Transformation Parameters and pp60^{src} Localization in Cells Infected with Partial Transformation Mutants of Rous Sarcoma Virus

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Rous sarcoma virus (RSV)-induced transformation is mediated by the action of the viral *src* gene product pp60^{src}. This transforming protein is found at several cytoplasmic locations, including the adhesion plaques of RSV-transformed cells. In these studies, we have focused on the adhesion plaque location of pp60^{src} and determined whether any of the induced transformation parameters correlate with the presence of pp60^{src} in the adhesion plaques. A series of partial transformation mutants of RSV that induce distinct transformation phenotypes were used, and infected chicken embryo cells were examined for (i) intracellular pp60^{src} location, (ii) vinculin localization, (iii) abundance of phosphotyrosine on vinculin, (iv) integrity of stress fibers, and (v) expression of cell surface fibronectin. The results indicate that, among the limited number of mutants studied here, the presence of pp60^{src} in adhesion plaques is independent of growth in soft agar and the increased phosphorylation of vinculin on tyrosine, but it does correlate with the loss of cell surface fibronectin. An elevated abundance of phosphotyrosine on vinculin is insufficient to cause stress fiber dissolution and is independent of the loss of fibronectin from the extracellular matrix. However, the increased relative amount of phosphotyrosine on vinculin is related to the ability of the cells to grow in soft agar. The adhesion plaque binding and tyrosine-specific kinase activities seem to represent two independent functions of pp60^{src}.

Neoplastic transformation results in numerous cellular alterations whose cumulative effect defines the total transformed phenotype. These cellular alterations often represent a rather diverse group of changes, and it is difficult to imagine a single cause to account for such diversity. In the case of Rous sarcoma virus (RSV)-transformed cells, however, both the initiation and the maintenance of the transformed state are controlled by a single viral gene (the *src* gene) whose protein product mediates the transformation events (8). This protein, pp60^{src}, is a 60,000-dalton phosphoprotein with a tyrosine-specific kinase activity (21, 25, 30), and although this is the only transforming protein detectable so far, genetic studies indicate that multiple independent targets seem to be required for transformation (4, 5, 7, 10, 47). Although the *src* gene may yet yield a second protein product (G. Mardon and H. E. Varmus, *Cell*, in press), pp60^{src} alone most likely phosphorylates multiple substrates and could possess additional uncharacterized functions.

pp60^{src} has been reported at various cellular locations within RSV-transformed cells. One principal location, however, appears to be the

cytoplasmic face of the plasma membrane and specialized regions of that structure such as cell-cell junctions and adhesion plaques (35, 36, 42, 50). These latter structures are small membrane regions (averaging 1.8 μm long and 0.75 μm wide) (1), situated on the ventral surface of cells in culture (1, 14, 23, 29). The adhesion plaques mediate adhesion between a cell and the substratum on which it grows. In addition, individual bundles of microfilaments, or stress fibers, traverse the intracellular space near the cytoplasmic face of the plasma membrane and terminate within adhesion plaques which anchor this complex to the substratum (2, 18, 23, 29, 33, 49). On the exterior of the cell, the extracellular matrix protein, fibronectin, is closely associated with adhesion plaques, at least during some phases of cell growth (9, 43). Alterations in these adhesion plaque-associated structures and functions, both intra- and extracellular, are well-characterized parameters of RSV-transformed cells (22). Therefore, the location of pp60^{src} within the adhesion plaques suggests that adhesion plaques are directly involved in the mechanism of transformation by RSV.

At the molecular level, transformation by

RSV most likely involves phosphorylation of host cell proteins on tyrosine residues (40). This tyrosine phosphotransferase activity is rare in uninfected cells, and it has been suggested that increased content of phosphotyrosine in proteins constitutes *prima facie* evidence for pp60^{src}-specific substrates (25, 39–41). Indeed, several phosphotyrosine-containing proteins, including three enzymes of the glycolytic pathway, have been identified in total cell extracts (12, 13; J. A. Cooper, N. A. Reiss, R. J. Schwartz, and T. Hunter, *Nature* [London], in press). Two cellular phosphoproteins of 36,000 and 130,000 (36K and 130K) daltons also have been extensively characterized and found to contain an elevated abundance of phosphotyrosine in RSV-transformed cells (16, 32, 39), and this phosphorylation is dependent on the activity of the *src* gene product. The 36K-dalton phosphoprotein may be associated with the cytoskeleton (11, 13), and the 130K-dalton phosphoprotein (vinculin) has been localized to the adhesion plaques of normal cells, where it is postulated to participate in anchorage of the stress fibers to the plasma membrane at these sites (18–20). The phosphorylation of vinculin on tyrosine and colocalization of vinculin and pp60^{src} within adhesion plaques (41, 42) further supports the notion that adhesion plaques represent important target sites in the mechanism of transformation by the *src* gene product.

We have previously characterized the biochemical interactions of pp60^{src} with adhesion plaque proteins (41; K. Shriver and L. Rohrschneider, manuscript in preparation), and in the present studies, we have focused on analyzing transformation parameters associated with pp60^{src} interacting with target cells. We have approached this problem by examining mutants of RSV containing partial defects within the *src* gene (4, 5, 17, 47). These partial mutations render some interactions of pp60^{src} inactive, whereas other interactions are retained and remain functional. Cells infected with such partial transformation mutants display some, but not all, properties or parameters of transformation. Studies with such mutants have confirmed the view that pp60^{src} is a multifunctional protein and that the pp60^{src} of each partial transformation mutant is defective in at least one function or interaction with the target cell (4, 47). For these studies, we have attempted to correlate transformation parameters with specific pp60^{src} location in infected cells. We have examined chicken embryo cells (CEC) infected with various partial transformation mutants of RSV to determine (i) the intracellular location of the mutant pp60^{src}, (ii) whether stress fibers remain intact, (iii) whether the distribution of vinculin is altered in these cells, (iv) the extent of phosphorylation of

vinculin on tyrosine, and (v) the distribution of fibronectin on these cells.

(These results were presented, in part, at the Cold Spring Harbor Symposium on Organization of the Cytoplasm [34]).

MATERIALS AND METHODS

Cells, viruses, and culture conditions. Chicken embryo fibroblasts (phenotype C/E) were cultured from 10-day-old embryos of White Leghorn chickens obtained from Heisdorf and Nelson Laboratories (Redmond, Wash.). Cells were grown in F10 medium (GIBCO Laboratories, Grand Island, N.Y.) containing 10% tryptose phosphate, 5% calf serum, 1% dimethyl sulfoxide, and 1% chicken serum.

The partial transformation mutants CU2, *ts*CU11, and CU12 were obtained from Mike Weber (University of Illinois, Urbana). The parental wild-type, Schmidt-Ruppin strain of RSV, subgroup A (SR-A), and *ts*NY68 were obtained from the same source. An additional virus exhibiting properties of a partial transformation mutant was isolated from a stock of Schmidt-Ruppin strain of RSV rescued from transformed rat cells (SR-NRK clone A4B5G4). The rescued virus was further cloned in quail fibroblasts and designated RD10 virus, clone 4A1L. Cells infected with RD10-4A1L exhibited a flat normal morphology, yet they formed foci and grew in soft agar. Chickens injected with the RD10-4A1L virus developed large tumors at the site of injection (unpublished data).

Immune reagents. Antiserum to the RSV transforming protein (pp60^{src}) was obtained from rabbits bearing tumors induced by SR-RSV-D. This tumor-bearing rabbit serum (B20) has been previously characterized and is virtually monospecific for pp60^{src} when preabsorbed with excess unlabeled SR-RSV-D (35, 36, 42). Vinculin was isolated from chicken gizzard by the method of Geiger (18). The final purification involved polyacrylamide gel electrophoresis and electroelution of the 130K-dalton vinculin band. Antiserum to the sodium dodecyl sulfate-denatured vinculin was prepared in rabbits (42) and affinity purified on electroeluted vinculin coupled to glutaraldehyde-activated Ultrogel AcA-44 (LKB Instruments Inc., Rockville, Md. [44]). Antiserum to native vinculin (i.e., before gel electrophoresis) was also prepared in a guinea pig and a rabbit. This latter rabbit anti-vinculin serum was very clean and was used for immune precipitating vinculin. Rabbit anti-filamin serum was a gift from Klaus Weber (Goettingen, West Germany). Affinity-purified antibodies to human fibronectin (cold insoluble globulin) were obtained from Bill Carter, Fred Hutchinson Cancer Research Center, Seattle, Wash.

Rhodamine- or fluorescein-conjugated antibodies to rabbit or guinea pig immunoglobulin G (IgG) were obtained from N. L. Cappel Laboratories, Inc. (Cochranville, Pa.) and affinity purified on the appropriate IgG coupled to glutaraldehyde-activated Ultrogel AcA-44 (42).

Indirect immunofluorescence microscopy. The indirect immunofluorescence technique for detecting a single antigen or for simultaneously detecting two antigens has been previously described (36, 42). For viewing fluorescence, cover slips were mounted in

TABLE 1. Properties of uninfected and virus-infected CEC used in these studies

CEC infected with:	Transformation phenotype	Morphology	Growth in soft agar
Uninfected	Normal, uninfected	Normal, flat	—
SR-A	Complete or wild type	Rounded, refractile	+++
<i>ts</i> NY68 (35°C)	Complete temperature sensitive	Rounded, refractile	+++
<i>ts</i> NY68 (42°C)	Complete temperature sensitive	Normal	—
CU2	Partial	Flat, blebby	+
<i>ts</i> CU11 (35°C)	Partial temperature sensitive	Near normal	+++
<i>ts</i> CU11 (42°C)	Partial temperature sensitive	Normal	—
CU12	Partial	Fusiform	+++
RD10	Partial	Flat	+++

^a CU2-infected CEC grew in soft agar but formed only very small colonies.

Elvanol (Monsanto). To observe both immunofluorescence and interference-reflection images (see below), cover slips were mounted in phosphate-buffered saline above a 3-mm-deep glass chamber. All photographs of fluorescence images were recorded with a Zeiss photomicroscope III on Tri-X film (Kodak) rated ASA 1600 and developed in Diafine (Acufine, Inc., Chicago, Ill).

Interference-reflection microscopy. A technique for viewing the adhesion plaques of cells by interference-reflection microscopy has been described previously (36, 42).

Phosphoamino acid analysis. Uninfected and virus-infected CEC (10⁶ cells) were plated onto 35-mm tissue culture dishes and incubated for 24 to 28 h at the appropriate temperature. The cells then were labeled for 16 h with 1.5 mCi of ³²P_i in 1.5 ml of phosphate-free minimal essential medium supplemented with 5% calf serum. The cells were lysed with RIPA buffer (35) containing 10 mM EDTA and were centrifuged for 15 min at 12,900 × *g*. Proteins in the clear lysate were extracted into phenol, precipitated with trichloroacetic acid, and washed with chloroform-methanol as described by Sefton et al. (40). Phosphoamino acids in the total cell protein were liberated by hydrolysis in constantly boiling HCl (Pierce Chemical Co., Rockford, Ill.) at 110°C for 2 h. The phosphoamino acids were separated by two-dimensional electrophoresis on plastic-backed cellulose thin layer plates (Kodak). Separation in the first dimension (3,000 V, 90 min) was in acetic acid-formic acid-water (75:20:905), and the second dimension (3,000 V, 30 min) was electrophoresed in acetic acid-pyridine-water (50:5:945). Initial experiments contained phosphoserine, phosphothreonine, and phosphotyrosine as internal ninhydrin-stainable standards. However, autoradiography with the aid of an intensifying screen (Cronex, Dupont) was sufficient to detect the three phosphoamino acids without standards. The spots containing the individual phosphoamino acids were scraped from the plate, and the radioactivity was measured by scintillation counting in Biofluor (New England Nuclear Corp.).

The abundance of phosphotyrosine in vinculin was determined by a modification of the method published by Sefton et al. (39). Cells were labeled with ³²P_i (2 mCi/ml, 16 to 18 h) in phosphate-free minimal essential medium containing 5% calf serum. Radioactive vinculin was immune precipitated from the cell lysate, using RIPA buffer containing 10 mM EDTA, and electro-

phoresed on an 11% polyacrylamide gel (35). The dried gel was autoradiographed, and vinculin was recovered by the method of Beemon and Hunter (6) or by extraction into 0.01 M Tris-hydrochloride buffer containing 1% sodium dodecyl sulfate and 1 mg of dithiothreitol per ml. Every 2 ml of extract was mixed with 3 ml of methanol-acetic acid (100:1), and the proteins were precipitated overnight at -70°C and collected by centrifugation (SW50.1 rotor) for 30 min at 50 krpm (-8°C) (Joe Brown, personal communication).

RESULTS

The virus-infected cells we studied are listed in Table 1, together with a few pertinent properties of the uninfected, wild-type-, and mutant-infected CEC. A description of the transformed phenotype induced by the individual viruses is also presented. Most of these properties (with the exception of the RD10-infected CEC) were taken from a publication by Anderson et al. (4).

pp60^{src} localization and adhesion plaques. CEC infected with the partial transformation mutant CU2 exhibit a flat morphology, often displaying numerous blebs on the dorsal cell surface (Table 1). Using antiserum from rabbits bearing tumors induced by the Schmidt-Ruppin strain of RSV, we examined the distribution of pp60^{src} within these mutant-infected CEC by indirect immunofluorescence. This anti-tumor serum was specific for pp60^{src} after absorbing out antibodies to viral structural proteins with unlabeled virus (35, 42). The photograph in Fig. 1A shows the distribution of pp60^{src} within a CU2-infected CEC, and the corresponding image of the ventral surface of the same cell is shown by interference-reflection microscopy in Fig. 1B. pp60^{src} was observed at several locations within the CU2-infected CEC. A pale fluorescence throughout the cytoplasm of these cells suggested a cytoplasmic location for some pp60^{src}. More intense fluorescence was observed at the advancing lamellipodium (Fig. 1A, right side of cell), and this appeared to trail into the newly formed adhesion plaques identified by the interference-

FIG. 1. Localization of pp60^{src} within the adhesion plaques of CU2-infected CEC. CEC infected with the partial mutant CU2 were grown on glass cover slips, fixed, and stained for pp60^{src} localization by indirect immunofluorescence, using tumor-bearing rabbit serum (A). The same cell was viewed by interference-reflection microscopy (B) to visualize the topology of the ventral cell surface. The adhesion plaques are seen as the darkest areas of the interference-reflection image; areas of greater separation between the cell and substratum appear lighter. An abnormal cluster of adhesion plaques is indicated by a single arrowhead. Bar in A = 20 μ m.

reflection image. Some of the larger adhesion plaques did not appear to contain pp60^{src}. We have observed this previously in other RSV-infected cells (36) but do not know what differentiates these adhesion plaques from those that do contain pp60^{src}. A remarkable characteristic of the CU2-infected CEC was the appearance of pp60^{src}-specific fluorescence as tiny speckles or dots and clusters of these dots on the ventral cell surface. Examination of the interference-reflection image indicated that these speckles corresponded to focal adhesion plaques. Such an abnormal array of adhesive plaques was not observed in other mutant-infected CEC and therefore was characteristic of CU2-infected CEC. The presence of such additional adhesion plaques under CU2-infected CEC could, in fact, explain the rather strong adhesion of these cells to the substratum (4).

Some locations of pp60^{src} within CU2-infected CEC were observed to depend upon cell-cell contact. For example, in dense cultures, the abnormal "dot-like" adhesion plaques shown to contain pp60^{src} (Fig. 1A) were not detectable; instead, pp60^{src} fluorescence was seen between cells in contact (not shown) as well as in more normal-appearing adhesion plaques at the periphery of the ventral cell surface.

CEC infected with the partial transformation mutant *ts*CU11 were temperature sensitive for transformation yet retained a near-normal morphology at both temperatures (Table 1). Indirect immunofluorescence experiments showed that when *ts*CU11-infected CEC were maintained at permissive temperature (35°C), pp60^{src} was detectable in streak and patch-like structures (Fig. 2A) that corresponded with the adhesion plaques detected by interference-reflection microscopy (Fig. 2B). Again, not all adhesion plaques contained pp60^{src}. Junctions between *ts*CU11-infected cells also stained with antibodies to pp60^{src}. A general cytoplasmic location for pp60^{src} was revealed by an overall cellular fluorescence. The localization of pp60^{src} within the cytoplasm was evident at permissive and non-permissive temperature, whereas the adhesion plaque localization was temperature dependent. When *ts*CU11-infected CEC were grown for at least 24 h at nonpermissive temperature, pp60^{src} was no longer detectable within the adhesion plaque structures (Fig. 2C and D). In this respect, the *src* gene product of *ts*CU11 behaves like the *src* gene product from *ts*NY68-infected CEC (36).

Cells infected with CU12 exhibit a classical fusiform morphology (very long and spindle shaped). These cells appear relatively "rigid," with few focal contact points to the substratum (Fig. 3). Fluorescence experiments indicated that pp60^{src} was predominantly in the cytoplasm

of the CU12-infected CEC (Fig. 3). In general, these cells contained fewer and smaller adhesion plaques, and a clear localization of pp60^{src} in these structures was not observed in these experiments (Fig. 2E and F). Likewise, cell-cell junctions did not stain with antibodies to pp60^{src}.

In addition to our usual tumor-bearing rabbit serum, we have also prepared antibodies to synthetic peptides of pp60^{src}. Again, by immunofluorescence, these highly specific antibodies did not detect pp60^{src} within the adhesion plaques of CU12-infected CEC, even though adhesion plaques were clearly visible by shifting the optics to interference-reflection (L. E. Gentry, L. R. Rohrschneider, J. Casnellie, and E. Krebs, manuscript in preparation; L. Gentry and L. Rohrschneider, unpublished data). Because both tumor-bearing rabbit serum and antibodies to the synthetic peptide of pp60^{src} easily detected pp60^{src} within adhesion plaques of SR-A-infected CEC, these results indicate that the *src* gene product of CU12 exhibits a greatly reduced ability to bind to the adhesion plaque structures of CU12-infected CEC.

RD10 virus was originally isolated from a stock of SR-D rescued from transformed NRK cells (36). Subclone 4A-IL of this virus was recognized as a partial transformation mutant because cells infected with this virus grew in soft agar yet displayed a relatively normal flat morphology. pp60^{src} within RD10-infected CEC was detectable within the cytoplasm and also at the cell periphery as discrete patches identified as focal adhesion plaques by interference-reflection microscopy (Fig. 2G and H). RD10-infected CEC were distinguishable from the other partial transformation mutant-infected cells on the basis of morphology as well as the appearance of rather broad pp60^{src}-containing adhesion plaques. Among all of the mutant-infected cells, these cells exhibited the most conspicuous localization of pp60^{src} within the adhesion plaque structures.

Distribution of vinculin and filamin. The location of vinculin and the disposition and integrity of stress fibers within the partial transformation mutant-infected cells were of interest because of their relationship to the pp60^{src}-containing adhesion plaque structures. Antibodies to these components were first tested in CEC infected with *ts*NY68, a complete temperature-conditional transformation mutant. The results in Fig. 4 show immunofluorescence experiments on *ts*NY68-infected CEC at either permissive temperature (panels A and B) or nonpermissive temperature (panels C and D). Affinity-purified rabbit antibodies to vinculin stained small focal patches on the ventral surfaces of cells maintained at either temperature (panels A and C).

FIG. 2. Localization of $pp60^{src}$ and the organization of adhesion plaques in CEC infected with $tsCU11$, $CU12$, and $RD10$ virus. CEC infected with the various partial transformation mutants were grown on glass cover slips and fixed, and $pp60^{src}$ was detected by indirect immunofluorescence, using tumor-bearing rabbit serum. The panels on the left (A, C, E, G) show the cell images recorded by fluorescence microscopy, and the panels on the right (B, D, F, H) show the respective adhesion plaques viewed by interference-reflection microscopy. A and B, $tsCU11$ -infected CEC grown at permissive temperature (35°C); C and D, $tsCU11$ -infected CEC grown at nonpermissive temperature (42°C); E and F, $CU12$ -infected CEC grown at 35°C ; G and H, $RD10$ virus-infected

These focal patches of fluorescence corresponded to adhesion plaques (18, 41, 42).

Filamin is a component of stress fibers (46), and antibodies to filamin were used to assess the organization of stress fibers within the mutant-infected CEC. The results (Fig. 4B and D) demonstrate that the anti-filamin detected stress fibers on *ts*NY68-infected CEC grown at the nonpermissive temperature. The same cells grown at permissive temperature exhibited little or no stress fiber organization (panel 4B). These results indicated that the anti-vinculin and anti-filamin sera were detecting the appropriate structures and established the standards of comparison for similar analyses on the partial transformation mutant-infected cells.

Because of the co-localization of pp60^{src} and vinculin within adhesion plaques (41, 42) and the possibility that vinculin is phosphorylated by pp60^{src} at this site (39), we examined the distribution of vinculin within the partial transformation mutant-infected cells (Fig. 5 and 6). CU2-infected CEC contained vinculin within peripheral adhesion plaques and also within the "dot-like" adhesion plaques directly under the cell (Fig. 5A). *ts*CU11-infected CEC at 35°C displayed very small adhesion plaques at the cell periphery. These adhesion plaques, as well as focal adhesion areas in the shape of scrolls, contained vinculin (Fig. 5C). The scroll-shaped adhesion areas also have been observed in CU2-infected CEC. When *ts*CU11-infected CEC were grown at 42°C, the scroll-shaped adhesion areas were no longer detectable, and vinculin was observed in more normal-appearing adhesion plaques. The adhesion plaques of CU12-infected CEC appeared very petite by interference-reflection microscopy (Fig. 2F), and anti-vinculin stained such structures on the ventral surface of these cells (Fig. 5G). In contrast, the adhesion plaques of RD10-infected CEC were very prominent and readily stainable with anti-vinculin antibodies (Fig. 6A). Vinculin was not observed in "rosette" structures described by David-Pfeuty and Singer (15). These results indicate that vinculin is a component of adhesion plaques, and although the size, shape (scrolls), and even number of the adhesion areas may change upon transformation, this does not appear to overtly alter the localization of vinculin within the adhesion area.

The organization of microfilament bundles (stress fibers) in the various mutant virus-infected CEC is illustrated by the results in Fig. 5 and 6. CU2-infected CEC stained with anti-filamin serum in indirect immunofluorescence experi-

FIG. 3. Localization of pp60^{src} in CU12-infected CEC. More confluent cultures of CU12-infected CEC displayed the characteristic fusiform morphology. These cells were stained by indirect immunofluorescence for detection of the location of pp60^{src}.

ments revealed extensive stress fiber formation. These fiber bundles terminated within adhesion plaques (not shown) at the termini of cellular processes (compare Fig. 5A with 5B). However, instead of traversing the entire cell length, these bundles appear to splay out over the nucleus. Anti-filamin serum also stained both dotlike and scroll-shaped adhesion areas in CU2-infected CEC. *ts*CU11-infected CEC did not contain stress fibers when maintained at permissive temperature (Fig. 5D); however, stress fibers were prominent structures within these cells when grown at nonpermissive temperature (Fig. 5F). Scroll-shaped adhesion areas on the cells at permissive temperature stained with anti-filamin serum. These abnormal adhesion areas (scrolls) were not found on *ts*CU11 cells grown at nonpermissive temperature. Within CU12-infected CEC, stress fibers were not detectable (Fig. 5H); however, some smaller fibers traversed the length of these fusiform cells and membranes stained with anti-filamin serum. This may suggest that actin fibers are associated with the cytoplasmic side of the plasma membrane. Finally, RD10-infected CEC exhibited stress fibers (Fig. 6B) which appeared qualitatively similar to those seen in CU2-infected CEC. Thick bundles of filamin-containing microfilaments terminated at peripheral adhesion plaque sites but did not

FIG. 4. The distribution of vinculin and filamin within CEC infected with the temperature-sensitive mutant *tsNY68*. Antibodies to vinculin and filamin were used to stain adhesion plaque structures and stress fibers in CEC infected with a mutant which was temperature sensitive but not a partial transformation mutant. A and B, *tsNY68*-infected CEC grown at the permissive temperature (35°C) and stained with antibodies to vinculin and filamin, respectively; C and D, *tsNY68*-infected CEC grown at the nonpermissive temperature (42°C) and stained with antibodies to vinculin and filamin, respectively. Vinculin-containing adhesion plaques were present at both temperatures, whereas abundant stress fibers were present only at the nonpermissive temperature. Bar in A = 20 μ m.

traverse the cell length. Again these fiber bundles played out over the nuclear area and appeared to rebundle as the stress fiber entered the distal adhesion plaque. We have not investigated further the loss of bundling over the nuclear area, but this could be related to loss of cellular tropomyosin (24).

To demonstrate more precisely the localization of *pp60^{src}* and vinculin, CU2-infected CEC were stained by double chromophore immunofluorescence to detect both antigens in the same cell. The results (Fig. 7) show that within CU2-infected CEC, vinculin (Fig. 7A) and *pp60^{src}* (Fig. 7B) are found at virtually identical intracellular locations. Both are contained within the same peripheral adhesion plaques and even exhibit a similar distribution within these structures. Also, clusters of dotlike adhesion areas (long arrows in Fig. 7A and B) contained both vinculin and *pp60^{src}*. In general, not only in CU2-infected CEC, but also in all other cells examined, vinculin and *pp60^{src}* show a remarkable co-localization which may reflect their putative enzyme-substrate relationship (39).

Phosphorylation of total cell proteins on tyrosine. Uninfected and mutant-infected CEC were labeled with 32 P_i (16 to 18 h), and the relative abundance of phosphotyrosine was determined in the total cellular protein by the method of Sefton et al. (40). CEC transformed by wild-type SR-A contained about a sevenfold increase in total cellular phosphotyrosine compared with uninfected CEC (Table 2). CEC infected with *tsNY68* exhibited a temperature-dependent level of phosphotyrosine with a five- to sixfold difference between the permissive (35°C) and nonpermissive (42°C) temperatures. These results are analogous to those provided by Sefton et al. (39) and suggest that the abundance of phosphotyrosine in total cellular protein is dependent on the action of *pp60^{src}*.

All partial transformation mutant-infected CEC contained an elevated level of phosphotyrosine in total cellular protein. CU2-infected CEC exhibited the smallest increase in abundance of phosphotyrosine (two- to threefold) relative to uninfected CEC. *tsCU11*-infected CEC were temperature sensitive with respect to

the total cellular phosphotyrosine level, and CU12- and RD10-infected CEC contained the highest levels of phosphotyrosine in total cellular protein (about sevenfold above uninfected CEC). These results are roughly in agreement with the phosphorylation state and kinase activity reported previously for pp60^{src} from these mutants (4).

Phosphorylation of vinculin on tyrosine. The relative abundance of phosphotyrosine in vinculin was examined in the set of virus-infected and uninfected CEC described above. Antiserum to vinculin was prepared in a rabbit and used to immune precipitate ³²P-labeled vinculin from cell lysates. Affinity-purified antibodies to vinculin also were used in a few cases and gave results identical to those obtained when the whole anti-vinculin serum was used. The immune precipitation results demonstrated that the anti-vinculin serum was specific for vinculin (unpublished data). This has also been demonstrated by the results of immunofluorescence experiments (Fig. 5 and 6).

Although the specific activity of ³²P in vinculin decreases upon transformation by RSV, the relative abundance of phosphotyrosine increases (39). This is also apparent from the results shown in Table 2. Vinculin from RSV-transformed CEC contained approximately a 10-fold increase in abundance of phosphotyrosine relative to vinculin from uninfected CEC. A similar effect was observed in the vinculin obtained from *ts*NY68-infected CEC at nonpermissive versus permissive conditions; however, the difference in abundance was not as large.

The vinculin from the partial transformation mutant-infected CEC was analyzed next and compared with the results described above. In these experiments, the results obtained with the partial transformation mutant-infected CEC are most comparable to the results obtained with *ts*NY68-infected CEC because each sustained a defect(s) within the *src* gene. Vinculin immune precipitated from CU2-infected CEC contained a relative abundance of phosphotyrosine not unlike that found in *ts*NY68-infected CEC at the nonpermissive temperature (Table 2). Also, this level of phosphotyrosine on vinculin was not temperature dependent, as expected from the nonconditional nature of other transformation parameters (4). *ts*CU11-infected CEC, on the other hand, exhibited a temperature-dependent abundance of phosphotyrosine on vinculin with an approximately fivefold difference between the permissive and nonpermissive temperatures. Vinculin from both RD10- and CU12-infected CEC contained a relative abundance of phosphotyrosine which approached the level seen in *ts*NY68-infected CEC at permissive temperature. CU12-infected CEC did not exhibit a

TABLE 2. Relative abundance of phosphotyrosine in total cellular protein and in vinculin from uninfected CEC and CEC infected with wild-type and mutant RSV

CEC infected with:	Relative % of phosphotyrosine in ^a :			
	Total cellular protein		Vinculin	
	35°C	42°C	35°C	42°C
Uninfected	0.038 (2)		1.6 (1)	
SR-A	0.267 (2)		20.4 (1)	
<i>ts</i> NY68	0.260 (1)	0.049 (1)	16.3 (1)	4.2 (2)
CU2	0.114 (2)	0.105 (2)	4.1 (2)	6.1 (2)
<i>ts</i> CU11	0.154 (3)	0.072 (2)	23.5 (3)	4.5 (3)
CU12	0.293 (2)	0.407 (2)	13.9 (2)	13.5 (1)
RD10	0.265 (2) ^b		15.0 (2) ^b	

^a Values are expressed as the percentage of radioactive phosphorus recovered in phosphotyrosine relative to the total recovered in phosphotyrosine, phosphoserine, and phosphothreonine. Most values are averages, and the number of separate determinations is indicated in parentheses. Temperatures are those at which infected CEC were maintained.

^b The RD10-infected CEC were maintained at 37°C.

temperature-dependent change in the abundance of phosphotyrosine on vinculin, and this result was consistent with other measurements, indicating that CU12 does not contain a temperature-sensitive *src* protein (4). Therefore, at permissive temperature, pp60^{src} from *ts*CU11, RD10, and CU12 appears directly or indirectly to cause the increased tyrosine phosphorylation of vinculin, whereas pp60^{src} from CU2 appears to be defective in this function.

Expression of cell surface fibronectin. A well-characterized parameter of RSV-transformed cells is the loss of the extracellular matrix protein fibronectin. Fibronectin is involved in both adhesion and locomotion of fibroblasts, and normal CEC express an elaborate matrix of this protein (27, 38). To determine what effect partial transformation mutations in pp60^{src} have on the expression of cellular fibronectin, we examined CEC infected with the various partial transformation mutant viruses for the distribution of this matrix protein. Fibronectin was detected by indirect immunofluorescence in fixed and detergent-treated cells; therefore, both extracellular and intracellular fibronectin could be seen (Fig. 8). Uninfected CEC expressed a normal amount of extracellular fibronectin (Fig. 8A), whereas CEC infected with the CU2 virus contained very little fibronectin on the outside of the cells (Fig. 8B). Instead of the fibrous extracellular array, only a weak, more diffuse pattern of intracellular fibronectin was present. The distribution of fibronectin in RD10-infected CEC (Fig. 8F) was similar to that seen in the CU2-infected cultures. *ts*CU11-infected CEC expressed extracellular

FIG. 5. The distribution of vinculin and organization of stress fibers within CEC infected with the partial transformation mutants CU2, *ts*CU11, and CU12. Antibodies to vinculin and filamin were used to probe by

FIG. 6. The distribution of vinculin and organization of stress fibers within CEC infected with the RD10 virus. RD10 virus-infected CEC were stained by indirect immunofluorescence with antibodies to vinculin (A) and filamin (B). Bar = 20 μ m.

fibronectin matrices at the nonpermissive temperature (Fig. 8D) and a dramatic absence of this pattern at the permissive temperature (Fig. 8C). The fusiform CU12-infected CEC exhibited extracellular matrices of fibronectin (Fig. 8E), even though other parameters of transformation indicated these cells were highly transformed (4).

DISCUSSION

In this study, we have analyzed possible relationships between pp60^{src} location and specific transformation parameters induced in RSV-transformed cells. We have concentrated on the adhesion plaque sites because the presence of pp60^{src} at these locations has correlated with the maintenance of the transformed phenotype in *ts*NY68-infected cells. In addition, the location of pp60^{src} within adhesion plaques seems to be a general phenomenon of RSV-induced transformation (36; Gentry and Rohrschneider, unpublished data), and this site is situated such that it could be linked to several cellular parameters characteristic of RSV-transformed cells (3, 36, 39, 42, 45, 48).

Table 3 shows a summary of the transformation parameters expressed in the various partial transformation mutant-infected CEC. The results with each virus-infected culture were quite clear; therefore, for simplicity, they are presented as either plus or minus. Because these parameters were measured in mass culture within a few passages of infection, they are all directly

related to the activity of pp60^{src} and are not selected cellular properties.

In the small sample of partial transformation defective mutants examined here, there was considerable independence in the expression of each transformation parameter measured in these studies. In fact, among cell cultures infected with these partial mutants, no two exhibited the same array of induced parameters. Such divergence is helpful and necessary in determining possible relationships between pp60^{src} location and a specific transformation parameter.

Most mutant-infected CEC contained pp60^{src} within the cellular adhesion plaques, and one mutant (CU2) appeared to induce the overproduction of abnormal pp60^{src}-containing adhesion plaques. RD10-infected CEC also contained an abundance of pp60^{src} in the adhesion plaques, and CU11-infected CEC (at 35°C), although at reduced levels, still contained discernible pp60^{src} bound to adhesion plaques. CU12-infected CEC, on the other hand, represented a distinct mutant class with respect to adhesion plaque localization because the pp60^{src} of CU12 was not concentrated at these cellular sites. CU12-infected CEC displayed a fusiform morphology with fewer and smaller adhesion plaques; however, the results of several fluorescence experiments with tumor-bearing rabbit serum as well as monospecific antiserum to a pp60^{src} peptide (Gentry et al., manuscript in preparation) indicated that pp60^{src} was greatly reduced or lacking from the adhesion plaques of

indirect immunofluorescence the organization of vinculin within adhesion plaques and filamin within stress fibers. A and B, CU2-infected CEC (35°C) stained with antibodies to vinculin and filamin, respectively; C and D, *ts*CU11-infected CEC (35°C) stained with antibodies to vinculin and filamin, respectively; E and F, *ts*CU11-infected CEC (42°C) stained with antibodies to vinculin and filamin, respectively; G and H, CU12-infected CEC (35°C) stained with antibodies to vinculin and filamin, respectively. Bar in A = 20 μ m.

FIG. 8. The distribution of fibronectin on cells infected with various partial transformation mutants. Cells were fixed and stained with affinity-purified antibodies to human fibronectin by indirect immunofluorescence. All cells were treated with Triton X-100 after formaldehyde fixation; therefore, both intracellular and extracellular matrix fibronectin are detectable. (A) Uninfected CEC; (B) CU2-infected CEC; (C) *ts*CU11-infected CEC grown at permissive temperature; (D) *ts*CU11-infected CEC grown at nonpermissive temperature; (E) CU12-infected CEC; (F) RD10 virus-infected CEC.

CU12-infected CEC. In these studies, CU12 represents the only mutant characterized by an absence of the adhesion plaque localization of pp60^{src}. CU12, however, is not alone in this category, as we have found other mutant viruses that display the same lack of adhesion plaque

localization for pp60^{src}. It is interesting that all of these mutants have a fusiform morphology (Rohrschneider and Gentry, unpublished data). There is no clear indication, however, whether the direct binding of pp60^{src} to adhesion plaques is prevented or whether some alternate action of

FIG. 7. Co-localization of vinculin and pp60^{src} within RD10 virus-infected CEC. RD10 virus-infected CEC were stained with guinea pig antibodies to vinculin and rabbit antibodies to pp60^{src}. Fluorescein-conjugated antibodies to guinea pig IgG stained the cellular location of vinculin (A), and rhodamine-conjugated antibodies to rabbit IgG stained the pp60^{src} location (B). Panel C shows a phase-contrast image of the same cell. Bar in A = 20 μ m.

TABLE 3. Summary of results of transformation parameters measured in the various wild-type- and mutant-infected CEC

CEC infected with ^a :	pp60 ^{src} in adhesion plaques	Intact stress fibers	Vinculin in adhesion plaques	Increased P-Tyr in vinculin	Cell surface fibronectin
CU2 (35°C)	++	+	++	—	—
tsCU11 (35°C)	+	—	+	+	—
tsCU11 (42°C)	—	+	+	—	+
CU12 (35°C)	—	—	+	+	+
RD10 (37°C)	++	+	+	+	—
Uninfected (37°C)	—	+	+	—	+
SR-A (37°C)	+	—	+	+	—
tsNY68 (35°C)	+	—	+	+	—
tsNY68 (42°C)	—	+	+	—	+

^a Temperatures are those at which infected CEC were maintained.

pp60^{src} modifies the ability of adhesion plaques to accept the *src* gene product.

With the distinction between mutants that bind pp60^{src} to adhesion plaques and mutants that do not, it was of interest to determine whether any of the transformation parameters were coordinately expressed or related to the presence or absence of pp60^{src} in the adhesion plaques. The expression of fibronectin in the extracellular matrix was of interest in this respect. In all wild-type- and mutant-infected CEC, whenever pp60^{src} was detectable in cellular adhesion plaques, fibronectin was absent from the extracellular matrix. Conversely, whenever pp60^{src} was decreased or absent from the adhesion plaque sites, fibronectin was present in the extracellular matrix. This relationship suggests that the two events are related, especially in view of the reported association between adhesion plaques and fibronectin (9, 43). The release of cell surface fibronectin is a well-known parameter of RSV-transformed cells (3, 22, 27), and we are exploring further the relationship between the binding of pp60^{src} to adhesion plaques and loss of matrix fibronectin.

The location of pp60^{src} within adhesion plaques was unrelated to the activity of the tyrosine-specific kinase activity. This was evident in the phosphotyrosine content of both total cell protein and the adhesion plaque protein vinculin. Under permissive conditions, pp60^{src} from CU2, tsCU11, and RD10 was in adhesion plaques, whereas no concentration of pp60^{src} from CU12 was observed at these sites. All mutants, however (with the exception of CU2), contained elevated abundance of phosphotyrosine in vinculin and the total cell protein. Therefore, vinculin and other cell proteins could become phosphorylated on tyrosine whether or not pp60^{src} was present in the adhesion plaque sites. It is not certain whether pp60^{src} of CU12 virus causes the increased phosphorylation of vinculin on tyrosine via rapid transit through adhesion

plaques, or whether pp60^{src} can phosphorylate soluble vinculin in the cytoplasm. Certainly other mechanisms are possible, but the results of Ito et al. (28) support the idea that a membrane-associated vinculin can be phosphorylated by a soluble pp60^{src}.

The phosphorylation state of vinculin and its relationship to other parameters of the transformed cell are important in assessing possible functions for this event. Vinculin has been proposed to act as an anchor protein attaching the ends of microfilament bundles to the membrane at the adhesion plaques (18–20). Others have suggested that vinculin mediates the transmembrane attachment of fibronectin to the extracellular matrix (26). Phosphorylation then would result in release of stress fibers or fibronectin or both. Although attractive models, the results with the partial transformation mutants would argue against both possibilities. First, there was no relationship whatsoever between the increased abundance of phosphotyrosine on vinculin and the absence of cell surface fibronectin (Table 3). CU2-infected CEC lacked cell surface fibronectin, but the abundance of phosphotyrosine on vinculin was not appreciably elevated. CU12-infected CEC, however, did contain matrices of fibronectin on the cell surfaces, yet the abundance of phosphotyrosine on vinculin approached that found in tsNY68-infected CEC at permissive temperature. The only event we have found to correlate with fibronectin loss is the increased presence of pp60^{src} in the adhesion plaques. Second, the phosphorylation state of vinculin was independent of stress fiber integrity. RD10-infected CEC in particular contained a six- to sevenfold increase in vinculin phosphotyrosine, yet intact stress fibers terminated within adhesion plaques of these cells. In separate experiments, we have also shown that the extent of phosphorylation of vinculin on tyrosine does not change, as stress fibers dissolve just before cell division in uninfected cells (37). Although

we have not measured specific compartments of intracellular vinculin, these results tend to suggest that the general increased phosphorylation of vinculin on tyrosine is unrelated to stress fiber integrity as well as to the loss of cell surface fibronectin. Certainly other pp60^{src}-related events could be necessary to induce these transformation parameters; however, in the mutant-infected cells studied here, merely increasing the abundance of phosphotyrosine on vinculin is not sufficient.

Morphology is another well-known parameter of the transformed state. The viruses used in these studies induced transformed morphologies ranging from very round (SR-A) to very flat (CU2 and RD10) and even fusiform (CU12). No single morphology was correlated with the increased abundance of phosphotyrosine on vinculin (compare Table 1 with Table 3).

The tyrosine-specific kinase activity is an inherent property of pp60^{src} (21, 30) reflected in the content of phosphotyrosine in both total cellular protein and in vinculin of RSV-transformed cells (39, 40). In the mutant-infected CEC studied here, the elevated abundance of phosphotyrosine on vinculin was correlated with the ability of the infected cells to grow in soft agar. Cells that exhibited low levels of phosphotyrosine on vinculin grew poorly in soft agar (i.e., CU2, *ts*CU11 grown at 42°C, *ts*NY68 grown at 42°C), and those with more elevated levels of phosphotyrosine on vinculin grew much better (*ts*CU11 grown at 35°C, CU12, RD10) regardless of pp60^{src} localization, stress fiber integrity, or expression of cell surface fibronectin. Similar results regarding the relationship of anchorage-independent growth to tyrosine-specific kinase activity have been obtained from other studies of the level of phosphotyrosine in the 36K-dalton substrate protein in the CU mutant-infected cells (31). Together, these results support the notion that the tyrosine-specific kinase activity of pp60^{src} is needed for transformation and that the specific phosphorylation of vinculin and the 36K-dalton protein is a necessary part of that mechanism.

The overall results with the various partial transformation defective mutants suggest that the two pp60^{src} activities, i.e., kinase activity and adhesion plaque binding, represent independent functions on the *src* gene product. We have previously proposed that these two activities are governed by independent domains on pp60^{src} and that each induces a separate set of transformation parameters (L. R. Rohrschneider, M. J. Rosok, and L. E. Gentry, *Prog. Nucleic Acid Res. Mol. Biol.*, in press).

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