Regulation of the enzymatic function of the lymphocyte-specific tyrosine protein kinase p56^ck by the non-catalytic SH2 and SH3 domains

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The enzymatic activity of the lymphocyte-specific tyrosine protein kinase p56^ck appears to be tightly regulated by phosphorylation of the conserved carboxy-terminal tyrosine residue 505. Indeed, substitution of this tyrosine residue by a non-phosphorylatable phenylalanine results in a constitutively activated version of p56^ck that can transform rodent fibroblasts. In this report, we evaluate the functions of the conserved non-catalytic Src homology (SH) domains 2 and 3 of p56^ck in the regulation of its enzymatic activity in NIH3T3 fibroblasts. We found that deletion of the SH2 or, to a lesser extent, the SH3 domain of p56^ck resulted in an increase in the tyrosine protein kinase activity of wild-type Lck polypeptides. The SH2 domain (but not the SH3 domain) was also required for full oncogenic transformation by Lck molecules activated through removal of tyrosine 505. This effect did not appear to be the result of a diminution of the enhanced catalytic activity of F505 Lck polypeptides. However, it may relate to the findings that the SH2 domain can bind and possibly enhance phosphorylation of specific phosphotyrosine-containing proteins. Taken together, these observations imply roles for the non-catalytic SH2 and SH3 domains in the regulation of the catalytic activity of p56^ck. They suggest that the enzymatic function of this Src-related polypeptide is physiologically repressed by processes dependent on the presence of the SH2 and SH3 sequences. Moreover, they indicate that the SH2 domain also plays a positive role in the function of activated p56^ck molecules in NIH3T3 cells.

Introduction

The lck gene product, p56^ck, is a 56-kDa Src-related tyrosine protein kinase expressed exclusively in cells of lymphoid lineage, most predominantly in T lymphocytes (Marth et al., 1985; Voronova & Sefton, 1986; Veillette et al., 1987; for reviews, see Veillette & Bolon, 1989; Sefton, 1991; Veillette et al., 1991) Accumulating data suggest that this internal membrane enzyme participates in the transduction and amplification of cell-surface receptor-induced signals in T cells. Indeed, through its unique amino-terminal domain, p56^ck physically associates with the cytoplasmic tails of the CD4 and CD8 T-cell-surface antigens, which respectively recognize class II and class I major histocompatibility complex (MHC) determinants on antigen-presenting cells (Rudd et al., 1988; Veillette et al., 1988a; Shaw et al., 1989; 1990; Turner et al., 1990). Moreover, the observation that antibody-mediated aggregation of CD4 rapidly stimulates the tyrosine protein kinase activity of p56^ck (Veillette et al., 1989a; Luo & Sefton, 1990a) and causes a prompt increase in intracellular tyrosine protein phosphorylation (Veillette et al., 1989a–c; Luo & Sefton, 1990a) shows that p56^ck can transduce intracellular tyrosine phosphorylation signals for CD4 and CD8. In addition, recent studies indicate that p56^ck may participate in other signal transduction pathways, as suggested by the finding of physical complexes involving Lck and other T-cell surface molecules such as the b chain of the interleukin 2 receptor (Hatakeyama et al., 1991) or glycophosphatidylinositol (GPI)-anchored molecules like Thy-1 (Stefanova et al., 1991).

A further characterization of the processes regulating the enzymatic function of p56^ck is critical for a better understanding of its role in normal cellular physiology. Previous studies have revealed that one key mechanism of regulation is tyrosine phosphorylation. p56^ck is extensively phosphorylated in vivo at a conserved carboxy-terminal tyrosine residue, tyrosine 505 (Tyr-505) (Amrein & Sefton, 1988; Marth et al., 1988; Abraham & Veillette, 1990; Abraham et al., 1991). Although the exact stoichiometry of Tyr-505 occupancy as well as the identity of the tyrosine protein kinase phosphorylating this site remain to be established, site-directed mutagenesis experiments strongly suggest that phosphorylation at Tyr-505 negatively regulates the enzymatic activity of p56^ck (Amrein & Sefton, 1988; Marth et al., 1988; Abraham & Veillette, 1990; Abraham et al., 1991). Indeed, substitution of Tyr-505 by a non-phosphorylatable phenylalanine results in a constitutively activated version of p56^ck which is capable of oncogenic transformation of rodent fibroblasts. While an alternative tyrosine residue of p56^ck, tyrosine 394 (Tyr-394), is the site phosphorylated during in vitro autophosphorylation reactions, this residue is not normally phosphorylated in vivo. Nevertheless, significant in vivo occupancy of Tyr-394 is observed in the context of activation of p56^ck by either mutation of Tyr-505 or antibody-mediated aggregation of CD4 (Veillette et al., 1989b; Luo & Sefton, 1990a). In both instances, replacement of Tyr-394 by phenylalanine prevents p56^ck activation (Abraham & Veillette, 1990; Veillette & Fournel, 1990), implying that Tyr-394 phos-

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phosphorylation may be involved in the positive regulation of the Lck enzymatic activity.

The two conserved non-catalytic domains of Src-related polypeptides, termed Src homology (SH) domains 2 and 3, have also been proposed to contribute to the regulation of these enzymes (for reviews, see Pawson, 1988; Cantley et al., 1991; Koch et al., 1991). The Src homology 3 (SH3) domain is a region of approximately 50 amino acid residues located beyond the amino-terminal unique domain of Src-related enzymes. As SH3 sequences are also found in cytoskeletal constituents such as the yeast actin-binding protein and fodrin, it is postulated that this domain mediates interactions with the cytoskeleton. The SH2 domain of Src-related tyrosine protein kinases is a sequence of roughly 100 amino acids located between the SH3 domain and the carboxy-terminal catalytic domain. This motif is also present in a variety of other signal transducers and amplifiers such as phospholipase C-γ, the 85-kDa subunit of the phosphatidylinositol (PI) 3' kinase and the GTPase-activating protein (GAP) of p21ras. SH2 domains can directly bind phosphotyrosine-containing proteins in vitro, implying that these non-catalytic sequences contribute to or regulate enzyme–substrate interactions. Additionally, it has been proposed that SH2 may bind intramolecularly to the phosphorylated carboxy-terminal tyrosine residue of Src-related enzymes, thereby possibly contributing to the maintenance of the low baseline catalytic activity of these products (Cantley et al., 1991; Koch et al., 1991). Consistent with these two proposed functions, mutations of the Src protein within SH2 can both elevate and reduce its catalytic activity, depending on the type of mutation created and the background in which it is introduced (Hirai & Varmus, 1990; O'Brien et al., 1990).

To evaluate further the functions of SH2 and SH3, we examined the contribution of these regions to the regulation of the tyrosine protein kinase activity of p56

We found that deletion of the SH2 sequence or, to a lesser degree, the SH3 sequence resulted in a measurable increase in Lck catalytic activity. Our studies also indicated that SH2 (but not SH3) provides a function(s) required for an activated version of p56

to fully transform rodent fibroblasts. This positive role may relate to the ability of the SH2 domain to bind and enhance tyrosine phosphorylation of certain cellular substrates such as the GAP-associated p62.

Results

Generation of p56

Enzymatically activated versions of Src-related enzymes (such as F505 p56
) are typically capable of oncogenic transformation of rodent fibroblasts (for reviews, see Hunter & Cooper, 1983; Veillette & Bolin, 1989). Hence, this property has been widely utilized in structure–function analyses aimed at understanding the regulation of these products. To evaluate the functions of the SH2 and SH3 domains of p56
, the murine lck cDNA NT18 (Marth et al., 1985) was altered by site-directed mutagenesis to create additional restriction endonuclease recognition sites allowing complete deletion of either of these regions in p56
(Figure 1; see Materials and methods). The boundaries of the SH2 and SH3 domains were defined according to Koch et al. (1991). To eliminate a valine-to-asparagine amino acid substitution created at position 66 of the SH3 Lck mutant, the deleted DNA was subjected to a second round of oligonucleotide-directed mutagenesis in order to regenerate the wild-type valine residue. Double mutants carrying the SH2 or SH3 domain deletion with a Tyr-505 to phenylalanine 505 substitution (F505 Lck) were also engineered.

Expression of mutant lck cDNAs in mouse NIH3T3 fibroblasts

Wild-type (wt), F505, ΔSH2 wt, ΔSH2 F505, ΔSH3 wt and ΔSH3 F505 lck cDNAs were cloned in the retroviral vector pLXSN and transfected in the ψ-2 retroviral packaging cell line. Mouse NIH3T3 cells were then infected with the appropriate viral supernatants and selected for growth in medium containing the aminoglycoside G418. Preliminary data indicated that, while the wt, F505, ΔSH3 wt and ΔSH3 F505 Lck proteins were efficiently expressed in these cells, the levels of expression of the ΔSH2 Lck polypeptides were significantly lower (data not shown). To ensure that cells with overlapping ranges of Lck protein were examined in our analyses, multiple monoclonal cell lines were generated from the polyclonal ΔSH2 wt and ΔSH2 F505 Lck-expressing cell populations using the trypsin filter paper method (Kniecik & Shalloway, 1987). The cell clones analysed in our studies were chosen based on levels of Lck expression.

To begin characterizing the mutant Lck polypeptides, cells were lysed in boiling sample buffer and lysates corresponding to equivalent cell numbers resolved on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels. A specific anti-Lck immunoblot was then performed using a rabbit antiserum raised against a synthetic peptide corresponding to amino acids 29–54 of the murine p56
sequence (Figure 2). Whereas wt (lane 1) and F505 (lanes 2–4) Lck polypeptides migrated at 56 kDa on these gels, the SH2 (lanes 5 and 6) and SH3 (lanes 7 and 8) domain deletion mutants were resolved at approximately 42 and 51 kDa respectively. Hence, these polypeptides were termed p42
and p51
.

Effects of SH2 or SH3 domain deletion on the biochemical properties of Lck

To test the biochemical function of the SH2 and SH3 Lck mutants, the effects of their expression on cellular
phosphotyrosine levels were assessed by anti-phosphotyrosine immunoblotting (Figure 3). In agreement with previous reports (Amrein & Seflon, 1988; Abraham & Veilleux, 1990), we noted that expression of F505 p56\(^{\text{a}}\) (Figure 3a, lanes 2–4), but not of wt Lck (lane 1), resulted in a marked increase in intracellular tyrosine protein phosphorylation. Expression of ΔSH2 wt (lane 5) or ΔSH2 F505 (lane 6) Lck also conferred elevated levels of cellular phosphotyrosine to NIH3T3 cells. The effects of expression of the two SH2 mutants on tyrosine protein phosphorylation were found to be quantitatively and qualitatively similar, as indicated by the analysis of panels of monoclonal cell lines expressing overlapping levels of these polypeptides (Figure 3b; compare lanes 6–10 with lanes 11–14). Moreover, these changes were similar to those induced by expression of equivalent amounts of F505 p56\(^{\text{a}}\) (Figure 3b, lanes 3–5), with the exception of a 62-kDa substrate (p62) which appeared to have a slightly faster electrophoretic mobility (migrating at approximately 61 kDa) in cells expressing the SH2 deletion mutants. In addition, cells containing the SH2 Lck deletion mutants expressed a novel 42-kDa tyrosine-phosphorylated polypeptide likely to represent p42\(^{\text{a}}\).

Introduction of ΔSH3 F505 Lck (Figure 3a, lane 8) in NIH3T3 cells resulted in changes in intracellular tyrosine protein phosphorylation that were similar to those induced by F505 p56\(^{\text{a}}\) (lanes 2–4). Moreover, unlike cells expressing wt Lck (lane 1), fibroblasts expressing ΔSH3 wt Lck (lane 7) showed slightly elevated levels of phosphotyrosine.

The 56-kDa tyrosine phosphorylation substrate (p56) which was formerly proposed to represent tyrosine-phosphorylated p56\(^{\text{a}}\) (Abraham & Veilleux, 1990) was also detected in cells expressing ΔSH2 and ΔSH3 Lck mutants. As fibroblasts do not express endogenous p56\(^{\text{a}}\), this finding implied that another tyrosine-phosphorylated substrate having the same apparent molecular weight as p56\(^{\text{a}}\) is expressed in these cells. The nature of this product remains to be established.

Effects of expression of SH2 or SH3 domain deletion mutants of Lck on growth properties of NIH3T3 cells

We also examined the effects of expression of the SH2 and SH3 Lck deletion mutants on the growth proper-
ties of NIH3T3 cells (Figure 4; Table 1). Whereas cells expressing wild-type p56Lck (Figure 4a) had a morphology similar to that of parental NIH3T3 fibroblasts (data not shown), those containing F505 p56Lck (Figure 4d) exhibited a typical transformed morphology. Indeed, these cells were rounded, refractile and showed multiple neuron-like processes. Cells expressing equivalent levels of ΔSH2 wt Lck (Figure 4b), ΔSH2 F505 Lck (Figure 4e) or ΔSH3 F505 Lck (Figure 4f) had morphologies similar to that of cells expressing F505 p56Lck (Figure 4d). In contrast, ΔSH3 wt Lck-expressing fibroblasts had a fusiform appearance (Figure 4c). This morphology was accentuated by prolonged periods in cell culture (2–3 weeks).

The ability of the fibroblast lines to form foci in monolayers was also tested, as described in Materials and methods (Table 1; data not shown). As reported previously (Amrein & Sefton, 1988; Marth et al., 1988; Abraham & Veillette, 1990), fibroblasts expressing wt p56Lck failed to produce foci in monolayer cultures. In contrast, cells expressing F505 p56Lck formed large foci with a swirl-like appearance. Similar foci developed from cells expressing equivalent amounts of ΔSH2 wt Lck, ΔSH2 F505 Lck or ΔSH3 F505 Lck. ΔSH3 wt Lck-expressing fibroblasts also formed foci in monolayers, although these occurred at a significantly lower frequency and tended to be less well demarcated.

We finally evaluated the ability of these cells to grow in an anchorage-independent fashion (Table 1). White cells expressing wt p56Lck or ΔSH3 wt Lck were incapable of growth in soft agar, fibroblasts expressing F505 p56Lck or ΔSH3 F505 Lck formed large and numerous colonies when grown in this medium. However, it was consistently noted that smaller and less numerous colonies were produced by cells expressing the ΔSH2 wt and ΔSH2 F505 Lck polypeptides.

Table 1 Biological properties of mutant Lck polypeptides expressed in NIH3T3 cells

<table>
<thead>
<tr>
<th>Lck polypeptide</th>
<th>Morphology</th>
<th>Focus formation (%)</th>
<th>Soft agar (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type Lck</td>
<td>normal</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ΔSH2 wt Lck</td>
<td>neuronal-like</td>
<td>16</td>
<td>n.d.</td>
</tr>
<tr>
<td>ΔSH3 wt Lck</td>
<td>fusiform</td>
<td>n.d.</td>
<td>0.45</td>
</tr>
<tr>
<td>F505 Lck</td>
<td>neuronal-like</td>
<td>17.6</td>
<td>5.8</td>
</tr>
<tr>
<td>ΔSH2 F505 Lck</td>
<td>neuronal-like</td>
<td>14</td>
<td>n.d.</td>
</tr>
<tr>
<td>ΔSH3 F505 Lck</td>
<td>neuronal-like</td>
<td>2.6</td>
<td>7.0</td>
</tr>
</tbody>
</table>

n.d. = not done. Focus formation and growth in soft agar assays were conducted as described in Materials and methods. Soft agar colonies were counted after three weeks of culture. ‘Small’ indicates colonies typically containing 5–10 cells, while ‘large’ represents colonies containing more than 100 cells.

Peptide-mapping studies

To characterize further the mutant Lck polypeptides, their state of in vivo phosphorylation was examined by tryptic peptide-mapping studies (Figure 5). Cell lines were metabolically labeled with 32P, lysed in boiling SDS-containing buffer, and the Lck polypeptides recovered by immunoprecipitation with a specific anti-Lck serum. The Lck proteins were resolved by SDS-PAGE and subsequently transferred to Immobilon-P membranes. After identifying the phosphorylated Lck polypeptides by autoradiography, trypsin digestion was performed directly on membranes. The products of this reaction were subsequently separated on thin-layer cellulose plates by successive electrophoresis and chromatography, and detected by autoradiography.

As described previously (Amrein & Sefton, 1988; Marth et al., 1988; Abraham & Veillette, 1990), wild-type p56Lck (Figure 5a) was phosphorylated in vivo on a single major phosphopeptide containing Tyr-505 (pep-
Tide 2). Low levels of Tyr-394 phosphorylation (peptide 1) were also noted. In contrast, F505 p56$^{cx}$ (Figure 5d) was primarily phosphorylated at Tyr-394 and lacked phosphorylation of peptide 2, consistent with the absence of Tyr-505 in these molecules. The increased Tyr-394 occupancy on these molecules is thought to reflect and be critical for their enhanced enzymatic activity (Amrein & Sefton, 1988; Marth et al., 1988; Abraham & Veillette, 1990). Strikingly, ΔSH2 wt Lck (Figure 5b) was more phosphorylated at Tyr-394 than at Tyr-505. In addition, while primarily phosphorylated at Tyr-505, ΔSH3 wt Lck polypeptides (Figure 5c) also showed increased in vivo phosphorylation at Tyr-394, when compared with wt p56$^{cx}$ (Figure 5a). Like F505 p56$^{cx}$, ΔSH2 F505 (Figure 5e) and ΔSH3 F505 Lck (Figure 5f) were prominently phosphorylated at Tyr-394 in vivo.

p56$^{cx}$ is also phosphorylated in vivo on as yet unidentified amino-terminal serine residues which are mostly represented by the tryptic peptide 6 (Veillette et al., 1988b,c; 1989b; Amrein & Sefton, 1988; Abraham & Veillette, 1990). As indicated in Figure 5, this phosphopeptide was still detected in digests of Lck mutants lacking SH2 (Figure 5b and e) or SH3 (Figure 5f). Thus, it is reasonable to postulate that at least some serine phosphorylation sites are localized within the amino-terminal unique domain of p56$^{cx}$. The basis for the lack of their detection in ΔSH3 wt Lck (Figure 5c) is uncertain at this point.

Tyrosine phosphorylation of GAP-associated proteins

We have previously reported that the GAP of p21$^{wa}$ as well as its associated proteins p62 and p190 are tyrosine phosphorylated in NIH3T3 fibroblasts expressing F505 p56$^{cx}$ (Ellis et al., 1991a). In contrast, these polypeptides are not detectably phosphorylated on tyrosine in cells expressing transformation-defective Lck polypeptides (Ellis et al., 1991a). The data presented in Figure 3 show that a 62-kDa tyrosine phosphorylation substrate may have a faster electrophoretic mobility (61 kDa) in cells expressing the SH2 mutants of Lck. To evaluate if this polypeptide is related to the GAP-associated p62 and directly examine its state of tyrosine phosphorylation in cells expressing the ΔSH2 Lck polypeptides, anti-GAP immunoprecipitates obtained from equivalent amounts of cellular proteins were resolved by SDS-PAGE and subjected to antiphosphotyrosine immunoblotting (Figure 6, top). An anti-GAP immunoblot was also performed on parallel immunoprecipitates to ensure that GAP was efficiently recovered from all cells (Figure 6, bottom).

Whereas cells expressing the neomycin resistance marker alone (lane 1) or wt Lck (lane 2) did not demonstrate detectable tyrosine phosphorylation of GAP or GAP-associated proteins, cells expressing F505 p56$^{cx}$ (lanes 3 and 4) showed tyrosine phosphorylation of the 190-kDa and 62-kDa proteins associated with GAP. Similar polypeptides were tyrosine phos-
were generated and incubated with lysates of NIH3T3 cells expressing F505 p56\textsuperscript{co} cells expressing F505 p56\textsuperscript{co}. After resolution on SDS-PAGE gels, SH2-binding proteins were identified by anti-phosphotyrosine immunoblotting (Figure 7). This experiment revealed that the SH2 domain of p56\textsuperscript{co} (lane 3) can stably associate \textit{in vitro} with tyrosine phosphorylation substrates of apparent molecular masses of 120, 80, 62 and 56 kDa. In contrast, trpE alone (lane 1) or trpE–Lck SH3 (lane 2) failed to bind detectably any of these tyrosine phosphorylated products. Further studies showed that the SH2 regions of the v-Crk oncoprotein (lane 4), the GAP of p21\textsuperscript{ras} (lane 5) and p60\textsuperscript{c-src} (lane 6) also bound several tyrosine-phosphorylated proteins contained in cells transformed by F505 p56\textsuperscript{co}. The nature of these various SH2-binding polypeptides remains to be clarified.

\textbf{SH2-binding proteins are tyrosine phosphorylated in cells expressing SH2 deletion mutants of Lck}

We next wished to test if the lower oncogenic potential of ASH2 wt Lck and ΔSH2 F505 Lck resulted from a lack of tyrosine phosphorylation of SH2-binding proteins in these cells. Therefore, lysates of NIH3T3 cells expressing equivalent amounts of the various Lck polypeptides were incubated with trpE–Lck SH2 fusion proteins, and SH2-binding proteins identified as described earlier. Whereas cells expressing wt Lck (Figure 8, lane 1) or ΔSH3 wt Lck (lane 5) did not contain tyrosine-phosphorylated SH2-binding proteins, all other cell lines expressed phosphotyrosine-

\textbf{The SH2 domain of p56\textsuperscript{co} binds phosphotyrosine-containing proteins}

SH2 domains can bind phosphotyrosine-containing proteins \textit{in vitro} (reviewed in Koch et al., 1991). Accordingly, it has been proposed that these sequences may be critical for the phosphorylation of cellular substrates by activated tyrosine protein kinases. To test if the SH2 domain of p56\textsuperscript{co} binds tyrosine-phosphorylated proteins, trpE–Lck SH2 bacterial fusion proteins

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6}
\caption{Anti-GAP immunoprecipitates. GAP was immunoprecipitated from NIH3T3 cells expressing various Lck polypeptides using equivalent amounts of cellular proteins (125 μg). Tyrosine protein phosphorylation and GAP abundance were assessed by anti-phosphotyrosine (top) and anti-GAP (bottom) immunoblotting respectively. Lanes: 1, neomycin-resistant NIH3T3 cells; 2, wt Lck; 3 and 4, F505 Lck; 5, ΔSH2 wt Lck and 6, ΔSH2 F505 Lck. Exposure: top, 36 h; bottom, 12 h. The positions of the 190-kDa and 62-kDa GAP-associated proteins, as well as that of the 120-kDa GAP, are shown. The heavy chain of rabbit immunoglobulin is identified [Ig(H)].}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure7}
\caption{SH2-binding tyrosine-phosphorylated proteins. The ability of phosphotyrosine-containing proteins from NIH3T3 cells expressing F505 p56\textsuperscript{co} to associate with various bacterial fusion proteins was tested using an anti-phosphotyrosine immunoblot assay. Lanes: 1, trpE alone; 2, trpE–Lck SH3; 3, trpE–Lck SH2; 4, trpE–Crk SH2; 5, trpE–GAP SH2 + SH3 + SH2; 6, trpE–Src SH3 + SH2; and 7, total cell lysate from F505 p56\textsuperscript{co}-expressing NIH3T3 cells. Exposure: 6 h. The positions of the major tyrosine-phosphorylated proteins detected are shown on the left, while those of pre-stained molecular mass markers are indicated on the right.}
\end{figure}
containing polypeptides capable of binding the SH2 domain of p56\(^{6k}\) (lanes 2–4 and 6). The most prominent tyrosine phosphorylation substrate detected in these cells migrated at approximately 62 kDa. As mentioned above for the small GAP-associated polypeptides (p62 and p61), the migration of the SH2-binding 'p62' appeared slightly faster (approximately 61 kDa) in cells expressing the SH2 deletion mutants of Lck (lanes 3 and 4). Moreover, we noted that the 120-, 80- and 56-kDa SH2-binding polypeptides appeared less abundant in ASH2 Lck-expressing cells (lanes 3 and 4), in spite of the fact that the overall abundance of phosphotyrosine-containing proteins in these cells was similar to that of F505 p56\(^{6k}\)-expressing fibroblasts. As the 56-kDa SH2-binding protein was also detected in cells expressing the SH2 or SH3 deletion mutants of Lck, it is unlikely that this product is solely p56\(^{6k}\). Together, these results show that the SH2 domain of p56\(^{6k}\) is not absolutely required for tyrosine phosphorylation of SH2-binding proteins. Nonetheless, it is possible that the SH2 region may be required for more extensive and/or more stable phosphorylation of these putative Lck substrates.

Discussion

Our results show that the SH2 domain of p56\(^{6k}\) contains structural elements that are critical for the suppression of the tyrosine protein kinase activity of this polypeptide. Indeed, deletion of the entire SH2 region resulted in a striking elevation of the catalytic activity of wild-type Lck. This was indicated by the ability of this mutant to transform NIH3T3 cells and elevate cellular phosphotyrosine levels. Moreover, this polypeptide showed increased in vivo phosphorylation at Tyr-394, a marker of p56\(^{6k}\) activation. One should note that, as is the case for F505 p56\(^{6k}\) (Amrein & Sefton, 1988; Abraham & Veillette, 1990), we failed to demonstrate an elevation of the in vitro tyrosine protein kinase activity of this mutant over that of wt p56\(^{6k}\) (data not shown).

The oncogenic potential and biochemical function of ASH2 wt Lck were identical to those of ASH2 F505 Lck. This observation suggests that, in the absence of SH2, the carboxy-terminal tyrosine residue 505 is inefficient at negatively regulating the enzymatic activity of Lck. Interestingly, it has been proposed that SH2 may bind intramolecularly to the phosphorylated carboxy-terminal tyrosine residue of Src-related enzymes (Cantley et al., 1991; Koch et al., 1991). In this context, the physical interaction between SH2 of p56\(^{6k}\) and phosphorylated Tyr-505 would be presumed to result in an inactive enzymatic conformation. While this physical interaction has not been formally demonstrated for any of the Src-related protein kinases, the equivalence of the functions of ASH2 wt Lck and ASH2 F505 Lck observed herein provides indirect in vivo support for its existence. Conceivably, lack of the SH2 domain would 'open' the conformation of Lck and facilitate interactions between the catalytic domain and tyrosine phosphorylation substrates. Alternatively, through physical association with phosphorylated Tyr-505, SH2 may stabilize phosphorylation at this site, possibly by preventing the action of tyrosine-specific phosphatases.

In addition to its critical role in the down-regulation of the catalytic activity of p56\(^{6k}\), our data indicate that SH2 is required for full oncogenic transformation of murine fibroblasts by an activated version of p56\(^{6k}\) (F505 p56\(^{6k}\)). Indeed, while capable of inducing most of the biological and biochemical changes caused by F505 p56\(^{6k}\), ASH2 F505 Lck consistently allowed NIH3T3 cells to form fewer as well as smaller colonies when grown in soft agar. Since deletion of the SH2 domain does not measurably reduce the elevated specific enzymatic activity of F505 Lck, it may be proposed that SH2 modulates the interaction of activated Lck molecules with their substrates. However, we have found no evidence that SH2 is required for in vivo recognition of tyrosine phosphorylation substrates by p56\(^{6k}\). More specifically, this domain appears dispensable for tyrosine phosphorylation of SH2-binding proteins by activated Lck. One possibility is that, through binding phosphotyrosine-containing proteins, the SH2 domain allows quantitatively or qualitatively different tyrosine phosphorylation of these products. This may provide an explanation for the apparently faster electrophoretic migration of SH2- and GAP-associated 'p62' noted in cells expressing the ASH2 Lck mutants.

Previous studies have suggested that tyrosine phosphorylation of a 62-kDa polypeptide is critical for oncogenic transformation of fibroblasts by activated tyrosine protein kinases (Koch et al., 1989; Moran et al., 1990; Bouton et al., 1991; Brott et al., 1991). Alterations in SH2 subdomains I or II of v-Src or v-Fps, while not affecting the elevated tyrosine protein kinase activity of these polypeptides, completely prevent tyrosine phosphorylation of p62 (Koch et al.,...
1989; Moran et al., 1990; Bouton et al., 1991; Brott et al., 1991). This rather specific alteration parallels the markedly diminished oncogenicity of these mutants. Therefore, differences between the tyrosine-phosphorylated 'p62' present in cells expressing fully transforming Lck mutants and the tyrosine-phosphorylated 'p61' detected in cells containing the ASH2 Lck polypeptides may be of mechanistic relevance to the failure of the ASH2 Lck mutants to fully transform NIH3T3 cells. The reason for the previous lack of detection of a tyrosine-phosphorylated 'p61' in cells expressing the SH2 domain deletion mutants of v-Src and v-Fps is unclear (Koch et al., 1989; Moran et al., 1990; Bouton et al., 1991; Brott et al., 1991). This may reflect differences in the extent of the SH2 mutations created, in the nature of the tyrosine protein kinases studied or in the cell lines used for gene transfer. Further understanding of the significance of these various observations awaits molecular cloning of cDNAs encoding p62 and p61.

We also noted that deletion of SH3 resulted in a detectable increase in the activity of wild-type p56^*^.

This change was exemplified by the fusiform appearance of the cells expressing this mutant, their ability to form foci in monolayers and their elevated phosphotyrosine content. Moreover, the ASH3 wt Lck protein showed increased in vivo occupancy at Tyr-394. However, ASH3 wt Lck-expressing cells failed to grow in soft agar, indicating that they were not fully transformed. These observations are consistent with the previously reported effects of SH3 domain mutations on the function of other cytoplasmic tyrosine protein kinases such as c-Abl (Franz et al., 1989; Jackson & Baltimore, 1989). They also raise the possibility that SH3 may contribute to the maintenance of the low baseline activity of p56^*^.

However, one should note that, in contrast to the SH2 sequence, the SH3 region seems to be less critical for the control of the enzymatic activity of p56^*^. Indeed, regulation of p56^*^ by Tyr-505 was still operationally in the absence of the SH3 sequence.

The SH3 domain was completely dispensable for oncogenic transformation by activated F505 p56^*^.

Nevertheless, as SH3 is conserved in all Src-related tyrosine protein kinases, it remains likely that this sequence is involved in the physiological function of these products. Its presence in a number of cytoskeletal elements suggests the unverified possibility that SH3 allows interactions with the cytoskeleton (Koch et al., 1991). It will be interesting to test if SH3 is also dispensable for F505 p56^*^ to enhance T-cell responsiveness to antigen (Abraham et al., 1991).

In summary, our findings show that the non-catalytic SH2 domain is critical for the negative regulation of the enzymatic activity of p56^*^.

One possible explanation for this phenomenon is that binding of phosphorylated Tyr-505 to the SH2 domain maintains p56^*^ in an inactive conformation. In addition to its function in the negative regulation of p56^*^, our data also suggest that the SH2 region modulates substrate phosphorylation by activated Lck molecules. In contrast, although deletion of the SH3 motif resulted in a detectable increase in Lck tyrosine kinase activity, this sequence is not absolutely required for regulation of p56^*^ by Tyr-505. Moreover, it is dispensable for activated Lck to interact with substrates involved in cellular transformation.

**Materials and methods**

### Cells

NIH3T3 and ψ-2 packaging cells were grown in alpha minimal essential medium (MEM) (Stanners et al., 1971) supplemented with 10% fetal calf serum (FCS), glutamine, penicillin and streptomycin. NIH3T3 cells expressing the neo gene alone, wild-type Lck or F505 Lck have been described elsewhere (Abraham & Veillette, 1990).

**Site-directed mutagenesis**

The mutant lck complementary DNAs (cDNAs) were generated from the murine lck cDNA clone NT18 (Marth et al., 1985; kindly provided by Roger Perlmutter, University of Washington, Seattle, WA, USA). For deletion of SH3 (amino acid residues 67–122 inclusive; Figure 1), an EcoRV site was introduced by site-directed mutagenesis (Kunkel, 1985) at nucleotide 361 of NT18 using the oligonucleotide 5'-GGCCGATCCAGGTG-3'; in addition, an XhoI site was created at nucleotide 527 using the oligonucleotide 5'-TCAOGCTCGAGGCTGT-3' (generating NT18/EcorV-XhoI). Deletion of SH3 was accomplished by sequential digestion of NT18/EcorV-XhoI with XhoI, blunt ending with Klenow polymerase and dCTP/dTTP and digestion with EcoRV. After purifying the appropriate DNA fragment by agarose gel electrophoresis, religation of ASH3 lck was performed using T4 DNA ligase. Deletion of the region encoding SH3 was confirmed by DNA sequencing. To correct a valine-to-asparagine mutation created at position 66 as a result of the introduction of the EcoRV site, the ASH3 lck DNA was subjected to a second oligonucleotide-directed mutagenesis using the oligonucleotide 5'-CAGGCTACAAGGCTTG-3'. Restoration of the wild-type nucleotide sequence was verified by DNA sequencing. The BglII–NcoI fragment of NT18 lacking the SH3-encoding sequences was sequenced and substituted for the equivalent fragment in wild-type NT18. No other mutations were identified (data not shown).

For deletion of SH2 (amino acid residues 122–234 inclusive; Figure 1), NT18/XhoI DNA was serially digested with XhoI, blunt ended with Klenow polymerase in the presence of all deoxynucleotides, further digested with NcoI and then blunt ended with DNA polymerase 1 and dATP/dCTP. After purifying the appropriate fragment on an agarose gel, the ASH2 lck DNA was religated with T4 DNA ligase. Deletion of SH2-encoding sequences was confirmed by DNA sequencing. The BglII–BglII fragment of NT18 missing the SH2-encoding sequences was sequenced and substituted for the equivalent region in the wild-type lck cDNA. No additional mutations were detected (data not shown).

Double-mutant lck cDNAs (ASH3 F505 lck and ΔASH2 F505 lck) were generated by exchanging the BglII–PstI fragment of F505 lck (Abraham & Veillette, 1990) with those of ASH3 lck and ΔASH2 lck respectively.

**Retroviral infection**

The Stu–Stu fragment of the various lck cDNAs was cloned in the appropriate orientation into the HpaI site of the retroviral vector pLXSN (Miller & Rosman, 1989; kindly provided by Dusty Miller, Fred Hutchinson Cancer Center, Seattle, WA, USA). This construct also contains the neomycin phosphotransferase gene (neo) from Tn5. DNA was transfected in ψ-2 packaging cells by calcium phosphate precipitation (Cartier et al., 1987) and retrovirus-producing cell lines selected for growth in medium containing the aminoglycoside G418 (400 μg ml⁻¹). Retroviral infection of NIH3T3 cells was performed as outlined elsewhere (Albritton et al., 1989). Infected NIH3T3 cells were selected for growth in medium supplemented with 250 μg ml⁻¹ G418.
Transformation assays

To study focus formation, 10^5 or 10^4 Lck-expressing NIH3T3 fibroblasts were mixed with 10^4 neomycin-resistant NIH3T3 cells and plated in six-well Costar plates with 250 μg ml^-1 G418. Foci were counted 10 days later. For growth in soft agar, 2 x 10^4 cells were plated in semisolid medium as previously described (Freedman & Shin, 1974). Fresh G418-containing medium was added every 4 days and colonies were counted after 10 and 21 days. Assays were done in duplicate and repeated at least twice (data not shown).

Immunoblots

ImmunobLOTS were performed as described previously (Veillette et al., 1988b; Kamps & Selton, 1988; Abraham et al., 1991), using either a specific rabbit polyclonal antiserum generated against a synthetic peptide corresponding to amino acids 29–54 of the murine p56^cc sequence (Veillette et al., 1988b), affinity-purified rabbit anti-phosphotyrosine antibodies (our unpublished data) or a previously described rabbit anti-GAP serum (Ellis et al., 1991a, b). Detection of immunoprecipitated polypeptides was done by using [125]l-protein A (Amersham) and subsequent autoradiography. For quantitation, bands were cut from nitrocellulose filters and counted in a gamma counter (data not shown). The presence of equivalent amounts of cellular proteins in each lane was confirmed by amido-black staining of nitrocellulose membranes (data not shown).

Peptide mapping

Fibroblasts were metabolically labeled for 4 h in phosphate-free Dulbecco’s modified Eagle medium (DMEM) supplemented with 2% dialyzed fetal calf serum (FCS) and 1.0 μCi ml^-1 ^32P (carrier free; New England Nuclear). After the labeling period, cells were immediately lysed in boiling 2 x TNE buffer (see below) supplemented with 2% sodium dodecyl sulfate (SDS). After heating the DNA, lysates were boiled again for 5 min and diluted fivefold in TNE buffer containing protease and phosphatase inhibitors (see below). Lck polypeptides were recovered by immunoprecipitation as described by Veillette et al. (1988b). Subsequent electrophoresis and tryptic peptide mapping studies using Immobilon-P membranes were done as outlined elsewhere (Luo & Selton, 1990b).

Anti-GAP immunoprecipitations

After lysis in a buffer containing 50 mM Tris pH 8.0, 1% NP-40, 2 mM EDTA pH 8.0 (TNE buffer) with 10 μg ml^-1 each of the protease inhibitors leupeptin, aprotinin, N-tosyl-L-phenylalalnine chloromethyl ketone, N-tosyl-L-lysine chloromethyl ketone and phenylmethylsulfonyl fluoride as well as the phosphatase inhibitors sodium fluoride (50 mM) and sodium orthovanadate (1 mM), post-nuclear supernatants containing 125 μg of total cellular proteins were immunoprecipitated with 2.5 μl of a previously described rabbit anti-GAP serum (Ellis et al., 1991a, b). Immune complexes were collected by the addition of Staphylococcus aureus protein A (Pansorbin; Calbiochem). After washing three times in lysis buffer containing 1 mM sodium orthovanadate, proteins were processed for anti-GAP immunoblotting.

In vitro binding to phosphotyrosine-containing proteins

To study the ability of the SH3 and SH2 domains of p56^cc to bind phosphotyrosine-containing proteins, SH3- and SH2-encoding sequences were cloned in the appropriate pATH vectors. These vectors encode trpE bacterial fusion proteins. pATH constructs encoding fusion proteins that join trpE to the SH2 domains of GAF, v-Crk or v-Src have been described elsewhere (Anderson et al., 1990). Induction and purification of bacterial fusion proteins were performed as outlined previously (Anderson et al., 1990). Recovery and analyses of bound phosphotyrosine-containing proteins were also done according to previously established protocols (Anderson et al., 1990).

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