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The Carboxy-Terminal Sequence of p56^{lck} Can Regulate p60$^{c\text{-}src}$

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A chimera containing the coding region for residues 1 to 516 of p60$^{c\text{-}src}$ and residues 495 to 509 (the carboxy terminus) of p56^{lck} was constructed and expressed in mouse fibroblasts. The chimeric protein appeared to be phosphorylated and regulated in the same fashion as p60$^{c\text{-}src}$.

p60$^{c\text{-}src}$ and p56^{lck} are the products of two members of a gene family that encodes cytoplasmic membrane-associated protein-tyrosine kinases of about 60,000 daltons. Other genes in this family (the *src* family) are c-*yes*, c-*fgr*, *fyn*, *hck*, *tkl*, and *lyn* (17, 25, 26, 29, 34, 36, 37, 40, 42, 43; for a review, see J. A. Cooper, *in* B. Kemp and P. F. Alewood, ed., *Peptides and Protein Phosphorylation*, in press). p60$^{c\text{-}src}$ has two major sites of tyrosine phosphorylation, Tyr-416 in the kinase domain and Tyr-527, which lies six amino acids from the carboxy terminus. Tyr-416 is the major site of phosphorylation in vitro (27, 35). Enzymatically active forms of p60$^{c\text{-}src}$ are also phosphorylated at Tyr-416 in the cell (3, 4, 15, 18, 20, 28, 30). Tyr-527 is the major site phosphorylated in repressed forms of p60$^{c\text{-}src}$ in fibroblasts (7, 19). The residues in p56^{lck} that correspond to Tyr-416 and Tyr-527 are Tyr-394 and Tyr-505, respectively (23, 38, 41). Their phosphorylation in vivo and in vitro follows the same pattern as for p60$^{c\text{-}src}$ (5, 22).

Lack of phosphorylation of Tyr-527 is associated with activation of c-*src* transforming potential and stimulation of in vitro kinase activity. The sequence surrounding and including Tyr-527 is absent in p60$^{v\text{-}src}$, a transforming variant of p60$^{c\text{-}src}$ that has much greater protein-tyrosine kinase activity than does p60$^{c\text{-}src}$ (13, 15, 20, 32, 38). Transformation by the polyomavirus middle T antigen leads to dephosphorylation of Tyr-527 and stimulation of p60$^{c\text{-}src}$ kinase activity (2, 4, 11). Alteration of c-*src* codon 527 to specify Phe, a residue which is not phosphorylated, activates the transforming potential of c-*src* (3, 18, 28, 30). Dephosphorylation of Tyr-527 in vitro results in a 10- to 20-fold stimulation of the specific activity of p60$^{c\text{-}src}$ in an in vitro kinase reaction (8). Negative regulation by tyrosine phosphorylation may apply generally to the products of the *src* family genes, since all contain a carboxy-terminal tyrosine residue (Cooper, in press).

Examination of the amino acid sequences of members of the *src* gene family shows them to be most different between residues 3 and 90 but to be very similar in the kinase domain (residues 240 to 516, p60$^{c\text{-}src}$ numbering). Alignment of sequences allows the division of the *src* family into two subfamilies, one including c-*src*, c-*yes*, c-*fgr*, and *fyn* (529 to 540 residue proteins) and the other including *lck*, *hck*, *tkl*, and *lyn* (505 to 512 residues). Pairwise sequence identities within each subfamily average 68 ± 4%; interfamily identities average 55 ± 3%. The sequences carboxy terminal to the kinase domain define the same two subfamilies, with *lck*, *hck*, *tkl*, and *lyn* being two residues shorter than the other four and intrafamily sequence relationships being closer than interfamily relationships (Fig. 1A). Variation at the carboxy terminus may indicate that despite the demonstrable importance of this region for regulation, the sequence requirements of the region are relatively flexible. In this case, one carboxy terminus should be able to substitute for another within the family to give normal regulation of kinase activity. Alternatively, divergence of the carboxy termini might either permit differential regulation of the two subfamilies or compensate for mutations elsewhere in the protein so as to maintain normal regulation. In these cases, the carboxy terminus of one protein might not be able to regulate the enzymatic activity of a member of the other subfamily.

We tested whether p60$^{c\text{-}src}$ could be regulated by the p56^{lck} carboxy terminus. The region of the c-*src* gene encoding the carboxy-terminal 16 amino acids was replaced by a fragment encoding the 14 carboxy-terminal amino acids of *lck* (Fig. 1). To do this, the c-*src* gene from p5H (20) was modified by the introduction of a *Bam*HI site upstream of the initiating methionine (9), and a *Bam*HI fragment containing c-*src* was inserted in the *Bam*HI site of a Bluescribe (Stratagene, San Diego, Calif.) derivative, pVZSfi (S. Henikoff, unpublished plasmid). A *Bgl*II site just downstream of the termination codon was then fused to the *Sal*I site in the polylinker by using an adapter that retained the *Bgl*II site. The c-*src* gene was then subjected to site-directed mutagenesis (39) to introduce a *Xho*I site at codons 516 and 517 and an *Spe*I site at codons 521 and 522, creating pMX. Neither of these changes alters the coding potential of the c-*src* gene (8a). The *lck* carboxy-terminal coding region was introduced into pMX by synthesizing complementary oligonucleotides encoding the desired residues (Fig. 1B). The oligonucleotides were phosphorylated with polynucleotide kinase and ligated into pMX that had been cut with *Xho*I and *Bgl*II. The ligation products were transformed into *Escherichia coli* TG1, and the colonies were screened for the loss of the *Xho*I site. The presence of the *lck* sequence was confirmed by dideoxynucleotide sequencing (31). To provide an activated version of c-*src* as a positive control for transformation, the Tyr-527 codon of pMX was changed to code for Phe by using oligonucleotide-directed mutagenesis (3, 18, 28, 30). The modified c-*src* genes were excised from the plasmids by using *Bam*HI and *Bgl*II and cloned into the *Bam*HI site of pLJ, a murine retroviral expression vector that carries a neomycin phosphotransferase minigene kindly provided by R. Mulligan (28).

Retroviral packaging lines were produced by a two-step technique (24). PA317 cells were transfected with the retroviral constructions, and supernatants were collected 48 h later. The supernatants, containing transiently expressed amphotropic virus, were used to infect ψ2 cells, which were subjected to G418 selection. Resistant populations of cells

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A

```
fyn    ...                    Ala                                  (537)
c-yes  ...                    Ala                          Asp Leu (540)
c-fgr  ...                 SerAla                          AspGlnThr(529)
c-src  ... LeuGluAspTyrPheThrSerThrGluProGlnTyrGlnProGlyGluAsnLeu(533)
             ∇     ∇        ∇       ∇                  ∇  ∇  ∇  ∇
lck    ... LeuAspAspPhePheThrAlaThrGluGlyGlnTyrGlnProGlnPro *  * (509)
hck    ...            Tyr            Ser        Gln            (505)
lyn    ...            Tyr                       Gln            (512)
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B

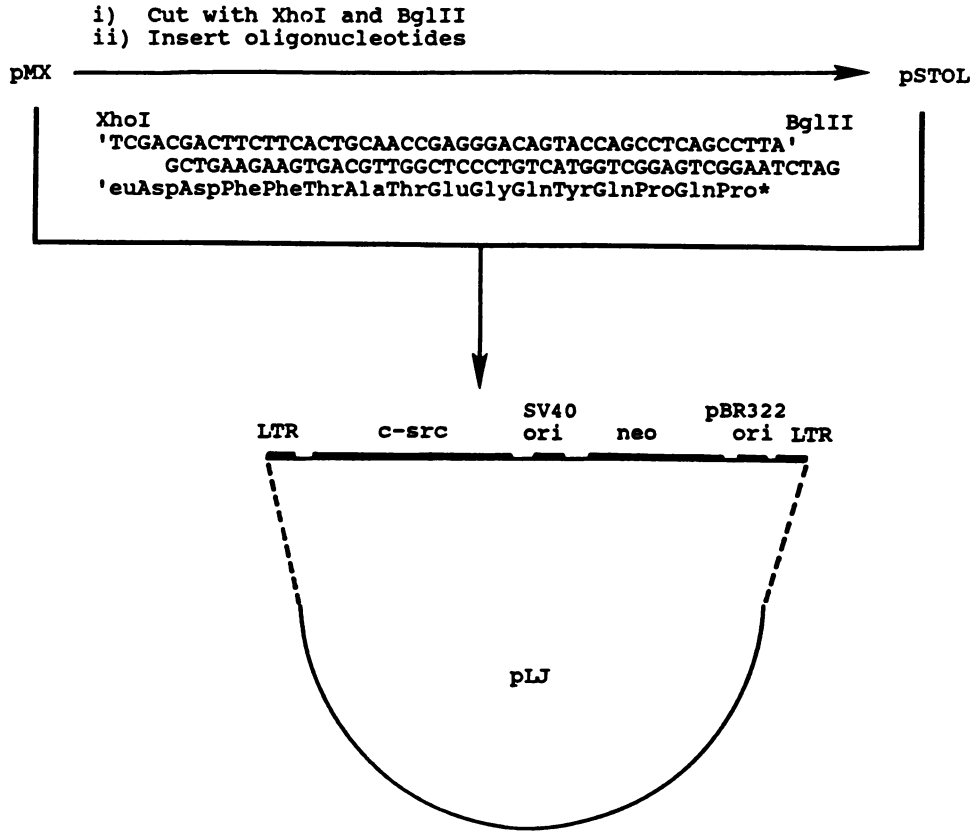

FIG. 1. (A) Sequences of carboxy termini of *src* family protein-tyrosine kinases. The complete sequences of c-*src* and *lck* are shown. ∇, Differences between c-*src* and *lck*. Only residues in *fyn*, c-*yes*, and c-*fgr* that differ from those in c-*src* and residues in *hck* and *lyn* that differ from those in *lck* are shown. (B) Construction of chimeric gene. Plasmid pMX has been described before (8a). pSTOL was derived by the insertion of the synthetic oligonucleotides shown between the *Xho*I and *Bgl*II sites of pMX. The c-*src*-coding regions of pMX and pSTOL were inserted into the *Bam*HI site of pLJ (28).

(100 to 1,000 colonies in each case) were expanded. These polyclonal cell lines, which produce ecotropic virus, were used as sources of the various $p60^{c\text{-}src}$-related proteins. These proteins are designated $p60^{WT}$ (avian $p60^{c\text{-}src}$ expressed from the pMX sequence), $p60^{src/lck}$ (chimera of avian $p60^{c\text{-}src}$ residues 1 to 516 and mouse $p56^{lck}$ residues 495 to 509), and $p60^{F527}$ (Phe-527 mutant of avian $p60^{c\text{-}src}$).

G418-resistant populations of ψ2 were labeled with $^{32}P_i$ and [^{35}S]methionine, and immunoprecipitates were prepared with monoclonal antibody 327 against $p60^{src}$ (21). A labeled band of M_r approximately 60,000 was apparent in the immunoprecipitates of both the $^{32}P_i$- and [^{35}S]methionine-labeled lysates of all cell lines infected with pLJ/*src* derivatives but not with the parental virus (data not shown). The similar intensities of the 60-kilodalton bands in the different lines suggested that $p60^{src/lck}$ and $p60^{F527}$ were expressed to levels similar to those achieved with $p60^{WT}$ and were phosphorylated to a comparable extent.

To examine the sites of phosphorylation of $p60^{src/lck}$, immunoprecipitated $^{32}P_i$-labeled $p60^{src/lck}$ was subjected to two-dimensional phosphopeptide mapping. For comparison, we also mapped $p60^{WT}$ and $p56^{lck}$. The latter was immunoprecipitated from fibroblasts expressing an introduced *lck* gene (22; R. Louie and J. A. Cooper, unpublished results).

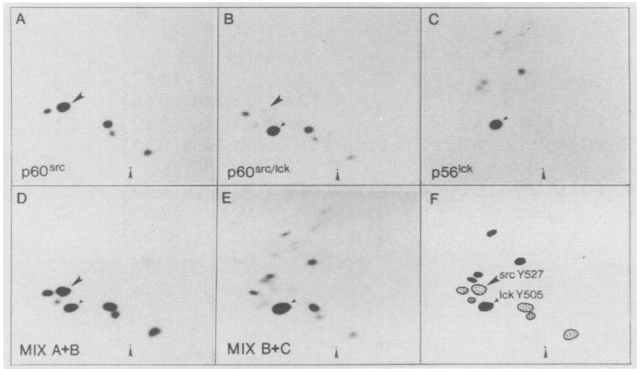

FIG. 2. Chymotryptic phosphopeptides of $p60^{c\text{-}src}$, $p60^{src/lck}$, and $p56^{lck}$ from $^{32}P_i$-labeled cells. Phosphopeptides of $p60^{WT}$ (A), $p60^{src/lck}$ (B), and $p56^{lck}$ (C) were separated by electrophoresis at pH 8.9 (anode at left) and ascending chromatography and detected by autoradiography. Mixtures of equal radioactive quantities of phosphopeptides from $p60^{c\text{-}src}$ and $p60^{src/lck}$ (D) and $p60^{src/lck}$ and $p56^{lck}$ (E) were also analyzed. Large arrowheads, phosphopeptide containing residues 521 to 533 of $p60^{c\text{-}src}$; small arrowheads, phosphopeptide containing residues 499 to 505 of $p56^{lck}$. (F) Diagram of major chymotryptic phosphopeptides detected. Solid areas, phosphopeptides of $p56^{lck}$; stippled areas, $p60^{c\text{-}src}$.

Subconfluent 50-mm dishes of cells were labeled for 4 h with 2 mCi of $^{32}P_i$ in 1.5 ml of Dulbecco modified Eagle medium minus phosphate and containing 3% fetal bovine serum. Immunoprecipitates were prepared by using monoclonal antibody 327 and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (33). The phosphoproteins were extracted, oxidized with performic acid, and digested with chymotrypsin. Phosphopeptides were separated on thin-layer cellulose plates by electrophoresis at pH 8.9 and chromatography (1). $p60^{src/lck}$ was phosphorylated in the same peptides as $p60^{WT}$ (Fig. 2A, B, and D), except that the chymotryptic peptide containing Tyr-527 was considerably reduced in quantity. The small amount of the Tyr-527 phosphopeptide is presumably derived from endogenous murine $p60^{c\text{-}src}$ which would be immunoprecipitated together with $p60^{src/lck}$. One major chymotryptic phosphopeptide from $p60^{src/lck}$ that was not found in $p60^{WT}$ comigrated with the major phosphopeptide of $p56^{lck}$ (Fig. 2B, C, E, and F). This phosphopeptide contained phosphotyrosine (data not shown), identifying it as the Tyr-505 peptide of $p56^{lck}$ (22) or the Tyr-527 peptide of $p60^{src/lck}$. These maps demonstrate that the major product in the immunoprecipitates from cells expressing the chimeric gene was $p60^{src/lck}$. Mapping of tryptic phosphopeptides (data not shown) confirmed that the patterns of phosphorylation of $p60^{src/lck}$ and $p60^{WT}$ were very similar, in that the sites of serine phosphorylation were identical and the predominant site of tyrosine phosphorylation was the most carboxy-terminal tyrosine, Tyr-527, not Tyr- 416. We conclude that $p60^{WT}$ and $p60^{src/lck}$ were phosphorylated like normal $p60^{c\text{-}src}$ (7).

The high ratio of Tyr-527 to Tyr-416 phosphorylation in $p60^{src/lck}$ suggested that $p60^{src/lck}$ was inhibited in the cell by Tyr-527 phosphorylation. To ascertain if this regulation occurred in kinase reactions in vitro, the specific activity of $p60^{src/lck}$ relative to $p60^{WT}$ and $p60^{F527}$ was determined. Immunoprecipitates were prepared from unlabeled cells with antibody 327, as described above. Kinase assays were performed in duplicate, incubating immunoprecipitate from 1/40 of a 50-mm dish culture of cells with [γ-^{32}P]ATP and a protein substrate, enolase, under conditions which give linear incorporation of ^{32}P (8). Reaction products were separated by SDS-PAGE and detected by autoradiography (Fig. 3). Sections of the gel containing p60 and enolase were excised, and the radioactivity was quantified. The relative amounts of the various forms of $p60^{c\text{-}src}$ were estimated from a Western blot (immunoblot) on samples of the same immunoprecipitate (Fig. 3B). $p60^{WT}$, $p60^{src/lck}$, and $p60^{F527}$ were expressed equally, to within an estimated 0.7- to 1.5-fold experimental error. Calculation of specific activity based on radioactivity incorporated relative to the quantity of p60 (Table 1, experiment 1) shows that the kinase activity of $p60^{src/lck}$ was similar to that of $p60^{WT}$ and both enzymes were five- to sevenfold less active than the $p60^{F527}$ enzyme.

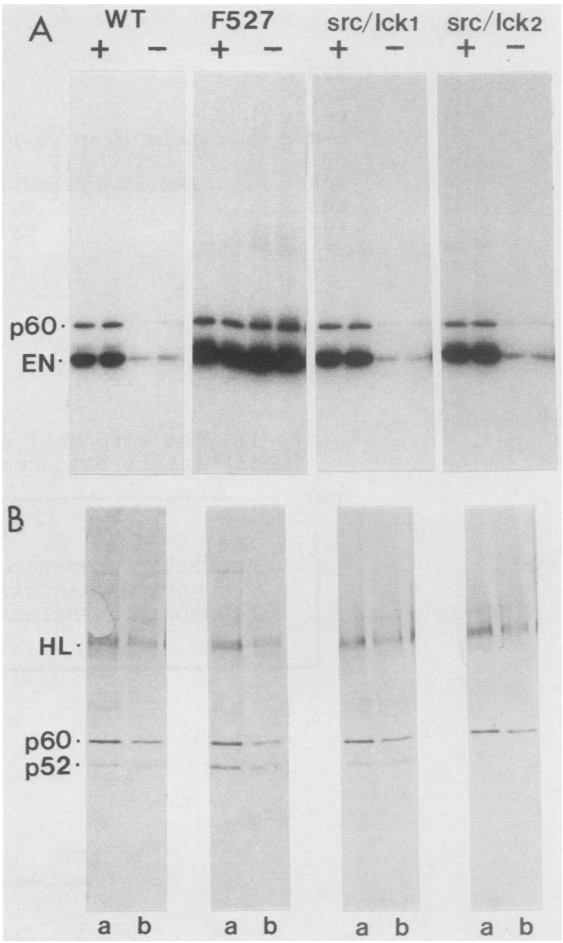

FIG. 3. Assay of $p60^{c\text{-}src}$ mutant proteins in vitro. $p60^{src/lck}$ was immunoprecipitated from two independent polyclonal ψ2 cell populations (src/lck1 and src/lck2) arising from transfection with the *src/lck* expression vector. $p60^{WT}$ and $p60^{F527}$ were immunoprecipitated in parallel. (A) Duplicate samples of the immunoprecipitates were incubated in pH 7 buffer with (+) or without (−) potato acid phosphatase (Table 1, footnote *b*) prior to incubation with [γ-^{32}P] ATP and acid-treated enolase. Reaction products were separated by SDS-PAGE and detected by autoradiography. The positions of the various p60 molecules and enolase (EN) are indicated. (B) Samples of similar immunoprecipitates corresponding to 5% (a) and 2.5% (b) of a 50-mm dish culture were analyzed by SDS-PAGE and immunoblotted with antibody 327 (21). Besides the various p60 molecules, this procedure detected the immunoglobulin used for immunoprecipitation (HL) and a 52-kilodalton breakdown product of p60 (p52) (12).

TABLE 1. Quantification of $p60^{src/lck}$ kinase activity

Expt	Protein[a]	Phosphatase[b]	Radioactivity incorporated (cpm)[c]		Relative activity[d]
			p60	Enolase	
1	$p60^{WT}$		2,460	9,860	1.0
	$p60^{src/lck}$		2,400	7,020	0.71
	$p60^{src/lck}$		3,080	7,850	0.80
	$p60^{F527}$		14,600	53,200	5.4
2	$p60^{WT}$	−	1,750	6,560	1.0
		+	19,600	82,140	12.5
	$p60^{src/lck}$	−	1,370	8,550	1.3
		+	19,000	101,000	15.4
	$p60^{src/lck}$	−	1,450	6,320	0.96
		+	15,600	95,000	14.5
	$p60^{F527}$	−	20,600	101,000	15.4
		+	20,300	96,400	14.7

[a] Protein kinase assays were performed with immunoprecipitates containing equal quantities (experiment 1) (Fig. 3B) or undetermined quantities (experiment 2) of p60 mutants. Two different polyclonal cell lines expressing $p60^{src/lck}$ were examined.

[b] Before the assay, the immunoprecipitates in experiment 2 were incubated with 0 (−) or 100 (+) μg of potato acid phosphatase per ml at 37°C for 10 min as described previously (8), except that the pH was 7.0. Immunoprecipitates in experiment 1 were not incubated before assay.

[c] ^{32}P incorporation was determined by counting Cerenkov radiation. Radioactivity incorporated in triplicate (experiment 1) or duplicate (experiment 2) assays was averaged and corrected for background. The incorporation of ^{32}P due to endogenous $p60^{c\text{-}src}$ is about 5 to 10% of that seen with cells expressing $p60^{WT}$.

[d] Phosphorylation of enolase by mutant p60 protein relative to enolase phosphorylation by $p60^{WT}$.

In vitro dephosphorylation of Tyr-527 has been shown to stimulate $p60^{c\text{-}src}$-specific activity 10-fold (8). Immunoprecipitates were incubated with potato acid phosphatase under conditions that remove most of the phosphate from serine and tyrosine residues in $p60^{WT}$ (8), and their kinase activities were determined. Phosphatase treatment of $p60^{F527}$ did not result in any significant change of activity. The specific activities of both $p60^{src/lck}$ and $p60^{WT}$ were stimulated approximately 10-fold by phosphatase (Fig. 3 and Table 1, experiment 2). These results suggest that the phosphorylated *lck* carboxy terminus was able to down-regulate the kinase activity of the c-*src*-encoded kinase domain to the same extent as the c-*src*-encoded carboxy terminus.

To test whether $p60^{src/lck}$ would induce morphological transformation, ecotropic virus stocks from ψ2 cells expressing the various $p60^{c\text{-}src}$ derivatives were used to infect NIH 3T3 cells. The virus encoding $p60^{F527}$ caused dramatic transformation. Consistent with the low kinase activity of $p60^{src/lck}$ in vitro and with the extensive phosphorylation of the novel Tyr-527 in vivo, the *src/lck* virus did not induce any morphological transformation (data not shown).

Since neither *lck* (22) nor *src/lck* transforms fibroblasts, it appears that $p56^{lck}$ and $p60^{c\text{-}src}$ can both be regulated by the $p56^{lck}$ carboxy terminus. Because mutations in the kinase domain of $p60^{c\text{-}src}$ reduce the level of phosphorylation at the carboxy terminus (15, 20), the carboxy-terminal tyrosine may be recognized in the context of the kinase domain. If the same fibroblast kinases and phosphatases act on the carboxy termini of $p60^{c\text{-}src}$, $p56^{lck}$, and $p60^{src/lck}$, they presumably recognize the carboxy termini in terms of features common to the whole proteins. The results of this study are from steady-state analyses, so it is possible that the rates of phosphorylation and dephosphorylation are changed, but the net effect is balanced to generate enzymes that are regulated indistinguishably.

The ability of the novel carboxy terminus to regulate $p60^{c\text{-}src}$ was surprising. Shortening the carboxy terminus by 2 residues and replacing 6 of the remaining 14 residues did not interfere with normal phosphorylation of Tyr-527 and consequential inhibition of kinase activity. Recently, it has been shown that a variety of residue changes at positions 524, 525, 526, and 528 in the $p60^{c\text{-}src}$ sequence are compatible with normal regulation (6). This plasticity implies that the primary sequence environment of Tyr-527 is not a prime determinant in regulating its phosphorylation. When expressed in yeast cells, $p60^{src/lck}$ and $p60^{WT}$ are phosphorylated at Tyr-527 to the same extent (data not shown). Tyrosine phosphorylation of $p60^{WT}$ in yeast cells is catalyzed by $p60^{WT}$ in an apparent intermolecular reaction (8a, 16). Therefore, the carboxy-terminal sequences encoded by either c-*src* or *lck* can be phosphorylated by $p60^{c\text{-}src}$.

There are few sequence differences between chicken and human $p60^{c\text{-}src}$ or between mouse and human $p56^{lck}$, implying that the sequence differences between $p60^{c\text{-}src}$ and $p56^{lck}$ (most extreme in the amino-terminal-proximal 70 residues) have evolved to allow functional specialization. The compatability of the $p56^{lck}$ carboxy-terminal tail with the body of $p60^{c\text{-}src}$ suggests that the carboxy terminus does not interact with regions of the kinase domain or amino terminus that differ between $p60^{c\text{-}src}$ and $p56^{lck}$. The differences in the carboxy termini might allow interactions with different effector proteins in different cell types. $p56^{lck}$ is expressed at a high level in T lymphocytes (23, 41), and $p60^{c\text{-}src}$ is expressed at a high level in neurons and blood platelets (10, 14). Expression of carboxy-terminal mutants in different host cells may reveal quirks of regulation not evident in fibroblasts.

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