Intracellular Localization and Processing of pp60$^{\text{v-}src}$ Proteins Expressed by Two Distinct Temperature-Sensitive Mutants of Rous Sarcoma Virus

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The transforming protein of Rous sarcoma virus, pp60$^{\text{v-}src}$, is known to be a tyrosine protein kinase, but the mechanism of cell transformation remains unclear. In further investigating pp60$^{\text{v-}src}$ structure and function, we have analyzed two temperature-sensitive (*ts*) Rous sarcoma virus *src* gene mutants, *tsLA*29 and *tsLA*32. The mutations in *tsLA*29 and *tsLA*32 map in the carboxy-terminal region and the amino-terminal half of pp60$^{\text{v-}src}$, respectively, and encode mutant proteins with either temperature-labile (*tsLA*29) or -stable (*tsLA*32) kinase activities. Here we examined the intracellular processing and localization of these pp60$^{\text{v-}src}$ mutants and extended our characterization of transformation parameters expressed by cells infected by the Rous sarcoma virus variants. No obvious defects in functional integrity of the *tsLA*32 pp60$^{\text{v-}src}$ could yet be demonstrated, whereas the *tsLA*29 pp60$^{\text{v-}src}$ was perturbed not only in kinase activity, but also in aspects of protein processing and localization. Analysis of transformation parameters expressed by infected cells demonstrated the complete temperature lability of both mutants.

The v-*src* gene of Rous sarcoma virus (RSV) encodes a phosphoprotein of molecular weight 60,000, pp60$^{\text{v-}src}$, which can induce in vitro neoplastic transformation of a variety of cells (24, 38). This transformation is a complex process, involving considerable structural and metabolic perturbations to the cell, and yet pp60$^{\text{v-}src}$ is capable of initiating and maintaining the pleiotropic changes which are seen (18). The mechanisms by which pp60$^{\text{v-}src}$ induces transformation remain unclear, although a considerable amount of knowledge has been gained in recent years concerning its structure, location, and functional activity within the cell.

The only well-defined function ascribed to pp60$^{\text{v-}src}$ is that of a tyrosine protein kinase (2, 6, 7, 16, 25, 30, 31, 42), for which a number of putative target proteins have been identified in the cell (1, 8, 34). These include two phosphoproteins, pp36 (15, 39) and pp50 (23), vinculin (41), and three glycolytic enzymes (10). However, in no case has specific phosphorylation of these substrates been shown to be necessary for transformation, suggesting that the critical cellular targets have yet to be identified.

An additional property of the pp60$^{\text{v-}src}$ protein is its association with the plasma membrane, where the majority of mature pp60$^{\text{v-}src}$ is found (for review, see reference 27). The importance of this localization is demonstrated by mutants showing defects in membrane binding that are also invariably defective in some or all aspects of cell transformation (11, 20, 28). The mechanism by which pp60$^{\text{v-}src}$ associates with the membrane is unclear, but several factors appear to be of importance in governing the transport to or association with this site. First, the lipid myristic acid is found covalently attached to the N-terminal glycine residue of pp60$^{\text{v-}src}$ (5, 43). Absence of this lipid prevents stable association with the membrane, thus preventing transformation, but not apparently affecting the kinase activity (13, 21, 26). The N-terminal 8 kilodaltons of pp60$^{\text{v-}src}$ is believed to be involved in close contact with the membrane (28) and

together with myristic acid may mediate the stable association. In addition to these factors, pp60$^{\text{v-}src}$ is known to form a transient complex with two cellular proteins, pp50 and pp90, soon after its synthesis on cytosolic polyribosomes (2, 4, 36). The precise role of the complex is unclear, but some involvement in transport of pp60$^{\text{v-}src}$ to the membrane has been suggested (11).

It has become clear that the functions ascribed to pp60$^{\text{v-}src}$ can be crudely mapped to domains of the protein (17, 29, 37, 44). The kinase function is believed to lie in the carboxy-terminal (C-terminal) half of the protein in an area with sequence homology to other known tyrosine kinases (40). The membrane binding is ascribed to the amino-terminal (N-terminal) region, and additional areas of the protein may provide specific binding points for the pp50-pp90 proteins (46). To dissect further the structure and functions of pp60$^{\text{v-}src}$, we have been studying temperature-sensitive (*ts*) mutants of the v-*src* gene in which different domains of the pp60$^{\text{v-}src}$ protein have been perturbed. We have focused on two mutants, *tsLA*32 and *tsLA*29, and our previous work has shown that these mutants have lesions which either do affect (*LA*29) or do not directly affect (*LA*32) the kinase activity of the protein (44). The *LA*32 mutant is of particular interest as it maintains elevated cellular phosphotyrosine levels and phosphorylates pp36 (44) and vinculin (S. Kellie, B. Patel, N. M. Wigglesworth, D. R. Critchley, and J. A. Wyke, Exp. Cell Res., in press) at restrictive temperature, suggesting that this mutant has a lesion in a novel domain necessary for the maintenance of transformation.

Our work did not indicate whether the lesion in *LA*32 was affecting aspects of pp60$^{\text{v-}src}$ localization or maturation, or whether the *LA*29 pp60$^{\text{v-}src}$ exhibited defects other than in the kinase function. To investigate these questions, we have studied both the intracellular processing and the localization of the mutant pp60$^{\text{v-}src}$ proteins. We show that the *LA*32 pp60$^{\text{v-}src}$ mutant is not significantly defective in the above parameters and contrast these findings with those of the *LA*29 mutant. In addition, having previously demonstrated

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the elevation of phosphotyrosine levels and the residual soft-agar growth capacity of *LA*32-infected chicken embryo fibroblasts (CEF) at 41°C (44), we were interested in examining whether additional parameters of transformation were expressed under restrictive conditions. We describe a detailed study of the dissolution of actin- and α-actinin-containing cables and the fibronectin matrix in the mutant-infected cells and demonstrate the full temperature lability of these transformation parameters.

MATERIALS AND METHODS

Cells and viruses. CEF were prepared from fertile eggs of a white leghorn and brown leghorn cross (Wickham Laboratories, Wickham, Hampshire, U.K.). Methods of cell culture and infection were as described previously (47). Isolation of viruses and initial characterization have been described before (17, 44, 49).

Antibodies and fluorescent reagents. Normal rabbit serum was collected as described previously (44). Anti-pp60$^{v\text{-}src}$ antibodies were kindly provided by the following people: JB327 monoclonal (J. Brugge [32]); anti-v-*src*-C (S. J. Courtneidge); anti-*src* rabbit serum raised against pp60$^{v\text{-}src}$ expressed and purified from *Escherichia coli* (R. L. Erikson [22]). Rabbit anti-mouse serum MH1 was kindly supplied by M. Hayman. Antisera against chicken gizzard α-actinin were raised in rabbits and affinity purified as described previously (26a). Fluorescein isothiocyanate-conjugated affinity-purified goat anti-rabbit and goat anti-mouse immunoglobulins were obtained from Sigma Ltd. Nitrobenzo-oxadiazole (NBD)-phallicidin was obtained from Molecular Probes Inc., Junction City, Ore., and was used as described by the supplier.

Immunoprecipitation and gel electrophoresis. Cell lysis, immunoprecipitations, and sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis were performed as described previously (44). The lysis buffers used were as follows: modified RIPA buffer (10 mM Tris hydrochloride, pH 7.2, 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.1 trypsin inhibitor unit per ml of aprotinin [Sigma], 20 mM NaPP$_i$; buffer A (150 mM NaCl, 10 mM Tris hydrochloride, pH 7.0, 1% Nonidet P-40, 20 mM NaPP$_i$).

Immunofluorescence. Cells were grown on 13-mm-diameter cover slips for at least 18 h and fixed in 3.8% formaldehyde in phosphate-buffered saline (PBS) for 30 min followed by extensive washing in PBS. After permeabilization by immersion in PBS containing 1% Nonidet P-40, the cover slips were washed and overlaid with 50 μl of the first antibody in PBS (anti-α-actinin, 50 μg/ml; antifibronectin, 1:100 dilution; JB327 anti-pp60$^{v\text{-}src}$, 1:20 dilution) for 30 min. After thorough washing, the cover slips were overlaid with 50 μl of a 1:20 dilution of fluorescein isothiocyanate-conjugated goat anti-rabbit or goat anti-mouse immunoglobulin for 30 min. Cover slips were washed and mounted in PBS-glycerol (1:9) containing 1 mg of *p*-phenylamine diamine per ml to reduce photobleaching and viewed with a Leitz Dialux microscope equipped with epifluorescence. Photomicrographs were taken with Ilford HP5 film.

Cell fractionation. Plates (35 mm) of RSV-infected CEF were incubated for 30 min in methionine-free medium and then labeled for 1 h with 200 μCi of [^{35}S]methionine per ml at 35 and 41°C. The label was removed and chased in cold, complete medium for 2 h at the respective temperatures. Uninfected CEF carrier cells (10^7) were suspended and washed twice in PBS. The labeled cells were rinsed twice

with PBS, scraped into the carrier cell suspension with a rubber policeman, and washed once with PBS. The cells were resuspended in 3 ml of hypotonic buffer (10 mM Tris hydrochloride, pH 7, 1 mM MgCl$_2$) and left to swell on ice for 4 min. NaPP$_i$ to 10 mM and 2 trypsin inhibitor units of Aprotinin (Sigma) were added, and the cells were lysed with 30 strokes of a tight-fitting Dounce homogenizer. The lysates were cleared of unbroken cells and nuclei with a low-speed spin (600 × *g*) for 10 min. Half of the resultant supernatant was not further treated (fraction MC), and the other half was further spun at 80,000 × *g* for 30 min to obtain a crude membrane pellet (fraction M) and a supernatant of soluble cytoplasmic constituents (fraction C). All fractions were adjusted to RIPA concentration and immunoprecipitated with normal rabbit serum and the anti-v-*src*-C antisera. This procedure provided 90 to 100% recovery of pp60$^{v\text{-}src}$ in the M and C fractions when compared with the total unfractionated MC sample. Immunoprecipitates were subjected to polyacrylamide gel electrophoresis, and the gels were impregnated with Amplify (Amersham International) and fluorographed with Kodak XAR-5 film. pp60$^{v\text{-}src}$ in each fraction was quantified by scanning densitometry of the resulting fluorographs, using a Joyce-Loebl densitometer.

pp60$^{v\text{-}src}$ complex analysis. (i) Complex immunoprecipitation. CEF infected with RSV were labeled for 18 h with 100 μCi of [^{35}S]methionine per ml in complete medium. Cells were washed twice with PBS and lysed in buffer A. The lysates were normalized for protein content and halves were immunoprecipitated with normal rabbit serum and monoclonal JB237, followed by rabbit anti-mouse serum MH1. Immunoprecipitates were subjected to gel electrophoresis on 10% acrylamide gels.

(ii) Complex stability analysis. Cells were labeled as above but using 200 μCi of [^{35}S]methionine per ml. Cells were washed twice in PBS and lysed in buffer A. Immunoprecipitation of pp60$^{v\text{-}src}$ was performed initially as in (i) above. Immune complexes were washed once with buffer A, and then each sample was split into two aliquots. Each aliquot was washed three times in either buffer A or RIPA buffer. The immune complexes were subjected to polyacrylamide gel electrophoresis, and after fluorography gel slices containing pp60$^{v\text{-}src}$ and pp90 were cut out and the ^{35}S counts per minute were quantitated in an LKB RackBeta scintillation counter. Counts were corrected for background counts per minute from control areas of the gel.

Myristic acid labeling. 9,10-[^{3}H]myristic acid (2 mCi/ml; Amersham International) supplied in toluene was dried and suspended in calf serum. CEF either infected with RSV or uninfected were labeled for 18 h with 40 μCi of [^{3}H]myristic acid per ml in complete medium at 35 and 41°C. Cells were lysed in RIPA buffer, and pp60$^{v\text{-}src}$ was immune precipitated with anti-*src* serum. After polyacrylamide gel electrophoresis, gels were impregnated with Amplify (Amersham International) and fluorographed with Kodak XAR-5 film for 28 days. pp60$^{v\text{-}src}$ bands were scanned with a Joyce-Loebl densitometer, and values were normalized for the respective lysate protein concentration.

RESULTS

pp60$^{v\text{-}src}$ localization: cell fractionation and immunofluorescence. After synthesis on cytosolic polyribosomes, and a short lag period of 10 to 15 min, wild-type pp60$^{v\text{-}src}$ can be found localized in the plasma membrane compartment of the cell (29). It is believed that the membrane-bound form of pp60$^{v\text{-}src}$ is the functional form of the protein, and thus it was

of importance to determine the localization of the mutant proteins. For this, a pulse-chase labeling protocol was used, followed by cellular fractionation and immunoprecipitation of pp60^{v-src}. Given the kinetics of wild-type pp60^{v-src} association with the plasma membrane, the majority of the labeled pp60^{v-src} proteins will be chased to their final intracellular location within the 2-h chase period.

Cellular fractionation of wild-type pp60^{v-src} reveals that 80% of the labeled protein is found in the crude membrane fraction at both 35 and 41°C (Table 1). The mutant protein encoded by LA32 behaves very similarly to the wild type at permissive temperature, with comparable levels of pp60^{v-src} in the membrane fraction. At nonpermissive temperature nearly 50% of the labeled pp60^{v-src} is still found in the membrane fraction. A different pattern is found with mutant LA29. At permissive temperature only 50% of the labeled pp60^{v-src} is found in the membrane, but this represents a level which is clearly sufficient for cell transformation. At nonpermissive temperature only 15% of the pp60^{v-src} remains in the membrane, with 85% as a cytosolic form.

We have not ruled out the possibility that the mutant proteins, particularly that of LA29, exhibit some instability in membrane binding during the fractionation procedure. Thus, to confirm the membrane association pattern, and in particular to demonstrate the association with plasma membranes, we analyzed pp60^{v-src} localization, using indirect immunofluorescence. Both LA29- and LA32-infected CEF display prominent plasma membrane staining of pp60^{v-src} at permissive temperature, similar in pattern to wild-type-transformed cells (Fig. 1). At nonpermissive temperature the pattern of fluorescence is quite different with LA29, the pp60^{v-src} distribution now being of a diffuse, cytoplasmic nature, correlating well with our cell fractionation data. In contrast to LA29, and again in agreement with the fractionation data, CEF infected with LA32 maintain staining in the plasma membrane at 41°C, with prominent signals at cell-cell boundaries. Although not quantitative, the membrane staining of LA32-infected CEF is reduced to some degree at restrictive versus permissive temperature, and this may in part be due to the flatter nature of the cells and thus the greater spreading of the plasma membrane. Membrane staining in wild-type RSV-transformed CEF is unaffected by growth temperature.

Association of mutant pp60^{v-src} proteins with the pp90-pp50 complex. Soon after synthesis pp60^{v-src} enters a cytoplasmic complex with cell proteins pp90 and pp50. Only a small percentage of the total cellular pp90 is incorporated in the complex, and newly synthesized pp90 enters a large cyto-

solic pool rather than the complex (4). Because of this, a long [^{35}S]methionine labeling period was performed to obtain the complex with high levels of labeled pp90 associated with pp60^{v-src} for quantitation purposes. RSV-infected CEF were labeled for 18 h with [^{35}S]methionine, and pp60^{v-src} was immunoprecipitated with the monoclonal JB327 which efficiently precipitates the complexed form of pp60^{v-src} (32). By this procedure, the efficiency of complex formation was judged from the ratio of pp90/pp60. The pp50 was not quantitated because of the difficulty of distinguishing this protein from pp60^{v-src} breakdown products and background proteins of a similar molecular weight.

The data in Fig. 2 demonstrate that the levels of pp90 coprecipitated with mutant pp60^{v-src} proteins were not greatly affected by growth temperature, and the levels seen were similar to those found with our wild-type pp60^{v-src} (quantitation of ^{35}S counts per minute from pp60 and pp90 gel areas was confirmatory; data not shown). We did note a small decrease in the amount of pp90 coprecipitated at 35°C in all cases, the largest decrease was seen with the wild type, but the significance of this is unclear. The sensitivity of pp90-pp60 binding to variations in detergent conditions is shown by the following ratios of pp90 remaining bound to pp60^{v-src} in buffer A/RIPA: with wild-type PrC as the infecting virus, 2.1; with tsLA29, 9.7; and with tsLA32, 3.3. The pp60^{v-src} proteins encoded by both mutants and wild type bind pp90 to similar degrees in Nonidet P-40-containing buffer, buffer A. However, in the more denaturing RIPA buffer, containing the ionic detergent sodium dodecyl sulfate, a three to fourfold greater instability in pp60-pp90 binding is found with LA29 than with LA32 or wild-type PrC. This in vitro instability is not dependent upon the growth temperature of the infected CEF from with the pp60^{v-src} proteins are isolated (data not shown). Under physiological conditions, however, such an instability may well be differentially expressed at 35 or 41°C and could have a significant effect upon pp60^{v-src} processing and function. We are presently investigating the in vivo kinetics of complex association with these mutants in an attempt to answer this question.

Myristylation of pp60^{v-src}. The lipid myristic acid is covalently attached to the N-terminal glycine residue of the pp60^{v-src} protein, and this modification to pp60^{v-src} has been shown to be essential for transforming activity. Given the importance of myristic acid attachment during pp60^{v-src} maturation, it was of interest to determine whether temperature shift significantly affected the myristylation states of the ts mutant pp60^{v-src} proteins.

CEF infected with the mutant viruses were labeled for 18 h with [^{3}H]myristic acid (providing steady-state levels of incorporation; data not shown), and pp60^{v-src} was immunoprecipitated from whole-cell lysates. The peak areas of labeled pp60^{v-src} were quantitated as described in Materials and Methods. The incorporation of labeled myristic acid into wild-type pp60^{v-src} shows little change between the two growth temperatures (35°C/41°C ratio = 1.5). The mutant pp60^{v-src} proteins were found to behave in a manner similar to the wild-type protein with respect to myristic acid incorporation (35°C/41°C ratios: LA32, 1.5; LA29, 1.7). Buss et al. (5) recently reported similar findings with the LA29 mutant, but with a shorter 2-h labeling period and using [^{3}H]palmitic acid.

It is clear from these data that the ts lesions in both mutants do not greatly affect the ability to incorporate myristic acid into the mutant pp60^{v-src} proteins and that a defect in myristylation, which is an early step in pp60^{v-src}

TABLE 1. Localization of pp60^{v-src} by cell fractionation

Infecting virus	Cell fraction[a]	% of pp60^{v-src} at given growth temp[b]	
		35°C	41°C
Wild-type	M	82	80
PrC	C	18	20
ts LA29	M	50	15
	C	50	85
ts LA32	M	78	46
	C	22	54

[a] M, Crude membrane fraction; C, cytoplasmic fraction.

[b] Each number represents the proportion of labeled pp60^{v-src} in each fraction as a percentage of the total M plus C densitometric value and is the average of two independent experiments.
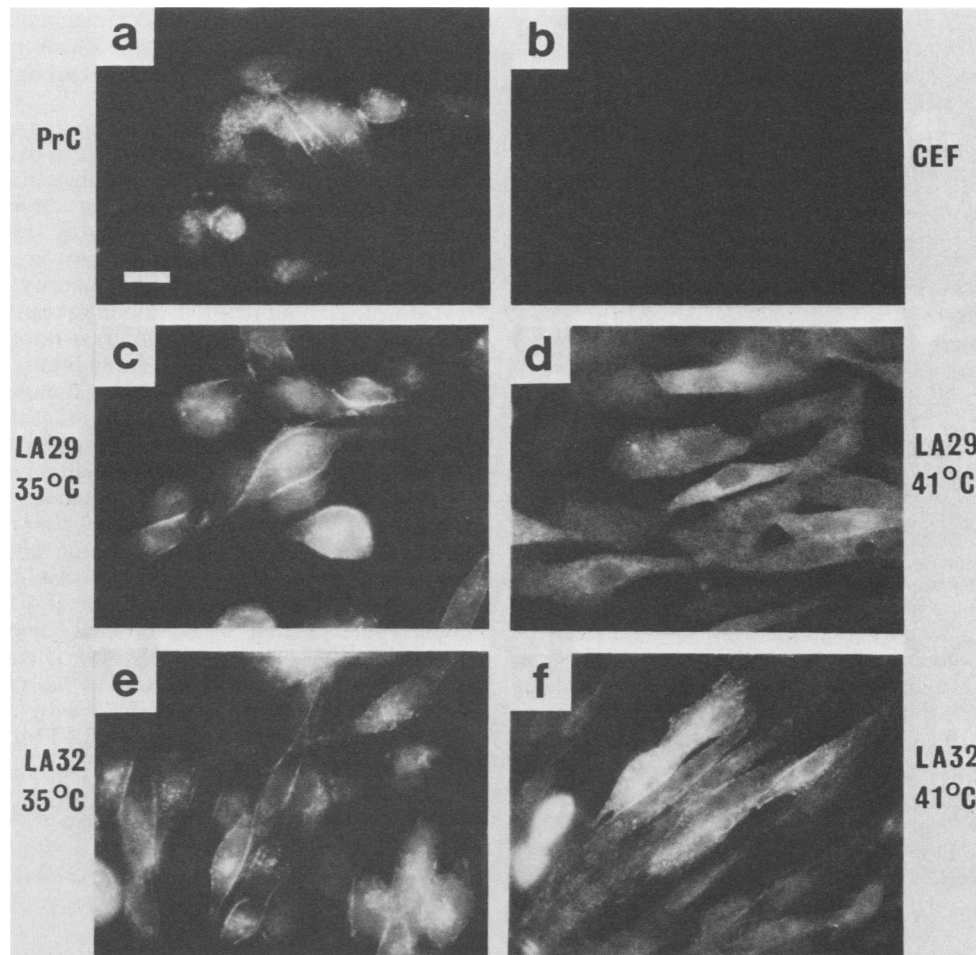
FIG. 1. Immunofluorescence localization of pp60$^{v\text{-}src}$ in transformed CEF. Cells were stained with monoclonal anti-pp60^{src} as described in Materials and Methods. (a) PrC transformed; (b) untransformed CEF; (c) *LA*29-transformed CEF at 35°C; (d) *LA*29-transformed CEF at 41°C; (f) *LA*32-transformed CEF at 35°C; (g) *LA*32-transformed CEF at 41°C. Bar = 10 μm.

maturation (5, 19), is not responsible for their temperature lability.

Fibronectin matrix production. The production of a fibronectin matrix is dramatically reduced in CEF transformed by RSV (35). When CEF infected with either *LA*29 or *LA*32 were grown at nonpermissive temperature, the cells produced a fibronectin matrix indistinguishable from that synthesized by uninfected CEF (Fig. 3i and k). At permissive temperature, the mutant virus-infected cells showed a large reduction in matrix production as expected. However, although the *LA*29-transformed CEF matrix was reduced to the residual level seen in wild-type-transformed cells, the *LA*32-transformed cells consistently produced a residual fibronectin matrix to a greater degree than either wild-type- or *LA*29-transformed cells (Fig. 3j and l). This residual level of fibronectin has not been analyzed quantitatively.

F-actin and α-actinin distribution. The two cytoskeletal proteins actin and α-actinin undergo gross intracellular redistributions upon cell transformation by RSV (14, 33). The temperature-dependent effects of the mutant RSV upon these proteins were examined. The distribution of F-actin in RSV-infected CEF was examined by fluorescence microscopy, using NBD-phallicidin. At 41°C both *LA*29- and *LA*32-infected cells grew as morphologically normal, flat cells with many prominent microfilament bundles running through the cytoplasm similar to those seen in uninfected CEF (Fig. 3a and c). At permissive temperature both mutants induced the expected dissolution of actin cables in the transformed cells. However, in the *LA*32-infected cells a sparse pattern of cables was consistently seen compared with *LA*29- or wild-type-transformed CEF (Fig. 3b and d). Although this observation is not quantitative, it may be related to the more fusiform morphology of the *LA*32-transformed cells at 35°C (44). The mutant- and wild-type-transformed cells all exhibited characteristic aggregates of F-actin within the cytoplasm at permissive temperature.

The distribution of α-actinin in the infected CEF closely paralleled that of F-actin. Upon temperature shift from 35 to 41°C, the diffuse, cytoplasmic pattern of α-actinin staining changed to the characteristic periodic staining along microfilament bundles as seen in uninfected CEF (Fig. 3e to h). The mutant-infected CEF thus exhibit full temperature sensitivity in the redistributions of F-actin and α-actinin, whereas the distributions of these proteins in uninfected or wild-type-transformed CEF were unaffected by growth temperature.

DISCUSSION

In this paper we extend our characterization of the mutant pp60$^{v\text{-}src}$ proteins encoded by *tsLA*32 and *tsLA*29 and ana-
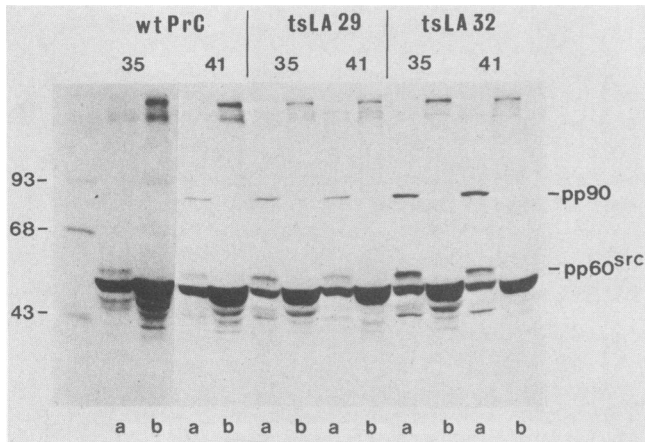
FIG. 2. Immunoprecipitation of pp60$^{v\text{-}src}$ and associated complex proteins. RSV-infected CEF were labeled with [^{35}S]methionine for 18 h at permissive (35°C) and nonpermissive (41°C) temperatures. pp60$^{v\text{-}src}$ was immunoprecipitated with monoclonal JB327. Lanes a, JB327 immunoprecipitates; lanes b, control normal rabbit serum immunoprecipitates.

lyze further parameters of transformation seen in CEF infected by these mutant RSV. Table 2 summarizes our current knowledge of the two mutants in this study.

The mutations in *LA*32 and *LA*29 have been approxi-

mately located by marker rescue mapping: *LA*32 contains a mutation(s) in the N-terminal 60% of pp60$^{v\text{-}src}$, whereas *LA*29 is mutated very near to the carboxy terminus of the protein (17).

*tsLA*29. Although we can detect no temperature sensitivity in either steady-state complex levels or myristylation of this mutant protein, it is clear that its intracellular localization is abnormal, particularly at restrictive temperature. A reduction in membrane binding is seen at both temperatures, although at 35°C there is no apparent impairment of transformation. Although at 41°C the majority of the pp60$^{v\text{-}src}$ is cytosolic, we note that the amount of pp90 bound does not increase proportionately, in contrast to other *ts* mutants of pp60$^{v\text{-}src}$, such as *NY*68 (11). In addition, we can detect an increased sensitivity of pp90-pp60 complexing to ionic detergents in vitro. We suggest, given that the *LA*29 mutation lies outside the membrane-binding region of pp60$^{v\text{-}src}$, that a defect in complex binding in the cell may be one aspect of this protein's dysfunction. This would appear to occur after initial complex binding, and possibly after myristylation, and may lead to premature dissolution of the complex. Such a malfunction could result in the observed failure of pp60$^{v\text{-}src}$ to associate well with the membrane at 41°C and, bearing in mind evidence that the C-terminal region of pp60$^{v\text{-}src}$ is involved with complex binding (46), it would not be unexpected. In addition, Wilkerson et al. recently described site-directed variants of pp60$^{v\text{-}src}$ with alterations in the C-terminal domain of the protein (48). These proteins behave
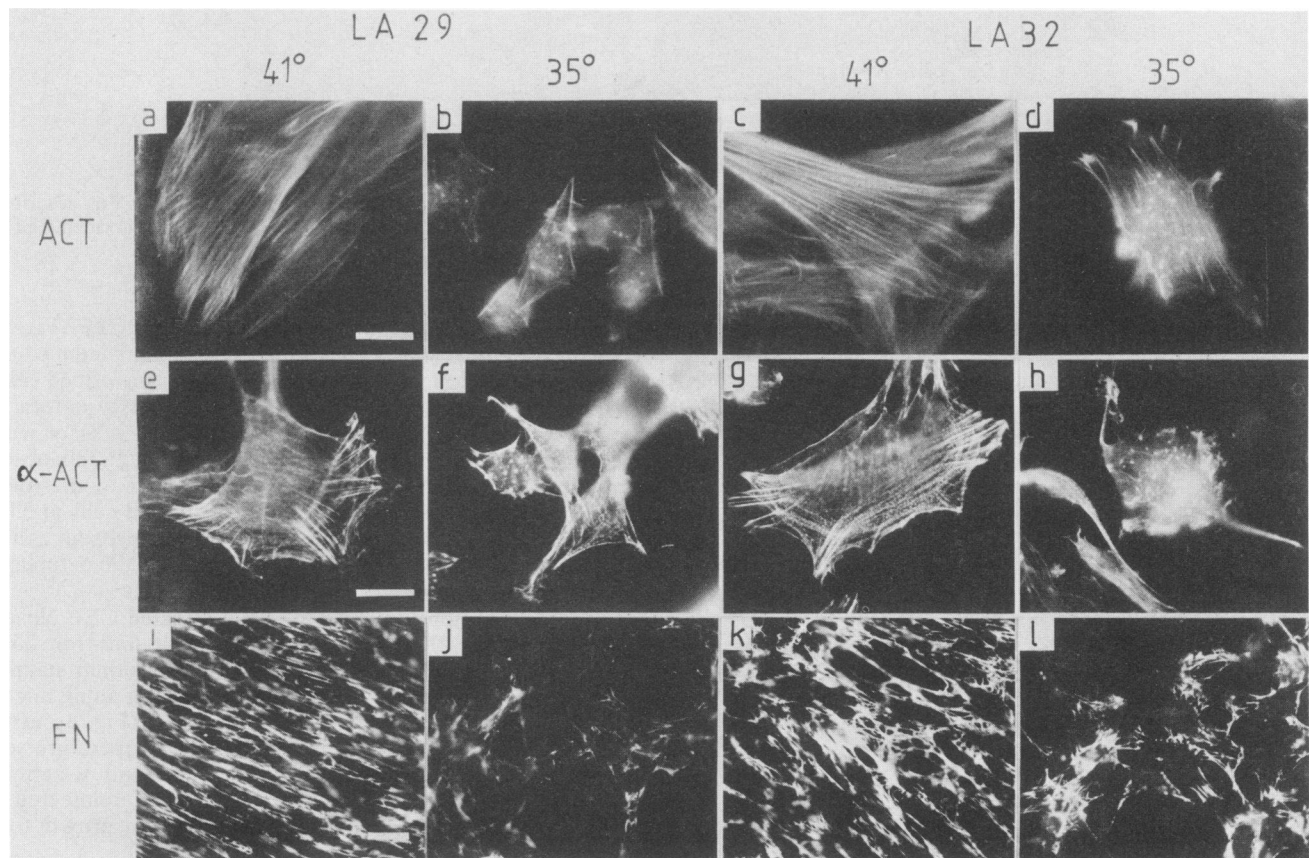
FIG. 3. Staining of actin (upper panel), α-actinin (middle panel), and fibronectin (lower panel) in transformed CEF. Cells were stained with NBD-phallicidin, rabbit anti-α-actinin, or rabbit anti-fibronectin as described in Materials and Methods. (a, e, i) *LA*29-transformed CEF at 41°C; (b, f, j) *LA*29-transformed CEF at 35°C; (c, g, k) *LA*32-transformed CEF at 41°C; (d, h, l) *LA*32-transformed CEF at 35°C. For (a) to (d) and (e) to (h), bar = 10 μm. For (i) to (l), bar = 100 μm.

TABLE 2. Parameters of transformation and pp60^{v-src} function in *ts* mutant-infected CEF[a]

Parameter	Infecting virus		
	Wild-type PrC	*ts*LA29	*ts*LA32
Transformation			
Morphological transformation	+[b]	*ts*[b]	*ts*
Soft-agar growth	+	*ts*	*ts*[c]
Fibronectin matrix production	+	*ts*	*ts*
Actin/α-actinin cable dissolution	+	*ts*	*ts*
Hexose uptake[d]	+	*ts*	*ts*
pp60^{v-src} function			
In vitro kinase activity	+	*ts*	+
In vivo phosphotyrosine elevation	+	*ts*	+
In vivo autophosphorylation	+	*ts*	*ts*
36K phosphorylation	+	*ts*	+
Vinculin phosphorylation[e]	+	*ts*	+
Complex formation	+	+[f]	+
Myristylation state	+	+	+
Localization[g]	+	*ts*	+

[a] Data are presented in this paper and Stoker et al. (44) unless otherwise stated.

[b] + and *ts* indicate absence and presence of temperature sensitivity, respectively.

[c] Some residual growth is seen at 41°C (44).

[d] Stoker, unpublished data.

[e] Kellie et al., in press.

[f] Although total levels of complex are unchanged, the protein may exhibit other defects in complex stability (see text).

[g] Localization data from both cell fractionation and immunofluorescence of pp60^{v-src} (see text).

(nonconditionally) in a manner similar to LA29 at 41°C. These mutants are kinase defective and predominantly cytosolic, and there is a low level of association with the complex proteins. The mutation in LA29 lies in the region affected in these site-directed variants, suggesting that both are defective in a similar, possibly regulatory domain of the protein. We cannot yet say whether the temperature-dependent kinase defect of LA29 results from, is causative in, or is unrelated to the transport defect. Given that genetic analysis indicates a single mutation towards the 3' end of this v-*src* gene, it is noteworthy that this mutant suffers from a number of processing and functional defects, suggesting that the lesion has pleiotropic effects on a structural or temporal basis.

*ts*LA32. The LA32 mutant pp60^{v-src} shows no obvious defect in complex formation or myristylation. The protein also remains membrane localized to a significant degree at nonpermissive temperature; in addition, we know the protein remains localized within adhesion plaques at 41°C (Kellie et al., in press). We have therefore ruled out several causes of the transformation defect in LA32 and suggest that the lesion in the protein disrupts its function at a late stage in maturation subsequent to membrane binding. It is likely that the protein is impaired in some specific interaction required at or near the plasma membrane, disrupting either kinase substrate specificity or a critical association with other cellular structures necessary for the maintenance of the transformed state. Of interest here are two recent reports by Garber et al. (19) and Cross et al. (12) describing pp60^{v-src} proteins with deletions between amino acids 15 and 169. This region is within the area to which the LA32 lesion has been mapped, and indeed the deletion mutants affect pp60^{v-src}

behavior in a similar way. The kinase functions are not apparently affected directly, and full morphological transformation of CEF expressing the proteins does not occur (cf. LA32 at 35°C). These mutants and our LA32 data together strongly suggest a critical functional role for domains in the N-terminal half of pp60^{v-src}.

It has recently been reported that pp60^{v-src} may be able to cause phosphorylation of phosphoinositide derivatives in the cell, either directly or indirectly, thus influencing a cell biochemical pathway of importance in cell growth control (45). Given that we have eliminated several sources of the lesion in LA32, we are at present analyzing the possibility that the mutant is defective in this function.

Although CEF infected with LA32 at 41°C show increased phosphotyrosine levels and retain some anchorage-independent growth (44), there are no residual structural features of transformation in these cells, nor is the hexose uptake rate elevated (A. Stoker, unpublished data). Indeed, even at permissive temperature, cells transformed by LA32 show a less dramatic loss of fibronectin matrix and redistribution of actin and α-actinin than similar cells transformed by wild-type or LA29 virus. This correlates with the somewhat fusiform morphology of LA32 transformants (44), and it is of interest that we have mapped the lesion in a fully fusiform variant of RSV (*td* SF/LO104) to the amino-terminal half of pp60^{v-src} (17). Sequencing of the v-*src* genes of LA32 (work in progress) and the fusiform virus should reveal whether the *ts* and fusiform phenotypes represent distinct mutations or manifestations of the same lesion.

In conclusion, we have analyzed two *ts* mutants of pp60^{v-src} which have lesions in different domains of the protein and which present defects in pp60^{v-src} function varying in both pattern and degree. The LA29 mutant is of interest considering the pleiotropic nature of the defects expressed in pp60^{v-src}. The mutation in LA32, in an as yet undefined domain of pp60^{v-src}, abolishes almost all transformation parameters at 41°C but has little effect on detectable facets of pp60^{v-src} maturation and function, with the exception of the previously demonstrated reduction in phosphorylation of pp60^{v-src} itself (44). This latter finding is under continuing investigation at present.

Further studies with such *ts* mutants and elucidation of the defects present should be of considerable value in advancing our understanding of the mechanism of pp60^{v-src}-mediated cell transformation.

LITERATURE CITED

1. **Beemon, K., T. Ryden, and E. A. McNelly.** 1982. Transformation by avian sarcoma viruses leads to phosphorylation of multiple cellular proteins on tyrosine residues. J. Virol. **42**:742–747.
2. **Brugge, J. S., E. Erikson, and R. L. Erikson.** 1981. The specific interaction of the Rous sarcoma virus transforming protein, pp60^{src}. Cell **25**:363–372.
3. **Brugge, J. S., P. J. Steinbaugh, and R. L. Erikson.** 1978. Characterisation of the avian sarcoma virus protein pp60^{src}. Virology **91**:130–140.
4. **Brugge, J. S., W. Yonemoto, and D. Darrow.** 1983. Interaction between the Rous sarcoma virus transforming protein and two cellular phosphoproteins: analysis of the turnover and distribution of this complex. Mol. Cell. Biol. **3**:9–19.
5. **Buss, J. E., N. P. Kamps, and B. M. Sefton.** 1984. Myristic acid

is attached to the transforming protein of Rous sarcoma virus during or immediately after synthesis and is present in both soluble and membrane-bound forms of the protein. Mol. Cell. Biol. 4:2697–2704.

6. **Collett, M. S., and R. L. Erikson.** 1978. Protein kinase activity associated with the avian sarcoma virus *src* gene product. Proc. Natl. Acad. Sci. USA 75:2021–2024.

7. **Collett, M. S., A. F. Purchio, and R. L. Erikson.** 1980. Avian sarcoma virus-transforming protein, pp60^{src} shows protein kinase activity specific for tyrosine. Nature (London) 285:167–169.

8. **Cooper, J. A., and T. Hunter.** 1981. Changes in protein phosphorylation in Rous sarcoma virus-transformed chicken embryo cells. Mol. Cell. Biol. 1:165–178.

9. **Cooper, J. A., and T. Hunter.** 1983. Identification of cellular targets for tyrosine protein kinases. J. Biol. Chem. 258:1108–1115.

10. **Cooper, J. A., N. A. Reiss, R. J. Schwartz, and T. Hunter.** 1983. Three glycolytic enzymes are phosphorylated on tyrosine in cells transformed by Rous sarcoma virus. Nature (London) 302:218–222.

11. **Courtneidge, S. A., and J. M. Bishop.** 1982. Transit of pp60^{v-src} to the plasma membrane. Proc. Natl. Acad. Sci. USA 79:7117–7121.

12. **Cross, F. R., E. A. Garber, and H. Hanafusa.** 1985. N-terminal deletions in Rous sarcoma virus p60^{src}: effects on tyrosine kinase and biological activities and on recombination in tissue culture with the cellular *src* gene. Mol. Cell. Biol. 5:2789–2795.

13. **Cross, F. R., E. A. Garber, D. Pellman, and H. Hanafusa.** 1984. A short sequence in the p60^{src} N terminus is required for p60^{src} myristylation and membrane association and for cell transformation. Mol. Cell. Biol. 4:1834–1842.

14. **David-Pfeuty, T., and S. J. Singer.** 1980. Altered distributions of cytoskeletal proteins vinculin and alpha-actinin in cultured fibroblasts transformed by Rous sarcoma virus. Proc. Natl. Acad. Sci. USA 77:6687–6691.

15. **Erikson, E., and R. L. Erikson.** 1980. Identification of a cellular protein substrate phosphorylated by pp60^{src}. Cell 21:829–836.

16. **Erikson, R. L., M. S. Collett, E. Erikson, and A. F. Purchio.** 1979. Evidence that the avian sarcoma virus transforming gene product is a cyclic-AMP-independent protein kinase. Proc. Natl. Acad. Sci. USA 76:6260–6264.

17. **Fincham, V. J., D. J. Chiswell, and J. A. Wyke.** 1982. Mapping of nonconditional and conditional mutants in the *src* gene of Prague strain Rous sarcoma virus. Virology 116:72–83.

18. **Friis, R. J., R. T. Schwartz, and M. F. G. Schmidt.** 1977. Phenotype of Rous sarcoma virus-transformed fibroblasts: an argument for a multifunctional *src* gene product. Med. Microbiol. Immunol. 64:155–165.

19. **Garber, E. A., F. R. Cross, and H. Hanafusa.** 1985. Processing of pp60^{v-src} to its myristylated membrane-bound form. Mol. Cell. Biol. 5:2781–2788.

20. **Garber, E. A., and H. Hanafusa.** 1983. Temperature sensitive membrane association of pp60^{src} in NY68-infected cells correlates with increased tyrosine phosphorylation of membrane-associated proteins. Virology 126:73–86.

21. **Garber, E. A., J. B. Krueger, H. Hanafusa, and A. R. Goldberg.** 1983. Only membrane-associated RSV *src* proteins have amino-terminally bound lipid. Nature (London) 302:161–163.

22. **Gilmer, T. M., and R. L. Erikson.** 1983. Development of anti-pp60^{src} serum with antigen produced in *Escherichia coli*. J. Virol. 45:462–465.

23. **Gilmore, T. D., K. Radke, and G. S. Martin.** 1982. Tyrosine phosphorylation of a 50K cellular polypeptide associated with the Rous sarcoma virus transforming protein pp60^{src}. Mol. Cell. Biol. 2:199–206.

24. **Hanafusa H.** 1977. Cell transformation by RNA tumor viruses, p. 401–483. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.), Comprehensive virology, vol. 10. Plenum Press, New York.

25. **Hunter, T., and B. M. Sefton.** 1980. Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc. Natl. Acad. Sci. USA 77:1311–1315.

26. **Kamps, M. P., J. E. Buss, and B. M. Sefton.** 1985. Mutation of NH$_2$-terminal glycine of pp60^{src} prevents both myristylation and morphological transformation. Proc. Natl. Acad. Sci. USA 82:4625–4628.

26a. **Kellie, S., B. Patel, E. Pierce, and D. R. Critchley.** 1983. Capping of cholera toxin-ganglioside GM$_1$ complexes on mouse lymphocytes is accompanied by co-capping of α-actinin. J. Cell. Biol. 97:447–454.

27. **Krueger, J. G., E. A. Garber, and A. R. Goldberg.** 1984. Subcellular localisation of pp60^{src} in RSV-transformed cells. Curr. Top. Microbiol. Immunol. 107:51–112.

28. **Krueger, J. G., E. R. Garber, A. R. Goldberg, and H. Hanafusa.** 1982. Changes in amino-terminal sequences of pp60^{src} lead to decreased membrane association and decreased in vivo tumorigenicity. Cell 28:889–896.

29. **Levinson, A. D., S. A. Courtneidge, and J. M. Bishop.** 1981. Structural and functional domains of Rous sarcoma virus transforming protein pp60^{src}. Proc. Natl. Acad. Sci. USA 78:1624–1628.

30. **Levinson, A. D., H. Opperman, L. Levintow, H. Varmus, and J. M. Bishop.** 1978. Evidence that the transforming gene of avian sarcoma virus encodes a protein kinase associated with a phosphoprotein. Cell 15:561–572.

31. **Levinson, A. D., H. Opperman, H. E. Varmus, and J. M. Bishop.** 1980. The purified product of the transforming gene of avian sarcoma virus phosphorylates tyrosine. J. Biol. Chem. 255:11973–11980.

32. **Lipsich, L. A., A. J. Lewis, and J. S. Brugge.** 1983. Isolation of monoclonal antibodies that recognize the transforming proteins of avian sarcoma virus. J. Virol. 48:352–360.

33. **Maness, P. F., and B. T. Levy.** 1983. Highly purified pp60^{src} induces the actin transformation in microinjected cells and phosphorylates selected cytoskeletal proteins in vitro. Mol. Cell. Biol. 3:102–112.

34. **Martinez, R., K. D. Nakamura, and M. J. Weber.** 1982. Identification of phosphotyrosine-containing proteins in untransformed and Rous sarcoma virus-transformed chicken embryo fibroblasts. Mol. Cell. Biol. 2:653–665.

35. **Olden, K., and K. M. Yamada.** 1977. Mechanism of the decrease in the major cell surface protein of chick embryo fibroblasts after transformation. Cell 11:957–969.

36. **Opperman, H., A. D. Levinson, L. Levintow, H. E. Varmus, J. M. Bishop, and S. Kawai.** 1981. Two cellular proteins that immune precipitate with the transforming protein of Rous sarcoma virus. Virology 113:736–751.

37. **Parsons, J. T., D. Bryant, V. Wilkerson, G. Gilmartin, and S. J. Parsons.** 1984. Site-directed mutagenesis of Rous sarcoma virus pp60^{src}: identification of functional domains required for transformation, p. 37–42. *In* G. F. Van de Woude, A. J. Levine, W. C. Topp, and W. C. Watson (ed.), Cancer cells, vol. 2. Oncogenes and viral genes. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

38. **Purchio, A. F., E. Erikson, J. S. Brugge, and R. L. Erikson.** 1978. Identification of a polypeptide encoded by the avian sarcoma virus *src* gene. Proc. Natl. Acad. Sci. USA 75:1567–1571.

39. **Radke, K., T. Gilmore, and G. S. Martin.** 1980. Transformation by Rous sarcoma virus: a cellular substrate for transformation-specific protein phosphorylation contains phosphotyrosine. Cell 21:821–828.

40. **Reddy, E. P., M. J. Smith, and A. Srinivasan.** 1983. Nucleotide sequence of Abelson murine leukemia virus genome: structural similarity of its transforming gene product to other *onc* gene products with tyrosine-specific kinase activity. Proc. Natl. Acad. Sci. USA 80:3623–3627.

41. **Sefton, B. M., T. Hunter, E. H. Ball, and S. J. Singer.** 1981. Vinculin: a cytoskeletal target of the transforming protein of Rous sarcoma virus. Cell 24:165–174.

42. **Sefton, B. M., T. Hunter, K. Beemon, and W. Eckhart.** 1980. Evidence that the phosphorylation of tyrosine is essential for cellular transformation by Rous sarcoma virus. Cell 20:807–816.

43. **Sefton, B. M., I. S. Trowbridge, J. A. Cooper, and E. M. Scolnick.** 1982. The transforming proteins of Rous sarcoma virus, Harvey sarcoma virus and Abelson sarcoma virus contain

tightly bound lipid. Cell **31**:465–474.

44. **Stoker, A. W., P. J. Enrietto, and J. A. Wyke.** 1984. Functional domains of the pp60^{v-src} protein as revealed by analysis of temperature-sensitive Rous sarcoma virus mutants. Mol. Cell. Biol. **4**:1508–1514.

45. **Sugimoto, Y., M. Whitman, L. C. Cantley, and R. L. Erikson.** 1984. Evidence that the Rous sarcoma virus transforming gene product phosphorylates phosphatidylinositol and diaglycerol. Proc. Natl. Acad. Sci. USA **81**:2117–2121.

46. **Tamura, T., H. Bauer, C. Birr, and R. Pipkorn.** 1983. Antibodies against synthetic peptides as a tool for functional analysis of the transforming protein pp60^{src}. Cell **34**:587–596.

47. **Tato, F., J. A. Beamand, and J. A. Wyke.** 1978. A mutant of Rous sarcoma virus with a thermolabile defect in the virus envelope. Virology **88**:71–81.

48. **Wilkerson, V. W., D. L. Bryant, and J. T. Parsons.** 1985. Rous sarcoma virus variants that encode *src* proteins with an altered carboxy terminus are defective for cellular transformation. J. Virol. **55**:314–321.

49. **Wyke, J. A., and M. Linial.** 1973. Temperature-sensitive avian sarcoma viruses: a physiological comparison of twenty mutants. Virology **5**:152–161.