pp60^{c-src} Has Less Affinity for the Detergent-Insoluble Cellular Matrix Than Do pp60^{v-src} and Other Viral Protein-Tyrosine Kinases

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A difference in affinity for a Nonidet P-40-insoluble cellular matrix was observed between the products of the viral and cellular *src* genes. It has previously been demonstrated that pp60^{v-src} is associated with a detergent-insoluble matrix containing the cellular cytoskeleton (J. G. Burr, G. Dreyfuss, S. Penman, and J. M. Buchanan, *Proc. Natl. Acad. Sci. USA* 77:3484-3488, 1980). We observed a similar association of the transforming proteins of Fujinami sarcoma virus (P130^{gag-fps}) and Yamaguchi 73 avian sarcoma virus (P90^{gag-yes}), both of which are tyrosine-specific protein kinases. However, we found that the endogenous *c-src* product, pp60^{c-src}, was not tightly bound to the detergent-insoluble matrix. This does not appear to have been due to differences in the cytoskeleton between transformed and nontransformed cells since pp60^{c-src} was also solubilized by nonionic detergent in cells transformed by Rous sarcoma virus. This difference in the affinities of the *v-src* and *c-src* products for cytoskeletal proteins may contribute to the inability of pp60^{c-src} to transform cells.

The neoplastic transformation of cells infected by Rous sarcoma virus (RSV) requires expression of a single viral gene, *v-src*, which codes for a 60,000-dalton phosphoprotein, pp60^{v-src} (8). A highly homologous cellular genetic locus, *c-src*, encodes pp60^{c-src} (14, 43). The amino acid sequences of these two *src* products differ only in a few scattered amino acids, except at their carboxyl termini, where 19 amino acids of pp60^{c-src} are replaced by 12 different amino acids in pp60^{v-src} (55). In addition to their structural homology, these proteins are functionally similar. Both exhibit tyrosine-specific protein kinase activity associated with their carboxyl halves (15, 24, 25), and each has an amino-terminal membrane-binding domain (30, 33, 34, 43, 44).

Despite these structural and functional similarities, only the *v-src* product is capable of readily transforming cultured cells and of tumorigenesis. Although the viral *src* gene is expressed at levels as much as 100-fold higher than the cellular *src* gene is in RSV-transformed cells (14, 43), the threshold level of *v-src* expression required for transformation is much lower (28). Further, overexpression of *c-src* at the level of *v-src* expression does not result in transformation of chicken or mammalian cells (27, 44, 53). The *c-src* gene can be converted to a transforming gene by single base substitutions or by replacement with various *v-src* sequences (11, 23, 27, 31, 32, 37, 46, 53). These results suggest that qualitative rather than quantitative differences between pp60^{c-src} and pp60^{v-src} are responsible for the difference in oncogenic potential. The only qualitative difference which has been noted is a lower specific activity of the purified pp60^{c-src} protein-tyrosine kinase in comparison with that of the pp60^{v-src} kinase (20, 26). However, if this is the only difference between the two proteins, it should be ameliorated by gross overexpression of *c-src*. Expression of *c-src* at very high levels in NIH 3T3 cells results in a low level of focus formation, 1% of that seen with *v-src*; however, these partially transformed cells are not tumorigenic (29).

The subcellular locations of pp60^{v-src} and pp60^{c-src} are clearly important in determining potential substrates for their kinase activities (24, 33, 50). The viral *src* protein has been shown by in situ immunological labeling to be associated with the cytoplasmic face of the plasma membrane (42, 48, 57). This plasma membrane localization was confirmed for both the viral and cellular proteins by differential centrifugation experiments (19, 26, 33). In addition, the *v-src* product has been found in adhesion plaques (49, 54), and both viral and cellular *src* products are also associated with intracellular perinuclear membranes (47, 48).

Using the Nonidet P-40 (NP-40) extraction procedure of Penman and co-workers (4, 35), Burr et al. (10) showed that pp60^{v-src} is associated with a detergent-insoluble matrix (DIM) or crude cytoskeletal fraction of cells. Several other viral transforming proteins, which are tyrosine-specific protein kinases encoded by *v-abl*, *v-fps*, and *v-fgr*, have been observed to fractionate similarly (1, 6, 40). We demonstrate here that the transforming proteins of Fujinami sarcoma virus (FSV) and Yamaguchi 73 (Y73) avian sarcoma virus (ASV) are also found in the DIM fraction. In striking contrast, the cellular *src* product was not tightly associated with the cytoskeletal matrix, regardless of the state of transformation of the cell. This difference in intracellular localization between the viral and cellular *src* proteins may be an important determinant of their different transforming abilities.

MATERIALS AND METHODS

Cells and viruses. Primary cultures of chicken embryo fibroblasts (CEFs) were prepared after 11 days of incubation of embryonated *gs⁻ chf⁻* eggs (SPAFAS, Inc.) as described previously (52) and infected with ASVs. Quail embryo fibroblasts were similarly prepared after 9 days of incubation of eggs from Truslow Farms. We obtained the CH119 temperature-sensitive mutant of Prague-A RSV from T. Parsons (9) and other ASVs as previously described (2). The RC cell line, which overexpresses the chicken *c-src* gene under control of the simian virus 40 early promoter in rat-2 cells (44), was obtained from R. Parker. We obtained Schmidt-Ruppin (SR) RSV-transformed NIH 3T3 cells from

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T. Patschinsky and mouse embryo fibroblasts from M. Eddin. Avian cells were maintained in medium 199 supplemented with 2% tryptose phosphate broth, 1% calf serum, and 1% chicken serum, and mammalian cells were grown in Dulbecco-Vogt modified Eagle medium supplemented with 10% fetal calf serum (all components were from GIBCO Laboratories).

Radiolabeling. Cells to be labeled with [^{35}S]methionine were preincubated for 1 h in methionine-free medium 199 supplemented with 4% calf serum and then radiolabeled for 18 h in the same medium containing 167 μCi of L-[^{35}S]methionine (Amersham Corp.) per ml. Radiolabeling with [9,10(*n*)- ^3H]myristic acid (Amersham) was done as described previously (3).

Detergent extraction. All extraction procedures were performed at 4°C. By a method similar to that of Burr et al. (10) and Adkins and Hunter (1), the cells were washed twice in phosphate-buffered saline containing Ca^{2+} and Mg^{2+} and once in detergent-free extraction buffer (10 mM 1,4-piperazinediethanesulfonic acid [PIPES] [pH 6.8], 100 mM KCl, 300 mM sucrose, 2.5 mM MgCl_2 , 1 mM phenylmethylsulfonyl fluoride, 0.5% Trasylol [FBA Pharmaceuticals]). The cells were then incubated for 10 min in extraction buffer plus 1% NP-40. The dishes were rocked gently every 2 min throughout the extraction. After extraction, the supernatant containing the soluble material (SOL) was removed and immediately clarified by microcentrifugation to remove any whole cells. The material remaining on the dish (DIM) was solubilized in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 10 mM sodium phosphate buffer [pH 7], 0.1% sodium dodecyl sulfate [SDS], 1% NP-40, 1% sodium deoxycholate, 1% Trasylol). SDS and sodium deoxycholate were added to the SOL supernatant to make the detergent concentration equivalent to that in the DIM fraction. Both DIM and SOL fractions were clarified by centrifugation at 100,000 $\times g$ for 30 min.

Immunoprecipitation and protein kinase assay. After normalization of uninfected cell lysates to 200 μg of total protein and infected cell lysates to 50 μg , determined by the method of Lowry et al. (39), pp60^{src} was immunoprecipitated by incubation of the lysates at 4°C for 1 h with either the murine monoclonal antibody 327 (MAb 327; a gift from J. Brugge), which recognizes both viral and cellular pp60^{src} (38), or antiserum derived from an RSV-injected, tumor-bearing rabbit (TBR serum) (52). In general, MAb 327 was used for immunoprecipitations from radiolabeled lysates and the TBR serum was used for protein kinase assays. Rabbit antiserum raised against avian myeloblastosis virus p19^{gag} (a gift from D. Bolognesi) was used to immunoprecipitate the transforming proteins of PRCII ASV, FSV, and Y73 ASV. All immunoprecipitations were performed with antibody in excess. Next, fixed *Staphylococcus aureus* cells (Calbiochem-Behring), preincubated with goat anti-mouse immunoglobulin G (IgG) (Cooper Biomedical, Inc.) for MAb 327 immunoprecipitates, were added to the samples. The samples were pelleted in a Microfuge (Brinkmann Instruments, Inc.) and then washed in a series of buffers similar to that of Gentry et al. (22): (i) RIPA buffer; (ii) low-salt buffer (10 mM Tris [pH 7.2], 0.5% sodium deoxycholate, 0.1% SDS); (iii) high-salt buffer (10 mM Tris [pH 7.2], 1 M MgCl_2); (iv) NP-40 buffer (10 mM Tris [pH 7.2], 50 mM NaCl, 0.5% NP-40); and (v) phosphate-buffered saline without Ca^{2+} or Mg^{2+} .

For the protein kinase assay, samples were suspended in 10 mM Tris hydrochloride (pH 7.4)–5 mM MgCl_2 –5 μCi of [γ - ^{32}P]ATP (Amersham; >3,000 Ci/mmol) and incubated at 25°C for 15 min. The kinase reaction was stopped by the

addition of 5 volumes of cold RIPA buffer, and the immune complex was pelleted in a Microfuge.

[^{35}S]methionine-labeled precipitates and kinase assay samples were both analyzed by SDS-polyacrylamide gel electrophoresis as described previously (52). Gels containing [^{35}S]methionine were enhanced with Amplify (Amersham). Gels were dried and exposed to preflashed Kodak XAR film at -80°C in the presence of an intensifying screen. Radiolabeled bands were quantified either by densitometry or by excision and liquid scintillation counting.

RESULTS

Transforming proteins of FSV and Y73 ASV are associated with the cytoskeletal matrix. Several viral oncogenes encode tyrosine-specific protein kinases which are associated with the DIM. These include the v-src product (10), P120^{gag-abl} of Abelson murine leukemia virus (6), P105^{gag-fps} of PRCII ASV (1), and P70^{gag-actin-fgr} of Gardner-Rasheed feline sarcoma virus (40). It was previously shown that the gag-fps product of FSV is weakly associated with the plasma membrane in a salt-dependent fashion (21, 41, 58). In contrast, the structurally related protein of PRCII, a poorly transforming virus, is associated with intracellular membranes independent of the presence of salt (58). We wished to extend the comparison of the subcellular localization of these two viral transforming proteins by comparing their association with cytoskeletal elements. The kinase activity associated with PRCII P105^{gag-fps} has been observed to fractionate with the DIM, whereas radiolabeled viral protein was distributed between the DIM and SOL fractions (1). The PRCII protein in the DIM fraction had a slightly slower electrophoretic mobility and was more highly phosphorylated than the species in the SOL fraction (1).

The solubility in NP-40 of the PRCII and FSV proteins, radiolabeled with [^{35}S]methionine, and their associated protein kinase activities are shown in Fig. 1. For both, essentially all of the in vitro autophosphorylation activity was present in the DIM fraction. As had been previously observed with PRCII P105 (1), a portion of the total [^{35}S]methionine-labeled FSV P130 was present in both fractions. However, the DIM fraction alone contained a gag-fps protein with a lower electrophoretic mobility. Thus, FSV and PRCII proteins demonstrated very similar patterns of association with the cytoskeleton.

Another viral tyrosine kinase, P90^{gag-yes} encoded by Y73 ASV, was also studied. Whereas its associated kinase activity was present primarily in the DIM fraction, [^{35}S]methionine-labeled immunoprecipitated P90 was present in both fractions, with most in the DIM (Fig. 1). However, in contrast to the gag-fps products, no difference in electrophoretic mobility was observed between the two fractions. In summary, the protein-tyrosine kinase activities associated with the transforming proteins of FSV and Y73 ASV both were present in the DIM of infected cells, as had previously been observed for the RSV v-src product and for several other viral tyrosine kinases (1, 6, 10, 40).

Endogenous pp60^{c-src} kinase activity is not tightly associated with the DIM. In the course of studies of the cytoskeletal association of several transforming proteins, we examined the nonionic detergent extractability of kinase activity in uninfected CEFs immunoprecipitated with TBR serum. The tyrosine kinase activity of several viral proteins had previously been observed to be present in a DIM containing cytoskeletal proteins. Therefore, we were surprised to observe that the kinase activity of the endogenous c-src prod-

FIG. 1. Detergent extraction of transforming proteins of PRCII ASV, FSV, and Y73 ASV. Infected cells were either metabolically labeled with [35 S]methionine for 18 h (A) or assayed for in vitro protein kinase activity, detected as autophosphorylation of *gag-onc* transforming proteins (B). Cells were extracted with 1% NP-40 for 10 min at 4°C and the DIM (D) was separated from the SOL fraction (S). Both fractions were immunoprecipitated with antiserum directed against avian myeloblastosis virus P19^{gag}. Proteins were electrophoresed through a 7.5% polyacrylamide gel containing SDS. The indicated transforming proteins are PRCII P105^{gag-fps}, FSV P130^{gag-fps}, and Y73 P90^{gag-yes}.

FIG. 2. Protein kinase activity of fractionated *v-src* and *c-src* products. The *v-src* product was analyzed in SR-A RSV-infected CEFs (RSV), and the chicken *c-src* product was analyzed in the overproducer RC rat cell line (RC). The endogenous *c-src* product was analyzed in CEFs (CEF) and in mouse embryo fibroblasts (MEF). All cells were extracted with NP-40, as described in Materials and Methods. Both DIM (D) and SOL (S) fractions were immunoprecipitated with TBR serum, and phosphorylation of the IgG heavy chain was determined in an in vitro protein kinase assay. Kinase samples were electrophoresed through a 10% polyacrylamide gel in the presence of SDS. The 32 P-labeled heavy-chain substrate is shown.

uct in CEFs was readily solubilized by treatment with NP-40.

The partitioning of the protein kinase activity of pp60^{v-src} and pp60^{c-src} into 1% NP-40 soluble (SOL) and insoluble (DIM) fractions is shown in Fig. 2. The detergent extractions were performed in parallel for 10 min at 4°C with uninfected and SR-A RSV-infected CEFs, and the *src* products were subsequently immunoprecipitated with TBR serum. Kinase activity was assayed by direct scintillation counting of IgG heavy-chain phosphorylation, which provided a very sensitive assay. Whereas 86% of the pp60^{v-src}-associated kinase activity was present in the DIM fraction, the pp60^{c-src} kinase activity was 79% solubilized. This striking difference in the fractionation of *c-src* and *v-src* kinase activity has been observed repeatedly. Similar fractionation results were obtained when immunoprecipitation of pp60^{v-src} and pp60^{c-src} was performed with MAb 327 and both autophosphorylation and phosphorylation of the exogenous substrate enolase were measured (data not shown).

To study the NP-40 solubility of the *c-src* product in mammalian cells, we performed additional experiments. First, the endogenous *c-src* product in secondary cultures of mouse embryo fibroblasts was subjected to detergent extraction, immunoprecipitation with TBR serum, and the IgG protein kinase assay as described above. In this experiment, >90% of the pp60^{c-src} kinase activity was present in the SOL fraction (Fig. 2). Similar results were obtained for the *c-src* product in quail embryo fibroblasts (data not shown).

To explore the possibility that the difference in the NP-40 solubility of the *c-src* and *v-src* products was due to a difference in levels of expression, we also studied the fractionation of overexpressed chicken pp60^{c-src} in the RC rat cell line of Parker et al. (44). Kinase activity associated with the *c-src* product was again predominantly in the SOL

FIG. 3. Time course of detergent extraction of pp60^{c-src} from CEFs. (A) CEFs were extracted in 1% NP-40 for the indicated times. pp60^{c-src} was immunoprecipitated from the SOL (S) and DIM (D) fractions with TBR serum, and in vitro protein kinase activity was assayed. The IgG heavy-chain kinase substrate is shown. (B) Kinase activity quantified by densitometric scanning and liquid scintillation counting. Averages from three or more time course experiments were plotted.

fraction (Fig. 2), as had been observed for the endogenous *c-src* product. Therefore, the kinase activity of endogenous pp60^{c-src} present in fibroblasts from three different species was found to be solubilized by brief treatment with NP-40, as was overexpressed pp60^{c-src}. In contrast, pp60^{v-src} kinase activity in RSV-transformed CEFs (Fig. 2) or RSV-transformed NIH 3T3 cells (data not shown) was predominantly in the DIM fraction, as observed previously (10).

Time dependence of NP-40 extraction of pp60^{c-src}. Most of our fractionation experiments involved a 10-min exposure of cells to 1% NP-40 at 4°C. However, we also investigated whether pp60^{c-src} kinase activity was associated with the DIM fraction when detergent extraction was performed for a shorter time, enabling detection of a weaker association. Several plates of uninfected CEFs were extracted in parallel for times ranging from 2 to 14 min (Fig. 3). In striking

contrast to pp60^{v-src}, which was still >80% associated with the DIM fraction after 10 min (Fig. 2), pp60^{c-src} was 53% solubilized after 6 min of NP-40 treatment (Fig. 3). However, after shorter detergent treatments, we found most pp60^{c-src} associated with the DIM fraction. As the time of extraction increased, so did the amount of solubilized *c-src* protein. If the cellular *src* protein is recognized by the same cytoskeletal component(s) that binds the viral protein, the *c-src* protein is bound with greatly diminished affinity. Therefore, any interaction between pp60^{c-src} and components of the DIM could be disrupted readily by exposure to 1% NP-40.

Fractionation of radiolabeled *c-src* and *v-src* products. The fractionation of [³⁵S]methionine-labeled pp60^{c-src} and pp60^{v-src} was examined next. It was important to rule out the presence of possible breakdown products of pp60^{src} which might fractionate differently than the intact protein while still retaining kinase activity, as has been observed in other types of cell fractionation experiments (33). The results of immunoprecipitation of [³⁵S]methionine-labeled *src* proteins from NP-40-extracted cells are shown in Fig. 4. pp60^{c-src} was immunoprecipitated both from uninfected CEFs and from the RC cell line, which expresses high levels of chicken *c-src*, whereas pp60^{v-src} was immunoprecipitated from SR-A RSV-transformed CEFs. In all instances, we observed a fractionation pattern very similar to that observed for protein kinase activity (Fig. 2). Most pp60^{c-src} protein was observed in the SOL fraction, and the bulk of pp60^{v-src} was found in the DIM fraction. Thus, there does not appear to be

FIG. 4. Fractionation of [³⁵S]methionine-labeled *v-src* and *c-src* products. Cells were metabolically labeled with [³⁵S]methionine for 18 h, and DIM (D) and SOL (S) fractions were generated by extraction with 1% NP-40. The fractions were immunoprecipitated with either MAb 327 (M), which recognizes both *v-src* and *c-src* proteins, or with nonimmune serum (N), and the precipitated proteins were electrophoresed through a 10% polyacrylamide gel containing SDS. pp60^{v-src} was immunoprecipitated from SR-A RSV-infected CEFs (RSV). Chicken pp60^{c-src} was immunoprecipitated from CEFs (CEF) and from the RC rat cell line (RC), which overexpresses it. CEFs infected with CH119 RSV were cultured at the permissive temperature for transformation and used to immunoprecipitate pp60^{c-src} and a deleted pp53^{v-src} simultaneously (CH119).

FIG. 5. Detergent fractionation of [^3H]myristate-labeled *v-src* and *c-src* products. After metabolic labeling was performed with [^3H]myristate for 18 h, cells were separated into DIM (D) and SOL (S) fractions by NP-40 extraction. $\text{pp60}^{v\text{-src}}$ was immunoprecipitated from SR-A RSV-transformed CEFs (RSV), and the endogenous $\text{pp60}^{c\text{-src}}$ was immunoprecipitated from uninfected CEFs (CEF), both with MAb 327. The $\text{pp60}^{v\text{-src}}$ and $\text{pp60}^{c\text{-src}}$ fractions were electrophoresed independently on 10% polyacrylamide gels containing SDS.

a pronounced alteration in protein kinase specific activity of either *src* protein between the SOL and DIM fractions.

A slightly slower electrophoretic mobility was observed for the portion of the *v-src* protein which fractionated in the DIM (Fig. 4 and 5). This could have been due to increased phosphorylation or other modification of this fraction of the *v-src* protein molecules. Similar results have been observed with PRCII ASV and FSV transforming proteins (Fig. 1; 1). The identity of the [^{35}S]methionine-labeled gel bands as $\text{pp60}^{c\text{-src}}$ or $\text{pp60}^{v\text{-src}}$ was confirmed by their excision and analysis after partial digestion with *S. aureus* V8 protease by the method of Cleveland et al. (13) (data not shown).

As a control for the fractionation procedure, a sample of fractionated total cell protein was also electrophoresed on an SDS-polyacrylamide gel. As reported previously (4, 7, 34, 35), the bulk of the cell protein was solubilized by NP-40 extraction. Several protein bands were seen exclusively in one fraction or the other, whereas a number of other bands were present in both the SOL and DIM fractions but showed quantitative differences between the two fractions (data not shown). Similar results were observed when unlabeled protein fractions were analyzed by staining with Coomassie blue (data not shown) as a control for the fractionation of experiments in which protein kinase assays were performed.

It has been observed that myristylation of the N terminus of $\text{pp60}^{v\text{-src}}$ is necessary for association of the protein with the cellular plasma membrane and for transformation (30, 45). We investigated whether [^3H]myristate-labeled $\text{pp60}^{v\text{-src}}$ and $\text{pp60}^{c\text{-src}}$ fractionated like the [^{35}S]methionine-labeled proteins. The bulk (70%) of the [^3H]myristate-labeled $\text{pp60}^{c\text{-src}}$ was observed in the SOL fraction, whereas most (76%) of the $\text{pp60}^{v\text{-src}}$ was in the DIM fraction (Fig. 5). This confirmed our results with [^{35}S]methionine-labeled *src* proteins (Fig. 4) and proteins detected by kinase activity (Fig. 2). Since the myristic acid moiety is at the N terminus of pp60^{src} (30, 45), this also showed that the presence of *c-src*

protein kinase activity in the SOL fraction was not due to the loss of the N terminus of the protein. Since both *c-src* and *v-src* proteins are myristylated at their N termini but fractionate differently, this modification does not appear to be sufficient for association with the DIM fraction.

Fractionation of $\text{pp60}^{c\text{-src}}$ in transformed cells. The data presented in the preceding sections show that $\text{pp60}^{c\text{-src}}$ in uninfected CEFs is predominantly present in an NP-40-soluble fraction, in distinct contrast to $\text{pp60}^{v\text{-src}}$ in RSV-infected CEFs. Since transformation by RSV leads to profound changes in cytoskeletal structure (50, 56), we also investigated the fractionation of $\text{pp60}^{c\text{-src}}$ in transformed cells. We studied the fractionation of endogenous *c-src* in CEFs transformed by the Prague-A RSV mutant CH119, constructed by Bryant and Parsons (9) by deleting the part of the *src* gene encoding amino acids 202 to 255, resulting in a 53-kilodalton (kDa) *src* product. This mutant is temperature sensitive for transformation and allows the detection of both *c-src* and *v-src* products in either transformed or untransformed cells. We analyzed the 53-kDa *v-src* product and the 60-kDa *c-src* product simultaneously, both by examination of [^{35}S]methionine-labeled protein and by a protein kinase assay, using MAb 327 to detect autophosphorylation of both *src* products.

When CH119-infected cells are cultured at 35°C, they are transformed, whereas at 41°C, they are not (9). We performed detergent fractionation experiments with cells grown at both temperatures. At the permissive temperature for transformation, the 53-kDa *v-src* product (Fig. 4) and its kinase activity (data not shown) were both predominantly in the DIM fraction, as had been seen with wild-type *v-src* products (Fig. 2 and 4) (10). In these same cells, $\text{pp60}^{c\text{-src}}$ could be seen in both the SOL and DIM fractions; however, most $\text{pp60}^{c\text{-src}}$ appeared in the SOL fraction (Fig. 4). Similar results were obtained when CH119-infected CEFs were cultured at 41°C, the nonpermissive temperature for transformation (data not shown). Therefore, the difference in the cytoskeletal association of the *v-src* and *c-src* products is not dependent on the transformation state of the cells but appears to be an intrinsic property of the *v-src* and *c-src* proteins themselves and of their different cellular interactions.

DISCUSSION

The mechanism of activation of nontransforming cellular proto-oncogenes to transforming oncogenes is not fully understood. For the *src* gene, activation of the tyrosine-specific protein kinase of $\text{pp60}^{c\text{-src}}$ appears to be necessary for its conversion to a transforming protein, but a difference in subcellular localization has not previously been thought to be involved in determining the functional difference between the *c-src* and *v-src* proteins (19, 33, 50). Both proteins have a myristic acid residue covalently bound to their amino termini, which has been shown to mediate attachment to the plasma membrane (30, 45), and both proteins are also associated with intracellular membranes (47, 48). In contrast, we show here that the two proteins do indeed have a different affinity for cytoskeletal proteins, perhaps resulting in a different subcellular localization. We think this difference could be crucial in activation of the full transforming potential of $\text{pp60}^{c\text{-src}}$ by helping to determine the substrate specificity of its activated tyrosine kinase.

Using a mild nonionic detergent extraction procedure developed by Lenk et al. for the examination of the cytoskeleton of HeLa cells (35) and used with a variety of

cell types, including CEFs (7), we examined the association of the *v-src* and *c-src* proteins with the cytoskeleton. In agreement with the results of Burr et al. (10), we saw a substantial majority of pp60^{v-src} associated with the DIM. In uninfected CEFs, however, most of the endogenous *c-src* protein was solubilized by the detergent. This treatment has been shown to leave adhesion plaques attached to the substratum (54). Although the *v-src* product has been observed in adhesion plaques (49, 54), our results suggest that the *c-src* product does not associate tightly with cytoskeletal proteins present in adhesion plaques.

The lack of association between pp60^{c-src} and the cytoskeletal matrix could contribute to its inability to transform cells by preventing the activation of its tyrosine kinase activity or by limiting its access to substrates critical for transformation. Alternatively, the association of the viral *src* product with the DIM fraction could be a result of changes the cytoskeleton undergoes during the process of transformation (50, 56). To address this, we examined the localization of viral and cellular *src* products simultaneously in cells infected with the RSV mutant CH119, which is temperature sensitive for transformation and encodes an internally deleted pp53^{v-src} (9). The viral *src* product was found to be associated with the cytoskeleton at both permissive and nonpermissive temperatures for transformation. However, the bulk of the cellular *src* protein was in the detergent-soluble fraction, even in transformed cells. Thus, the difference observed in localization of the viral and cellular *src* products appears to be an intrinsic property of the proteins rather than a consequence of the transformed state of the cells.

The intracellular localization of pp60^{v-src} has been shown previously to be closely correlated with its transforming potential. Mutations which alter the amino terminus of pp60^{v-src} so that it is not myristylated result in nontransforming *v-src* products which are not bound to the plasma membrane (30, 34, 45). We propose that association of pp60^{v-src} with the cytoskeletal matrix may also be essential for its transforming ability, in light of our finding that pp60^{c-src} is not associated with this matrix. In support of this hypothesis, studies of a number of mutant *src* proteins, including nonmyristylated mutants, has shown a good correlation between *src* product association with the DIM fraction and cell transformation (22a).

Association of pp60^{v-src} with the cytoskeleton may be involved in activation of its tyrosine kinase activity by a mechanism analogous to the activation of the pp60^{c-src} kinase by association with polyomavirus middle T antigen (5, 12, 18). It is possible that the apparent negative regulation of pp60^{c-src} kinase activity in vivo by phosphorylation at tyrosine-527 (16–18) and the lack of phosphorylation at tyrosine-416 (the major site of pp60^{v-src} phosphorylation) requires pp60^{c-src} to be in the detergent-soluble subcellular compartment. Association with the DIM may promote phosphorylation of *src* products at tyrosine-416 rather than at tyrosine-527 by a mechanism like that involved in the association of pp60^{c-src} with polyomavirus middle T antigen (5, 12, 18). It may be significant that the fraction of polyomavirus middle T antigen that is active in a kinase reaction (due to its association with pp60^{c-src}) is preferentially associated with the DIM (51). Alternatively, association of *src* proteins with the DIM fraction may require an activated tyrosine kinase.

Recently, three laboratories examined the activation of the transforming potential of pp60^{c-src} by mutation of tyrosine-527 to an unphosphorylatable phenylalanine residue (11, 32, 46). All three groups observed increased phospho-

rylation at tyrosine-416, as well as an increase in protein-tyrosine kinase activity. Although cells expressing these mutant *c-src* genes were transformed, foci were smaller and less efficiently induced than those seen in wild-type RSV-infected cells. Kmiecik and Shalloway (32) found these transformed cells to be tumorigenic in nude mice; however, the latent period for tumor formation was longer than for pp60^{v-src}-transformed cells. These data seem to indicate that pp60^{c-src} activated by dephosphorylation of tyrosine-527 and increased phosphorylation at tyrosine-416 is not as efficient a transforming protein as is pp60^{v-src}. This could be due to suboptimal phosphorylation of a critical substrate resulting from altered subcellular localization. Examination of the association of these new transforming mutants of pp60^{c-src} with the DIM will be important in clarifying this point.

A number of other viral oncogenes encode proteins having tyrosine-specific protein kinase activity (24) which are also associated with the cytoskeletal matrix. After the study of Burr et al. with pp60^{v-src} (10), it was found that the Abelson murine leukemia virus P120^{gag-abl} (6), the PRCII ASV P105^{gag-fps} (1), and the Gardner-Rasheed feline sarcoma virus P70^{gag-actin-fgr} (40) are all in the DIM fraction. We present similar data here for the cytoskeletal association of FSV P130^{gag-fps} and Y73 ASV P90^{gag-yes}. Despite their shared enzymatic activity and association with the cytoskeleton, these proteins vary in other aspects of their subcellular localization. Although most of these viral proteins are associated with the plasma membrane and have also been observed in adhesion plaques, there are some exceptions. The FSV *gag-fps* product is not present in adhesion plaques (50). Furthermore, both the PRCII *gag-fps* product and a fraction of the *v-src* product are associated with cellular membranes other than the plasma membrane (47, 48, 58). The FSV *gag-fps* product is rather weakly associated with the plasma membrane in a salt-dependent manner (21, 41, 58); however, the *c-fps* proto-oncogene product NCP98 is not associated with the plasma membrane (59). This is another instance in which cellular and viral oncogene products have different subcellular locations. In contrast to the results with peripheral membrane transforming proteins, Hamaguchi and Hanafusa have recently observed that two viral protein-tyrosine kinases that are transmembrane proteins, UR2 p68^{gag-ros} and avian erythroblastosis virus p68^{erb-B}, are solubilized by extraction with nonionic detergent (22a).

In summary, we found a qualitative difference between the cellular *src* product and a number of viral transforming proteins, including the *v-src* product, which are tyrosine kinases. The cellular *src* product is distinct in being readily solubilized by NP-40, whereas the viral transforming proteins are associated with a DIM. This altered affinity for cytoskeletal proteins may play a role in the conversion of the *c-src* proto-oncogene product to a transforming protein by altering its interaction with cellular regulatory factors and substrates.

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