatively immunoprecipitate all of the ³²P-labelled pp56^{lck}. This phenomenon may result from the low overall affinity of our antisera (see Figure 5). Antiserum lck-2102 was affinity purified by covalently linking the free peptide to an Affi-gel 15 support resin (BioRad, Richmond, CA) according to the manufacturer's instructions. Binding of specific antibodies was performed in 0.1 M MOPS buffer at pH 7.4. The antibodies were subsequently eluted in 50 mM glycine at pH 2.5.

RNA preparation and analysis

Using the guanidine thiocyanate method (Chirgwin *et al.*, 1979), total RNA was isolated from tissues, cell lines and T-cell cultures. RNA blotting was performed as previously described (Marth *et al.*, 1985). RNA blots were analyzed using a human *lck* cDNA probe that was obtained by screening a human peripheral blood mononuclear cell cDNA library (R.M. Perlmutter, R. Peet, J.D. Marth, D.B. Lewis, S.F. Ziegler and C.B. Wilson, unpublished). The human IFN and IL-2 probes were the kind gifts of Drs Patrick Gray (Genentech) and Alf Larson (Immunex) respectively.

Quantitation of RNA levels by solution hybridization

In a modification of the method devised by Durnam and Palmiter (1983), *lck* mRNA levels were assayed in total RNA samples by solution hybridization using *lck* complementary RNA probes synthesized *in vitro* with radiolabeled [³⁵S]-UTP (New England Nuclear). These probes, either a 0.6-kb *Bam*HI–*Eco*RI fragment of the human *lck* cDNA or a 0.3-kb *Bgl*II–*Bam*HI fragment of the murine *lck* cDNA (Marth *et al.*, 1985), and their complementary strands were prepared using the pBS vector (Stratagene, San Francisco, CA). Hybridization at 68°C overnight was performed in a reaction volume of 20 µl containing 20 mM Tris (pH 7.4), 0.6 M NaCl, 4 mM EDTA, 0.1% SDS, 10 mM DTT, 25% formamide and 10–40 µg of total cellular RNA. After hybridization, samples were each treated with 2 µg RNase T₁ and 40 µg of RNase A (Sigma) in 1 ml in the presence of 100 µg of salmon sperm DNA for 45 min at 37°C. The RNase insensitive material was then precipitated onto glass-fiber filters (GF/C, Whatman) with 100 µl of 6 M trichloroacetic acid (TCA) and residual ³⁵S activity detected by scintillation counting. Hybridization levels of samples were compared with those of a standard curve constructed using known amounts of the *in vitro* synthesized *lck* 'sense' RNA. The results are expressed as picograms of *lck* mRNA (total size: 2300 bases) per microgram of total RNA. Expression as molecules of *lck* mRNA/cell can be obtained by assuming that our observed yields of total RNA/lymphoid cell probably reflect ~50% recovery based upon previously published results (Kronenberg *et al.*, 1983). The detection limit of our solution hybridization technique is 8 pg of *lck* mRNA, and hence we can detect less than one copy per cell of *lck* transcripts by assaying 20 µg of total RNA (resting human T cells yield ~1 pg total RNA per cell by our analysis). Samples were typically analyzed at least three times with <5% variance between reactions.

Immunoblotting of pp56^{lck}

Protein samples were denatured in SDS-PAGE sample buffer and electrophoresed on 12.5% SDS-polyacrylamide gels. Proteins were transferred onto nitrocellulose (Biorad) for 4 h at 60 V in a buffer containing 25 mM Tris, 192 mM glycine and 15% methanol at pH 9.0. Following transfer, protein levels remaining in the gel were visualized by Coomassie Blue staining, both to verify transfer and to ascertain that relative protein concentrations were indeed similar in all sample lanes. The nitrocellulose blot was pretreated with BLOTTO (5% non-fat dry milk and 0.05% Tween-20 in PBS) for 1 h at room temperature (Johnson *et al.*, 1984), prior to overnight incubation at room temperature with 15 µg/ml of affinity-purified lck-2102 in BLOTTO. Blots were subsequently washed three times for 30 min in BLOTTO and then incubated with a facilitating antiserum (alkaline phosphatase conjugated goat anti-rabbit immunoglobulin from BioRad) diluted 1:3000 in BLOTTO for 1 h. Following facilitation, the blots were washed three times in BLOTTO, twice in distilled water and twice in TBS (20 mM TRIS 7.5, 100 mM NaCl). Blot development was performed with the BCIP/NBT color development reagents (BioRad) in 100 mM Tris–HCl (pH 9.5), 100 mM NaCl and 50 mM MgCl₂.

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