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Association of $p60^{src}$ with Triton X-100-resistant cellular structure correlates with morphological transformation

(Rous sarcoma virus/cytoskeleton/protein kinase)

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ABSTRACT **More than 70% of wild-type Rous sarcoma virus $p60^{v\text{-}src}$ was found to be associated with a cellular structure resistant to nonionic detergent extraction that consists primarily of cytoskeletal proteins. On the other hand, nontransforming src proteins, including cellular $p60^{c\text{-}src}$, nonmyristoylated forms, and those inactive in protein kinase, were found in the fraction solubilized by the detergent extraction. $p60^{c\text{-}src}$ was detergent-soluble even in transformed cells, suggesting that the association of $p60^{v\text{-}src}$ is not a result of cell transformation. Analyses with a variety of Rous sarcoma virus mutants showed a good correlation between the degree of association with the detergent-resistant structure and the extent of cell transformation caused by mutant src proteins, suggesting that this association may be significant for the process of cell transformation by Rous sarcoma virus.**

The src gene product of Rous sarcoma virus (RSV) is a phosphoprotein known as $p60^{v\text{-}src}$, which has tyrosine-specific protein kinase activity that appears to be essential for cell transformation. A number of cellular proteins are phosphorylated at tyrosine residues upon transformation by RSV (1–5), but targets of phosphorylation by $p60^{v\text{-}src}$ that are critical in initiating the transformation process remain to be identified.

One approach to this problem is to identify a subcellular structure with which only actively transforming $p60^{src}$ associates. Recent studies with nonmyristoylated forms of $p60^{src}$, which are active protein kinases but defective in membrane association, indicated that the association of $p60^{v\text{-}src}$ with the plasma membrane plays an important role in transformation (6–9). However, the src proteins of some other mutants, such as NH_2-terminal deletion mutants (10), are active in plasma membrane association and protein kinase and yet are very limited in cell transformation. In addition, the src proteins of two recovered avian sarcoma viruses (rASVs), which are active in cell transformation, associate with focal adhesion plaques rather than with the plasma membrane (11). These results suggest that additional function(s) of $p60^{src}$, such as interaction with a subcellular structure present in the vicinity of the plasma membrane, may be required to achieve cell transformation.

Burr *et al.* (12) demonstrated association of $p60^{v\text{-}src}$ with a nonionic detergent-resistant subcellular structure that consists largely of cytoskeleton (13–19), but the importance of this association was not immediately clear. To evaluate its significance for cell transformation, we have studied the association of various mutant src proteins, including overexpressed and endogenous cellular $p60^{c\text{-}src}$, with the detergent-resistant structure. Cellular $p60^{c\text{-}src}$ is known to be inactive in transformation even when expressed at levels comparable to that of $p60^{v\text{-}src}$ in infected cells (20–23). Here we report that there is a striking difference between $p60^{v\text{-}src}$ and $p60^{c\text{-}src}$ in their association with the detergent-resistant subcellular structure, despite their similarity in plasma membrane association. Further analyses extended to a variety of mutant $p60^{src}$ proteins showed a good correlation between the association of $p60^{src}$ with this structure and its ability to induce cell transformation.

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MATERIALS AND METHODS

Cells and Viruses. Chicken embryo fibroblasts (CEF) were prepared, maintained, and infected as described (24). The viruses analyzed include wild-type strains of RSV, Schmidt–Ruppin strain (SR-RSV), Prague B strain (PR-RSV), and RSV-29 (25); a series of mutants of SR-RSV including NY308, NY309, NY311, NY310, NY315, NY314, and NY300 (6, 10, 26); a temperature-sensitive mutant, tsNY68 (27); c-*src*-containing virus NY5H (28); transforming mutants, NY501T7 and NYCHB, derived from the c-*src*-containing virus (28); chimeric viruses containing c-*src* and v-*src*, NY701 (20), NY851, and NY951 (29); rASV157 and rASV-1702 (11); and other avian retroviruses, Fujinami sarcoma virus (FSV) (30), avian sarcoma virus Yamaguchi 73 (Y73) (31), avian sarcoma virus UR2 (32), and avian erythroblastosis virus (AEV) (33). NY501B, a c-*src* virus with the *env* gene derived from SR-RSV subgroup B, was obtained from CEF transfected with pTT501 (20) and pREP-B (34). SR-RSV-infected rat 3Y1 cells have been described (35). The kinase-negative mutants of PR-RSV, CHpm9 and CHpm26 (36), were provided by J. T. Parsons (University of Virginia, Charlottesville, VA).

Cell Fractionation. CEF were fractionated into detergent-soluble and -resistant fractions with CSK buffer (10 mM Pipes, pH 6.8/100 mM KCl/2.5 mM $MgCl_2$/1 mM $CaCl_2$/0.3 M sucrose/1 mM phenylmethylsulfonyl fluoride/1% Trasylol/1 mM Na_3VO_4/10 μM Na_2MoO_4/1% Triton X-100) (19). Cells grown in 60-mm plates were washed with Tris-buffered saline and incubated in 0.5 ml of CSK buffer for 3 min, unless otherwise stated, on ice with gentle rocking every 30 sec. The supernatant was collected and the insoluble structure that remained on the dish was again incubated for 1 min with 0.5 ml of fresh CSK buffer. The supernatants from the two incubations with CSK buffer were pooled and used as the "detergent-soluble fraction." The residual structure remaining on the dish was collected with RIPA buffer (6) and used as the "detergent-resistant fraction." Both fractions were clarified by centrifugation at 15,000 × *g* for 30 min and, after adding 1% sodium deoxycholate/10 mM Tris·HCl, pH 8.0, to the soluble fraction, both fractions were subjected to immu-

Abbreviations: RSV, Rous sarcoma virus; rASV, recovered avian sarcoma virus; CEF, chicken embryo fibroblasts; SR-RSV, Schmidt–Ruppin strain of RSV; PR-RSV, Prague strain of RSV; FSV, Fujinami sarcoma virus; Y73, avian sarcoma virus Yamaguchi 73; AEV, avian erythroblastosis virus.

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noprecipitation. Preparation of P100 fractions of cells was as described (6).

Immunoprecipitation of Radiolabeled Proteins and Protein Kinase Assay. [^{3}H]Leucine labeling, kinase assay, and immunoprecipitation of the src proteins were as described (37). For immunoprecipitation of src proteins, tumor-bearing rabbit serum (for NY309, NY310, NY311) (38), antiserum against $p60^{src}$ synthesized in bacteria (for CHpm9 and CHpm26) (39), and monoclonal antibody 327 (a gift of J. Brugge, State University of New York, Stony Brook, NY) (40) were used. The transforming proteins of FSV, Y73, UR2, and AEV were immunoprecipitated with anti-virion antiserum (32) or antiserum against the kinase domain of epidermal growth factor receptor provided by S. Decker (Rockefeller University, New York) (41). The labeled proteins were analyzed by $NaDodSO_4$/PAGE as described (19). ^{3}H- or ^{32}P-labeled protein bands were quantitated by densitometer tracing.

Marker Enzyme Assay. Activities of three enzymes, 5'-nucleotidase, acid phosphatase, and glucose-6-phosphatase, were assayed as described (42).

RESULTS

Viral and Cellular $p60^{src}$ Differ in Binding to Detergent-Resistant Structure. Cells were fractionated into soluble and resistant fractions by exposing the monolayer to CSK buffer. We found that >80% of marker enzymes, 5'-nucleotidase as a plasma membrane protein, acid phosphatase as a lysosomal protein, and glucose-6-phosphatase as an endoplasmic reticulum protein, were rendered soluble by treatment with CSK buffer for 4 min, indicating that this treatment solubilized not only the bulk of plasma membrane but also intracellular vesicles.

There were no significant differences in the composition of major proteins in the soluble and resistant fractions of cells infected with SR-RSV or with NY5H, containing v-*src* and c-*src*, respectively (data not shown), except that a band of about 220 kDa, presumably of fibronectin, is decreased in transformed cells (43). However, a striking difference was found between $p60^{v\text{-}src}$ and $p60^{c\text{-}src}$ in their association with structures fractionated by the CSK buffer. CEF infected with virus containing either v-*src* or c-*src* were labeled with [^{3}H]leucine, exposed to CSK buffer for various times, and fractionated into soluble and resistant fractions. The labeled $p60^{src}$ in each fraction was examined by immunoprecipitation, gel electrophoresis, and autoradiography. As shown in Fig. 1, >70% of $p60^{v\text{-}src}$ remained in the resistant fraction after extraction for up to 10 min. This is consistent with the previous observation by Burr *et al.* (12). In contrast, ≈90% of $p60^{c\text{-}src}$ was readily solubilized during the first 2 min of extraction. Essentially the same difference in the solubility of the two src proteins was found under a variety of conditions, including absence of $CaCl_2$ or $MgCl_2$, presence of 5 mM EDTA, or presence of KCl in concentration from 0 to 500 mM (data not shown). Furthermore, when the cells were suspended in CSK buffer and centrifuged briefly (1000 rpm, 3 min), $p60^{v\text{-}src}$ was pelleted together with resistant cellular structures (data not shown). A similar difference in the distribution of $p60^{v\text{-}src}$ and $p60^{c\text{-}src}$ was found with rat 3Y1 cells and 3Y1 cells transformed with SR-RSV (Fig. 2, lanes g and h).

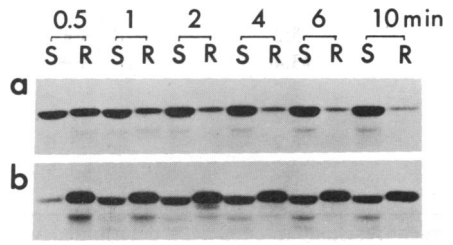

FIG. 1. Distribution of $p60^{src}$ between detergent-soluble and -resistant fractions of NY5H- or SR-RSV-infected cells. CEF infected with NY5H (*a*) or SR-RSV (*b*) were labeled with [^{3}H]leucine, extracted with CSK buffer for 0.5, 1, 2, 4, 6, or 10 min, and fractionated into soluble (S) and resistant (R) fractions. $p60^{src}$ of each fraction was immunoprecipitated with monoclonal antibody 327 and analyzed by $NaDodSO_4$/PAGE and fluorography.

$p60^{v\text{-}src}$ and $p60^{c\text{-}src}$ in the resistant fractions were slower in mobility than the respective $p60^{src}$ in the soluble fractions (Figs. 1 and 2). Several observations suggest that this difference is generated *in vitro* during fractionation and immunoprecipitation. With longer extraction time (30 sec–2 min), as more $p60^{src}$ was solubilized, there was a corresponding increase only in the faster moving $p60^{src}$ band in the soluble fraction. Furthermore, when the soluble and resistant fractions of SR-RSV-transformed cells were mixed and immunoprecipitated, $p60^{v\text{-}src}$ migrated as a single fast-moving band (data not shown). When SR-RSV-transformed cells were solubilized directly with RIPA buffer and immunoprecipitated, $p60^{v\text{-}src}$ was present as only a single fast-moving band (data not shown).

Lack of Association of $p60^{c\text{-}src}$ with Detergent-Resistant Structure in Transformed Cells. The possibility exists that resistance of $p60^{v\text{-}src}$ to the nonionic detergent extraction is a consequence of cell transformation rather than an intrinsic property of $p60^{v\text{-}src}$, since organization of certain cytoskeletal structures is known to be altered by transformation (44). If this were the case, then $p60^{c\text{-}src}$ might bind to the resistant structure in transformed cells. To examine this possibility, cultures were infected with NY308, which encodes a mutant $p60^{v\text{-}src}$ containing a deletion of amino acids 15–49 and which is fully active in transformation (6). As shown in Fig. 2 (lane b), the 51-kDa src protein of NY308 was resistant to the detergent extraction, whereas a faint band of endogenous $p60^{c\text{-}src}$ was seen primarily in the soluble fraction. Similarly, when CEF fully transformed with NY308 were superinfected with NY501B, a subgroup B virus containing c-*src*, overexpressed $p60^{c\text{-}src}$ was primarily soluble, whereas the bulk of NY308 src protein was resistant (Fig. 2, lane c).

We also found that the endogenous $p60^{c\text{-}src}$ was primarily soluble in a clonal line of FSV-transformed 3Y1 cells (data not shown).

Association of $p60^{src}$ of Various Mutants with the Detergent-Resistant Structure. To determine whether any correlation exists between the association with the detergent-resistant

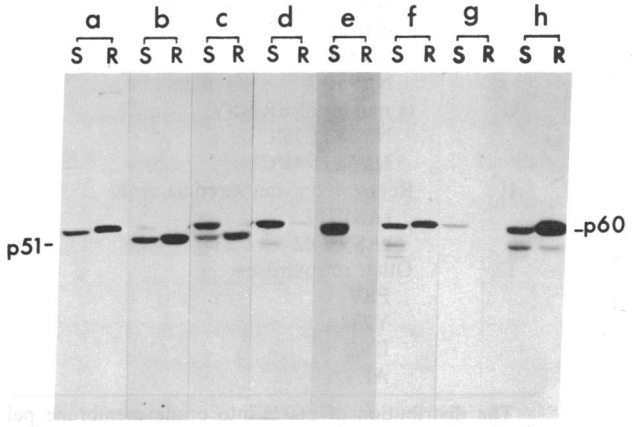

FIG. 2. Distribution of various src proteins between soluble and resistant fractions of infected cells. Cultures were labeled with [^{3}H]leucine and fractionated into soluble (S) and resistant (R) fractions. The src proteins of each fraction were analyzed as described in the legend to Fig. 1 for CEF infected with SR-RSV (a), NY308 (b), NY308 and NY501B together (c), NY501B (d), NY315 (e), or NYCHB (f); 3Y1 cells: uninfected (g) or SR-RSV-infected (h).

structure and the phenotypic changes in transformed cells, we have examined the distribution of $p60^{src}$ of various mutants and wild-type strains of RSV. The analysis of various categories of mutants is described below and results are summarized in Table 1. Some data are shown in Fig. 2. The relative amounts of src proteins in P100 fractions containing plasma membrane and the degree of morphological transformation of infected cells are also included in Table 1. Group A: like the SR-RSV src protein, the bulk of $p60^{v\text{-}src}$ of two other wild-type RSV strains, PR-RSV and RSV-29, was found in the resistant fraction. Group B: the $p60^{src}$ of two v-*src* point mutants constructed by Bryant and Parsons (36) that lack kinase activity due to single mutations in the kinase domain of $p60^{src}$ was found to be in the plasma membrane by subcellular fractionation and in the soluble fraction by CSK buffer extraction. Group C: $p60^{src}$ of NY501T7 and NYCHB, two strongly transforming viruses containing single point mutations in the c-*src* sequence, was mostly in the detergent-resistant fraction, although the extent was somewhat less than that of wild-type SR-RSV protein. Group D: the src proteins of three RSV mutants, NY315, NY314, and NY300, that lack the NH_2-terminal myristoylation were released into the soluble fraction by the extraction. Group E: examination of a series of mutants, NY308, NY309, NY311, and NY310, in which varying portions of the NH_2-terminal half of $p60^{v\text{-}src}$ (amino acids 15–49, 15–81, 15–149, and 15–169, respectively) are deleted revealed that the amount of src protein remaining in the detergent-resistant fraction decreased as the deletion became longer. Although these mutants have similar levels of kinase activity, the more extensive is the deletion the less distinct is the morphological alteration they induce in infected cells (10). Group F: three mutants that have chimeric *src* genes (NY701, NY851, NY951) were examined. $p60^{src}$ of the transforming virus NY701, which consists of the NH_2-terminal four-fifths of c-*src* and the COOH-terminal one-fifth of v-*src* (20), was found primarily in the resistant fraction. However, $p60^{src}$ of two transforming derivatives, NY851 and NY951 (29), was significantly reduced in the association with the resistant fraction. These two mutants are chimeric constructs containing largely the c-*src* sequence and small v-*src*

Table 1. Relative amounts of oncogene products in detergent-resistant fraction of infected cells

Group	Virus strain	Relative amount, %		Morphological transformation
		P100	Resistant fraction	
A	Wild-type RSV			
	SR-RSV	81.0	74.1	+++
	SR-RSV-3Y1		90.0	+++
	RSV-29	85.5	87.5	+++
	PR-RSV	64.0	68.8	+++
B	c-*src* virus and kinase-negative mutant of PR-RSV			
	NY5H	80*	7.3	−
	CHpm9	54.4	2.6	−
	CHpm26	53.8	3.6	−
C	Point mutant of c-*src* virus			
	NY501T7	87.9	62.0	+++
	NYCHB	80.0	65.5	+++
D	Nonmyristoylation mutant of SR-RSV			
	NY315	6.0	5.7	−
	NY314	10†	2.6	−
	NY300	20‡	3.8	−
E	Deletion mutant of SR-RSV			
	NY308	83†	78.8	+++
	NY309	84†	65.8	++
	NY311	75§	35.5	+
	NY310	75§	21.6	±
F	Chimera of c-*src* and v-*src*			
	NY701	68.5	76.1	+++
	NY851	64.6	28.5	+
	NY951	60.0	25.0	+
G	ts mutant of SR-RSV			
	NY68 at 36°C	68¶	64.2	+++
	NY68 at 41°C		51.3	−
H	Recovered avian sarcoma virus			
	rASV157	13.3	35.2	++
	rASV1702	11.4	43.4	++
I	Other retroviruses			
	FSV		82.2	+++
	Y73		78.2	+++
	UR2		4.3	+++
	AEV		5.0	++

The distribution of $p60^{src}$ into crude membrane pellet (P100) and detergent-resistant fraction was determined with [^{3}H]leucine-labeled cells. Relative amounts of the radioactivity in P100 or in the detergent-resistant fractions are shown by percentage of the total radioactivity. The degree of morphological transformation is expressed arbitrarily from − (negative) to +++ (most distinct). ts, Temperature sensitive.

*Ref. 37.
†Ref. 6.
‡Ref. 38.
§Ref. 10.
¶Ref. 45.

sequence substitutions. They produce less distinct morphological changes in infected cells compared with wild-type RSV. Group G: the src protein of the temperature-sensitive mutant tsNY68 was associated with the resistant fraction to a similar extent as the wild-type protein at permissive temperature, and this association was reduced at nonpermissive temperature. However, the extent of reduction was not substantial. This may be due to the treatment of cultures at low temperature during the detergent extraction that might allow reassociation of the src protein with the resistant structure. Group H: the src proteins of rASV157 and rASV-1702, which were shown to be nonmyristoylated and uniquely present in focal adhesion plaques rather than evenly distributed throughout the plasma membrane (11, 26), were found to be intermediate in the association with the resistant fraction. However, association of these src proteins with the resistant structure was salt sensitive, with almost complete detergent solubilization occurring in the presence of 300 mM KCl.

Distribution of src Proteins Assayed by Protein Kinase Activity. The *in vitro* protein kinase activity of each fraction was also examined following immunoprecipitation. Autophosphorylation of each src gene product or phosphorylation of enolase added as an exogenous substrate was measured. The relative level of kinase activity in each fraction reflected the amount of [^{3}H]leucine-labeled p60^{src} proteins, except for p60$^{c\text{-}src}$, which showed substantially higher kinase activity in the resistant fraction (Fig. 3). Thus, these results suggest that, except for p60$^{c\text{-}src}$, there is no activation of kinase activity by association with the resistant structure. The significance of the elevated p60$^{c\text{-}src}$ kinase activity is presently unclear.

Interaction of Other Oncogene Products with the Detergent-Resistant Structure. CEF infected with FSV, Y73, UR2, and AEV were examined. As shown in Table 1 and Fig. 3, P140$^{gag\text{-}fps}$ of FSV and P90$^{gag\text{-}yes}$ of Y73 associated with the resistant fraction by >78%. However, in contrast to wild-type p60$^{v\text{-}src}$, these transforming proteins were fully recovered in the soluble fraction in the presence of 300 mM KCl (data not shown). On the other hand, the transmembrane proteins, P68$^{gag\text{-}ros}$ of UR2 (ref. 46; S.-M. Jong and L.-H. Wang, personal communication) and P68$^{erb\text{-}B}$ of AEV (33), distributed to the soluble fraction using CSK buffer containing 100 mM KCl. Thus, these transmembrane proteins are similar to the membrane protein 5′-nucleotidase in their solubility to Triton X-100.

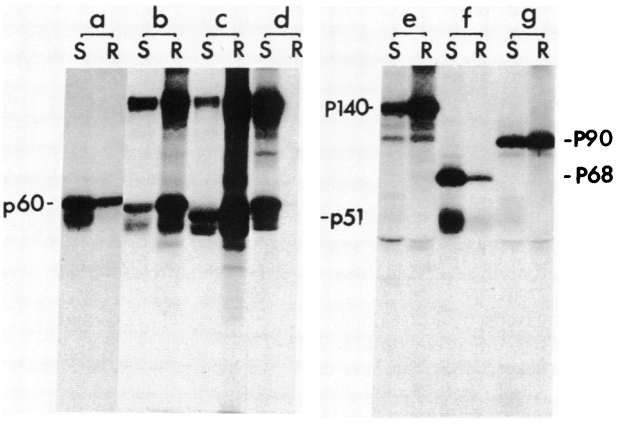

FIG. 3. Phosphorylation *in vitro* of various oncogene products from soluble and resistant fractions of infected cells. Cells infected with NY5H (a), SR-RSV (b), NY308 (c), NY315 (d), FSV (e), UR2 (f), or Y73 (g) were fractionated into soluble (S) and resistant (R) fractions. Each oncogene product was immunoprecipitated and subjected to kinase reaction. The reaction products were analyzed by NaDodSO$_4$/PAGE and gels were exposed to x-ray film for 4 hr at −70°C (a) or 45 min at room temperature (b–g).

DISCUSSION

Treatment of cells with nonionic detergents has been used to reveal the architecture of the cytoskeletal framework and its relation to other subcellular components (13–19). Gentle treatment of mouse fibroblast cells with Triton X-100 was shown to remove >90% of lipids and about three-fourths of total protein (16). We found that the protein components left behind by the treatment are enriched for cytoskeletal proteins but are not grossly different between nontransformed and transformed CEF.

Results of various experiments in this study strongly suggest that the association of p60$^{v\text{-}src}$ with the detergent-resistant structure is not artifactual. First, not all mutant p60^{src}s associate with this structure. For example, nonmyristoylated p60^{src} proteins failed to associate even though they are active in protein kinase activity. Transforming proteins of UR2 and AEV that are known to be transmembrane proteins with tyrosine protein kinase activity also did not associate with the detergent-resistant structure. In addition, we showed that p60$^{c\text{-}src}$ is solubilized by the detergent extraction even in fully transformed cells. Second, we found that the association of p60$^{v\text{-}src}$ with the detergent-resistant structure is stable. When cells were suspended, washed, and centrifuged in the CSK buffer, p60$^{v\text{-}src}$ was pelleted together with the resistant fraction. Furthermore, when the detergent-resistant fraction was treated with a mixture of 1% Triton X-100/0.5% sodium deoxycholate, the cytoskeletal framework was disrupted, but p60$^{v\text{-}src}$ sedimented in a sucrose gradient as a large complex with some cellular proteins (data not shown).

Previous studies have suggested that the level of protein kinase activity is a major difference between p60$^{v\text{-}src}$ and p60$^{c\text{-}src}$ that may contribute to their difference in transforming activity (37, 47). The results described in this paper show another possibly critical difference between these two proteins. To assess the significance of their difference in association with the detergent-resistant structure with respect to cell transformation, we have analyzed the behavior of the src proteins of various mutants in the detergent extraction. Salient features revealed from the analysis are as follows. (*i*) The src proteins defective in membrane association do not stably interact with the detergent-resistant structure. The src proteins of the nonmyristoylated mutants, which are known to be incapable of associating with plasma membranes, were soluble by the Triton X-100 extraction. The results suggest that the interaction with the detergent-resistant structure may take place in the vicinity of the plasma membrane. (*ii*) The protein kinase activity is required but not sufficient for stable interaction. Two kinase-negative CHpm mutants and p60$^{c\text{-}src}$ were poorly associated with the detergent-resistant structure. On the other hand, the src proteins of many mutants, including those of nonmyristoylated mutants, were active in kinase and yet were very limited in the association. (*iii*) No specific domain of p60^{src} is solely involved in the stable association. Proteins with a mutation in the kinase domain were weak in the association. By contrast, the NH_2-terminal deletion mutants NY310 and NY311 were active in kinase and in plasma membrane association but relatively weak in association with the resistant structure. It is conceivable that a certain conformation of p60^{src} is required for the association with the detergent-resistant structure. (*iv*) There is a good correlation between the degree of association with the detergent-resistant structure and the extent of morphological change. Perhaps the most dramatic cases were those of the highly transforming p60$^{c\text{-}src}$ point mutants NY501T7 and NYCHB. These mutants were active in the association, unlike the parental c-*src* virus NY5H. Other mutants constructed from c-src DNA, NY851 and NY951, were less distinct in transformation and more limited in the association.

In the case of the NH_2-terminal deletion mutants, transforming activity and association with the resistant structure are found to be inversely correlated with the length of the deletion.

The transforming proteins of FSV and Y73 and $p60^{src}$ of two isolates of rASV have been shown to be extractable by high-salt buffers from infected cells (30, 48). We found that these proteins are removable from the detergent-resistant structure by high-salt buffers, whereas $p60^{v\text{-}src}$ is not. We do not know whether their difference in salt sensitivity reflects a difference in the nature of interaction with the resistant fraction or whether an additional component, such as residual membranes, is involved in their interactions. Manger *et al.* (49) showed association of $P70^{gag\text{-}actin\text{-}fgr}$ of Gardner–Rasheed feline sarcoma virus with a similar detergent-resistant matrix. The results obtained with the UR2 and AEV proteins suggest that association with the detergent-resistant structure is not required for transformation by all of the tyrosine protein kinase oncogene products. Further studies are required, however, to determine whether this means that the subcellular structure(s) with which different oncogene products interact are not common or whether they are simply differentiated by the conditions used for the extraction.

Because of the correlation with morphological alteration, the association of $p60^{src}$ with the detergent-resistant structure seems to be required for the process leading to cell transformation. However, we do not know how this association is related to the interaction of $p60^{src}$ with substrate proteins. Thus far, we have no definitive evidence whether this detergent-resistant structure itself contains primary targets for $p60^{v\text{-}src}$. Identification of the protein(s) in the detergent-resistant structure to which $p60^{src}$ directly binds is another important problem that remains to be studied.

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