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A Short Sequence in the p60^{src} N Terminus Is Required for p60^{src} Myristylation and Membrane Association and for Cell Transformation

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We have constructed mutants by using linker insertion followed by deletion in the region of cloned Rous sarcoma virus DNA coding for the N-terminal 9 kilodaltons of the *src* protein. Previous work implicated this region in the membrane association of the protein. The mutations had little effect on *src* tyrosine kinase activity. Substitution of a tri- or tetrapeptide for amino acids 15 to 27, 15 to 49, or 15 to 81 had little effect on the in vitro transforming capacity of the virus. Like wild-type p60^{src}, the *src* proteins of these mutants associated with plasma membranes and were labeled with [^{3}H]myristic acid. In contrast, a mutant whose *src* protein had the dipeptide Asp-Leu substituted for amino acids 2 to 81 and a mutant with the tripeptide Asp-Leu-Gly substituted for amino acids 2 to 15 were transformation defective, and the mutant proteins did not associate with membranes and were not labeled with [^{3}H]myristic acid. These results suggest that amino acids 2 to 15 serve as an attachment site for myristic acid and as a membrane anchor. Since deletions including this region prevent transformation, and since tyrosine kinase activity is not diminished by the deletions, these results imply that target recognition is impaired by mutations altering the very N terminus, perhaps through their effect on membrane association.

Rous sarcoma virus (RSV) transforms chicken embryo fibroblasts (CEF) in culture and causes tumors in chickens as a result of the expression of the *src* gene (19). The *src* gene encodes a phosphoprotein tyrosine kinase, p60^{src} (4, 8, 9, 14, 22, 30, 31, 37). The tyrosine kinase activity is associated with transforming activity (8, 30, 43).

The C-terminal portion of p60^{src} isolated by proteolysis retains tyrosine kinase activity (2, 29), suggesting that this portion of the molecule is a domain capable of independent function. Additional evidence consistent with mapping the kinase activity to the C terminus of the molecule comes from sequence homologies detected between the amino acid sequence of p60^{src} in this region and regions of various other tyrosine kinases (24, 38, 46), as well as the catalytic subunit of cyclic AMP-dependent protein kinase (1), and from the study of a revertant cell line producing a *src* protein with an N-terminal deletion possessing tyrosine kinase activity (36).

Wild-type (wt) p60^{src} is known to be associated with the inner surface of the plasma membrane (11, 26–29, 34, 39, 40, 48). Proteolytic digestion experiments (29) suggest that the N-terminal 13 kilodaltons (kd) are involved in this association. Also, there is covalently bound fatty acid in the N-terminal 16 kd (17, 45). Some recovered avian sarcoma viruses (rASVs) (20) (recombinants between viruses with large *src* deletions and cellular *src* sequences) have deletions or insertions or both in the N-terminal 8 kd (23). Unlike the wt *src* protein, the *src* proteins of these rASVs can be extracted from a crude membrane pellet by isotonic salt (26), and they are not labeled with fatty acid (17). This suggested a direct involvement of the N-terminal 8 kd in membrane association and fatty acid attachment, consistent with the proteolytic data and the location of the fatty acid (17, 27, 29, 45). Therefore, we decided to make deletion mutants in the N-terminal 9 kd to try to perturb membrane association, and to examine the effects of such deletions on the attachment of fatty acid to p60^{src} and on the biological activity of the mutant viruses.

MATERIALS AND METHODS

Plasmid constructions. Figure 1 shows a map of the *src* regions of most of the plasmids used in this study, with relevant restriction sites indicated. Figure 2 shows the DNA sequences deduced for the mutant clones studied.

Digestions with restriction enzymes and BAL 31, linker insertion, and recombinations between clones with linkers inserted at different locations were as described previously (13, 33).

The constructions of pSR-XD2, -XD11-0, -XD11-1, -XD11-4, -XD11-7, and -REP were described previously (13). pSR-XD11-7 has been renamed pSR-XD307 in this publication.

pSR-XD11-0 and pSR-XD11-1 have *Bgl*II linkers inserted in the codon for the arginine residue at position 15, with a one-base-pair difference in the site of insertion (13). pSR-XD308 and pSR-XD309 were constructed from pSR-XD11-0 by *Bgl*II digestion and BAL 31 treatment, followed by *Bgl*II linker insertion and recombination in vitro of the 3′ arm of the deletion with the 5′ arm of either pSR-XD11-1 (for pSR-XD308) or pSR-XD11-0 (for pSR-XD309). This was accomplished by digesting the parental plasmids with *Bgl*II and *Sac*I and ligating together the 6.7-kilobase fragment from pSR-XD11-1 or -XD11-0 (containing the DNA coding for amino acids 1 to 14 of p60^{src}) with the 2.5-kilobase fragment (containing the 3′ portion of the BAL 31-deleted *src* gene) from one of the clones with the BAL 31-generated deletions. This was the same procedure as the one used for the construction of pSR-XD307 (13). pSR-XD11-0 and pSR-XD11-1 were chosen to create in-frame deletions after the

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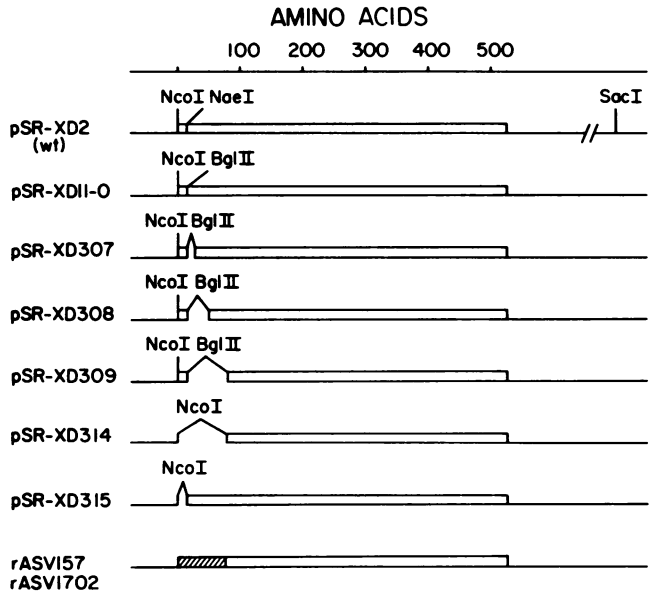

FIG. 1. Structures of mutant plasmids. The open boxes represent *src* coding sequences. The bridges drawn with a thin line represent the linker information bridging the deletions. The positions of restriction sites used in the constructions are shown. The hatched box indicates the region containing the alterations in sequence in the *src* coding sequences of rASVs 157 and 1702 (23).

DNA sequencing (32) of the clones containing the BAL 31-generated deletions.

pSR-XD314 was constructed from pSR-XD309 as follows. pSR-XD309 DNA was cut with *Nco*I and *Bgl*II. The cut DNA was gap filled with Klenow fragment of DNA polymerase I as described previously (33) and self ligated in a concentrated blunt-ended reaction. This mixture was digested with *Nco*I and *Sac*I. Since the original *Nco*I sites were gap filled, the only *Nco*I sites that are predicted to exist should be derived from ligation of a filled *Nco*I site and a filled *Bgl*II site. The 2.3-kilobase band was isolated from this reaction and ligated to the 6.7-kilobase band from pSR-XD2 digested partially with *Nco*I and completely with *Sac*I. Plasmids were screened for the loss of the *Bgl*II site of pSR-XD309, for the deletion of the DNA coding for amino acids 2 to 14 in pSR-XD309, and for the presence of the *Nco*I site. pSR-XD314 DNA was found to satisfy these criteria and, upon DNA sequencing (32) from the *Nco*I site was found to have the desired structure.

pSR-XD315 was constructed from a clone called pSR-XD11-6. pSR-XD11-6 is a derivative of pSR-XD11-0 (13), with one base pair extra inserted between the 3′ end of the linker and the *src* sequence (this clone was constructed in the same way as pSR-XD11-1 and pSR-XD11-4, by *Bgl*II digestion of pSR-XD11-0, followed by nuclease S1 treatment and *Bgl*II linker reinsertion [13]). The steps in the construction of pSR-XD315 were the same as those used in the construction of pSR-XD314, except pSR-XD11-6 was the starting material instead of pSR-XD309.

Cell culture and virus. Cultures of secondary CEF were maintained, transfected, and infected as described previously (13). Experiments were done either with transfected cultures or with cultures infected with virus derived from transfection with essentially identical results. When infected cultures were used, experiments were generally performed within 1 week after infection. Transfected cultures were maintained for at least 2 weeks with two to three transfers to guarantee complete infection before virus was harvested or the cells were used for experiments.

Protein biochemistry and cell fractionation. Labeling cultures with $^{32}P_i$ and [^{3}H]leucine and immunoprecipitation of *src* protein were essentially as described previously (23). Labeling of cultures with [^{3}H]myristic acid was performed as follows. [9,10-^{3}H]myristic acid (kindly provided by Alan Schultz and Stephen Oroszlan) was dissolved in a minimum volume of 70% ethanol and diluted to 0.5 mCi/ml with Ham F-10 medium containing 10% tryptose phosphate broth, 5% calf serum, and 1% dimethyl sulfoxide. Petri plates (60 mm) of infected cells were labeled for 4 h with 1 ml of this medium.

Cells were fractionated by differential centrifugation and equilibrium centrifugation on discontinuous sucrose gradients essentially as described previously (16), except that: (i) buffer D contained 1 mM EDTA and had no $MgCl_2$, and (ii) membranes floating at interfaces in discontinuous sucrose gradients were collected, diluted in buffer D, pelleted by a 1-h centrifugation at 100,000 × *g*, and suspended in buffer D-RIPA (16) for analysis.

Fractionation of extracts of cells on glycerol gradients for

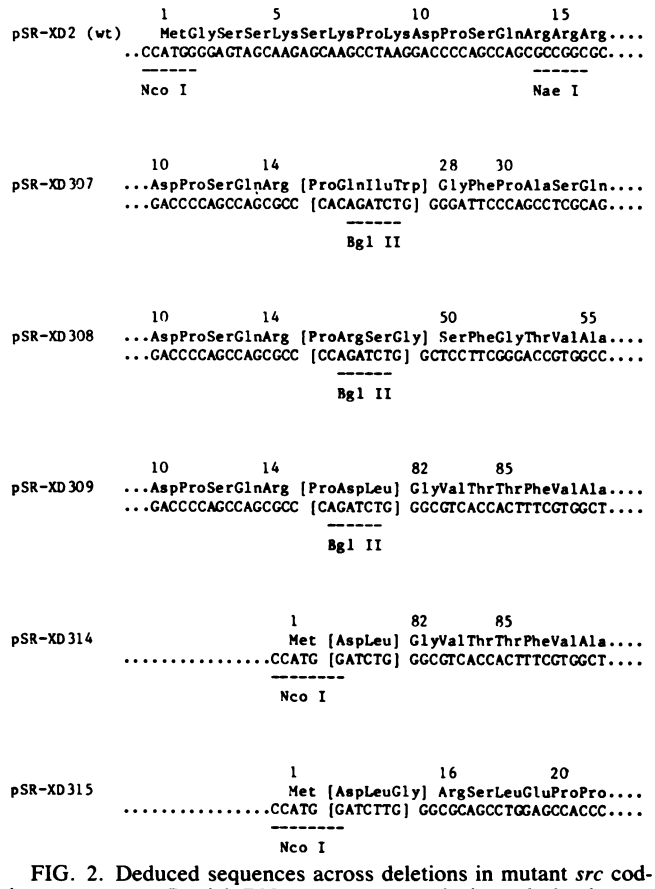

FIG. 2. Deduced sequences across deletions in mutant *src* coding sequences. Partial DNA sequence analysis and the known sequence of the wt *src* gene (47) were used to predict the sequence across the deletions in the mutants. Amino acids are numbered according to the wt sequence. The positions of restriction enzyme cleavage sites used in the constructions are shown. Sequences that are inserted or altered relative to the wt sequence are delimited by brackets. pSR-XD2 is the parental plasmid containing the wt *src* sequence (13). Virus derived from this plasmid is referred to as SRA. Viruses derived from the other plasmids are referred to as NY307, NY308, NY309, NY314, and NY315.

analysis of the sedimentation behavior of *src* protein was as described previously (3).

The immunoglobulin G (IgG) phosphorylation assay for determination of tyrosine kinase activity was performed as described previously (16).

Determination of cellular phosphoamino acid levels was performed as described previously (10, 22), using extracts from infected CEF labeled with $^{32}P_i$ for 4 h.

RESULTS

Construction of deletion mutants and recovery of virus. The construction of deletion mutants started with *Bgl*II linker insertion at various sites in the *src* gene in the plasmid pSR-XD2 (13). This plasmid contains the *src* gene of the Schmidt-Ruppin subgroup A strain of RSV (SRA).

Insertions and deletions were introduced and characterized as described above. Figure 1 shows the structure of the *src* coding regions in the various plasmids, as well as relevant restriction sites used in the constructions. Figure 1 also shows the approximate location of the alterations in the *src* genes of rASVs 157 and 1702 (23).

Figure 2 shows the deduced amino acid sequences around the deletions in the mutant plasmid. (See Takeya and Hanafusa [47] for the complete wt *src* sequence.) The N-terminal 8 kd, which has been suggested to play a role in membrane association (23, 26), consists of approximately amino acids 1 to 70 (47). The entire N-terminal 9 kd was removed in the predicted *src* protein encoded by the plasmid pSR-XD314, with amino acids 2 to 81 deleted (Fig. 1 and 2).

Virus was recovered from these plasmids by transfection of CEF with the *Sal*I-cut plasmids bearing the mutant *src* genes ligated to the *Sal*I-cut pSR-REP plasmid, as described previously (13). Focus formation after transfection proceeded with essentially identical efficiency and speed for all clones described, except for pSR-XD314 and pSR-XD315, which induced no foci, despite the replication of the mutant virus in transfected cells (see below).

Biological characterization of mutant viruses. pSR-XD2, pSR-XD307, pSR-XD308, and pSR-XD309 all induced foci of morphologically transformed cells after transfection. pSR-XD307 and pSR-XD308 induced foci of round cells similar to those induced by the wt pSR-XD2. pSR-XD309 induced foci of fusiform cells. Transformed cells grew to high density and yielded transforming virus in the medium. We refer to viruses derived from transfection with these clones as NY307, NY308, and NY309. The virus derived from transfection of pSR-XD2 is identical to wt SRA (13). Cells transformed by these viruses grew in soft agar suspension with about the same efficiency as cells transformed with wt SRA, at 37 or 41°C.

pSR-XD314 and pSR-XD315 did not induce foci, at either 37 or 41°C. However, infectious virus stocks, designated NY314 and NY315, were recovered after transfection with these DNAs. These stocks were shown to be infectious by the synthesis in infected cultures of the viral structural protein precursor Pr76 and of a deleted *src* protein (see Fig. 5 and 6). We standardized the infectivity of the virus stocks used in these experiments by measuring the ability of a small portion of the stock to induce the expression of the *src* tyrosine kinase activity (8, 30) 36 h after infection. Since the kinase activities and rates of synthesis of the various *src* proteins described here are approximately equivalent (see Table 1 and below), this assay is a fair one for comparing the different viruses. By this standardization, the titers of the various stocks were equivalent to within a factor of 10.

Focus formation by NY314 and NY315 stocks was reduced by at least 10,000-fold relative to SRA or NY309 stocks (data not shown). SRA, NY309, NY314, and NY315 were tested quantitatively for their ability to induce soft agar colony formation at various dilutions of virus. After the infectivity of the virus stocks was standardized with the kinase assay, SRA induced 1.0 colony per unit of virus (this represents 5.5×10^5 CFU/ml of virus). NY309 was essentially like SRA in its ability to induce soft agar colony formation (2.2 colonies per unit of virus). NY314 and NY315 induced a low level of soft agar colony formation (7×10^{-4} and 3×10^{-4} colony per unit of virus, respectively). The few colonies induced by NY314 and NY315 were small and took over 1 month to become clearly visible, as compared with the colonies induced by SRA and NY309, which were apparent within 2 weeks. We are currently testing to find out whether the low level of colony formation is due to a small mutant population in the NY314 and NY315 virus stocks or represents a rare weak transformation by the original constructs. In either case, it is clear that the transforming potential of NY314 and NY315 is severely reduced compared with the other viruses tested, which are essentially similar to wt SRA.

Expression of mutant *src* genes. All of the mutants produced *src* proteins of the sizes expected from the DNA sequence analysis (47; see Fig. 1, 2, 5, and 6). The stability of the wt and mutant *src* proteins was assayed by pulse-chase analysis with [^{3}H]leucine. wt *src* protein had a half-life of between 8 and 12 h (44). The *src* proteins of NY309, NY314, and NY315 had half-lives of about 7, 4, and 7 h, respectively (data not shown). We do not consider these small alterations in half-life to be likely to significantly affect the biological activity of the mutant viruses. The half-life of the *src* protein of the Prague strain of RSV was reported to be only about 2 h, and the Prague strain is able to transform cells (44).

Tyrosine kinase activities of mutant *src* proteins. p60^{src} has been shown to phosphorylate the heavy chain of IgG in immunoprecipitates with serum from tumor-bearing rabbits (TBR) (4, 8, 30). We used this in vitro assay to measure the kinase activity of the mutant *src* proteins (Table 1). All the mutant viruses encoded *src* proteins with tyrosine kinase activities comparable to that of wt *src* protein. A slight reduction in the level of tyrosine kinase activity from cells infected with NY314 may be related to the reduced half-life of the NY314 *src* protein.

In addition, we labeled uninfected cells and cells infected with NY309, NY314, and SRA with $^{32}P_i$ to determine the amount of tyrosine phosphorylation of cellular proteins (22). SRA-infected cells and NY309-infected cells had about seven times more phosphotyrosine than uninfected CEF,

TABLE 1. Tyrosine kinase activities of mutant *src* proteins

Virus	IgG phosphorylation[a] (fraction of SRA)
SRA	1.0
NY307	1.0
NY308	1.1
NY309	0.8
NY314	0.6
NY315	1.0
None	0.002

[a] Immunoprecipitates were assayed for their content of kinase activity, as described previously (16). A wt SRA sample was included as a standard in each experiment. The ^{32}P counts per minute incorporated into IgG heavy chain in this sample averaged abou 2×10^5 cpm, over a background of about 200 cpm.

and NY314-infected cells had about four times more (Table 2).

These results support the in vitro measurements of kinase activity, on the assumption that the increase in cellular phosphotyrosine in RSV-infected cells is catalyzed directly by the *src* kinase. Our data show that most of the increase in phosphotyrosine is not a secondary consequence of RSV transformation, since NY314-infected cells are not transformed, yet phosphotyrosine is increased. NY314 increases the phosphotyrosine level less than does SRA. This correlates with a slight reduction in the total tyrosine kinase activity measured in vitro (Table 1).

Membrane association of mutant *src* proteins. To examine *src* membrane association, infected cells were Dounce homogenized, and a postnuclear supernatant was fractionated into an S100 and a P100 (27). *src* protein was immunoprecipitated from these fractions. The distribution was quantitated by determining the radioactivity associated with the *src* protein gel band, where labeled extracts were fractionated, or by assaying the tyrosine kinase activity in the immune complexes. Consistent with previous reports (11, 26–29), wt *src* protein fractionated mostly with the membrane pellet (Table 3). The *src* proteins of NY307, NY308, and NY309 also fractionated with the membrane pellet. However, about 90% of the *src* proteins of NY314 and NY315 fractionated in the cytosolic supernatant.

To extend these results, we performed a fractionation of the crude membrane pellet by flotation in discontinuous sucrose gradients (Table 4). The distributions of the SRA, NY308, and NY309 *src* proteins were similar to the distribution of plasma membrane markers in this type of fractionation, showing a large enrichment in the plasma membrane fraction (10%/30% interface). The small amounts of the NY314 and NY315 *src* proteins that were associated with the crude membrane pellet (Table 3) showed much less enrichment in the plasma membrane fraction (Table 4).

Complex formation by mutant *src* proteins. $p60^{src}$ is known to associate with two cellular proteins, pp50 and pp90, shortly after its synthesis and before it reaches the plasma membrane (3, 5, 12, 35). The *src* protein of an SRA mutant that is temperature sensitive for transformation, tsNY68, is mostly found in the complexed form at nonpermissive temperature (5, 12). The restriction of this protein to the complex and its lack of membrane association at the nonpermissive temperature might be related (5, 12, 18). We wanted to investigate whether the lack of membrane association of the NY314 *src* protein might also correlate with increased binding to pp50 and pp90.

We detected pp90 in immunoprecipitates from extracts of $^{32}P_i$-labeled cells infected with NY314, NY309, and wt SRA (Fig. 3), and the level of pp90 was comparable in these immunoprecipitates. (The mutant *src* proteins are underphosphorylated relative to wt *src* protein, and the intensity of the *src* bands in Fig. 3 probably indicates an approximately equal level of *src* protein in all three immunoprecipitates. Using partial proteolytic mapping with *Staphylococcus aureus* V8 protease [7; data not shown], we have found that most of the ^{32}P label in these proteins was in the C-terminal 26 kd. Some reduction in N-terminal phosphorylation of these proteins is expected because of the deletion of serine 17 [13].) We have confirmed that the pp90 in all three immunoprecipitates was identical by partial proteolytic mapping with *S. aureus* V8 protease (data not shown). pp50 was also detectable in these immunoprecipitates, although it migrated very close to the *src* proteins of NY314 and NY309 (predicted molecular masses are 50 and 51 kd, respectively).

The formation of the complex can be demonstrated by sedimentation of extracts of infected cells through glycerol gradients (3). The complex of *src* protein, pp50, and pp90 sediments faster than the monomer *src* protein. When gradient fractions are immunoprecipitated with TBR serum, pp50,

TABLE 2. Phosphoamino acid content of infected cells[a]

Virus	% P-Ser	% P-Thr	% P-Tyr
SRA	95.0	4.9	0.50
NY309	96.2	3.8	0.55
NY314	96.2	4.2	0.27
None	96.2	3.8	0.07

[a] See the text. The total ^{32}P counts per minute in phosphoamino acids analyzed for each sample were 20,000 to 30,000. The background (a region of the thin-layer chromatography plate adjacent to the phosphotyrosine spot), varying between 10 and 20 cpm, was subtracted.

TABLE 3. Distribution of mutant *src* proteins

Virus	% *src* protein in P100[a] determined by:	
	[^{3}H]leucine[b]	Kinase activity[c]
SRA	81	78
NY307	ND[d]	84
NY308	83	78
NY309	84	76
NY314	10	9
NY315	ND	13

[a] Infected cells were broken by Dounce homogenization. After removal of nuclei, a crude membrane pellet (P100) and cytosolic fraction (S100) were prepared. When checked, recovery was greater than 80%. The percentage values of the *src* protein found in the P100 are shown, assuming 100% recovery.

[b] If cells were labeled, the amount of [^{3}H]leucine in immunoprecipitated *src* protein was quantitated after gel electrophoresis. Approximately 1,000 cpm were incorporated into each *src* protein, distributed between the two fractions, over a background of 40 cpm.

[c] The amount of tyrosine kinase activity in the fractions was determined after immunoprecipitation, as in Table 2. Approximately 3×10^5 cpm were incorporated into IgG in immunoprecipitates from the S100 and P100 fractions for each virus, over a background of about 200 cpm.

[d] ND, Not determined.

TABLE 4. Fractionation of membrane-bound *src* kinase activity on discontinuous sucrose gradients

Virus	Sp. act of IgG kinase in membrane fractions[a]:			
	10%/30%	30%/35%	35%/45%	45%/60%
SRA	117.3	48.7	14.2	1.0
NY308	78.7	33.1	13.5	0.8
NY309	95.2	44.7	13.2	0.9
NY314	1.0	0.5	0.1	0.1
NY315	1.5	0.6	0.9	1.0

[a] Cells infected with the indicated viruses were labeled with [^{3}H]leucine for 4 h and Dounce homogenized, and a crude membrane pellet was prepared, fractionated on a sucrose gradient, and analyzed for specific activity of *src* kinase as described in the text. The data are normalized to the specific activity of the wt SRA kinase activity found in the 45%/60% interface. Specific activity is defined as the ^{32}P counts per minute in IgG in a kinase assay divided by the total amount of protein in the fraction as indicated by trichloroacetic acid-precipitable [^{3}H]leucine label. The distribution of total protein was essentially invariant for the various fractionations and was ca. 2:10:40:48 in the 10%/30%, 30%/35%, 35%/45%, and 45%/60% interfaces, respectively. The specific activity of 5′-nucleotidase fractionates in this procedure approximately as follows: 240:30:4:1 (arbitrary units). The total ^{32}P counts per minute in the fractions were around 10^6 in each case, and the total ^3H counts per minute were around 10^4. The amount of kinase activity in the fractions resulted in at least 3,000 cpm of ^{32}P over a background of ca. 200 cpm, and the amount of ^3H counts per minute in the fractions was at least 300 cpm over a background of ca. 10 cpm.

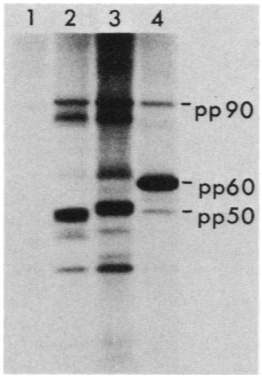

FIG. 3. Complex formation by *src* proteins of NY314, NY309, and wt SRA. Cells were labeled with $^{32}P_i$, and extracts were immunoprecipitated with TBR serum. The positions of pp90, pp50, and wt pp60^{src} are indicated. The *src* proteins of NY309 and NY314 run slightly behind and slightly ahead of the pp50 protein, respectively. Lane 1, Uninfected CEF; lanes 2 through 4, CEF infected with NY314, NY309, and wt SRA, respectively.

pp90, and a small proportion of the total *src* protein are detected in faster-sedimenting fractions. The results of such an assay on $^{32}P_i$-labeled cells infected with SRA and NY314 are shown in Fig. 4. pp50 and pp90 are detectable in both gradients at a position separated from the bulk of the SRA and NY314 *src* proteins. It is somewhat difficult to interpret this assay quantitatively in the case of NY314, since the NY314 *src* protein labeled poorly with $^{32}P_i$ and almost comigrated with the pp50 protein (Fig. 3). However, it is clear that the majority of the NY314 *src* protein is not complex bound. This has been confirmed by glycerol gradient analysis of extracts from [^{3}H]leucine-labeled cells (Fig. 4C). This behavior differs from that of the tsNY68 *src* protein at nonpermissive temperature (5, 12; data not shown). The glycerol gradient analysis of the *src* protein of NY315 from cells labeled with [^{3}H]leucine gave essentially the same result as is shown in Fig. 4C (data not shown). Therefore, the NY314 and NY315 *src* proteins behave like wt *src* protein with respect to complex formation, rather than like the tsNY68 *src* protein at nonpermissive temperature.

Attachment of myristic acid to *src* proteins. The wt *src* protein has been shown to contain covalently bound palmitic acid (17, 45). Recently, we have found that *src* protein can be labeled with myristic acid much more efficiently than with palmitic acid, and the fatty acid bound to *src* protein after this labeling was identified as myristic acid (E. Garber, A. Schultz, H. Hanafusa, and S. Oroszlan, unpublished data). Cells infected with SRA, NY307, NY309, NY314, and NY18-3, a transformation-defective virus with an in vitro-constructed *src* deletion of amino acids 169 to 264 (unpublished data), were labeled with [^{3}H]leucine or [^{3}H]myristic acid (Fig. 5). All of the *src* proteins were labeled well with myristic acid except for the NY314 *src* protein, which was not labeled detectably (even after long exposure).

Figure 6 shows the result of a similar analysis of the *src* protein of NY315. This protein also was not labeled with myristic acid. Therefore, myristic acid attachment requires amino acids 2 to 15. In addition, only amino acids 1 to 14 of the entire N-terminal 9 kd are required for myristylation, as is shown by the [^{3}H]myristic acid labeling of the *src* protein of NY309 (Fig. 5 and 6).

The lack of myristic acid attachment to the NY314 and NY315 *src* proteins cannot be explained by the transformation-defective nature of the encoding virus, since the *src* protein of the transformation-defective NY18-3 was labeled with myristic acid about as well as wt *src* protein (Fig. 5, lane 1).

DISCUSSION

The biological characterization of the mutant viruses described here and the biochemical properties of the *src* proteins they encode are summarized in Table 5.

A critical sequence in the p60^{src} N terminus. In this set of mutants, three aspects of *src* behavior are correlated: mem-

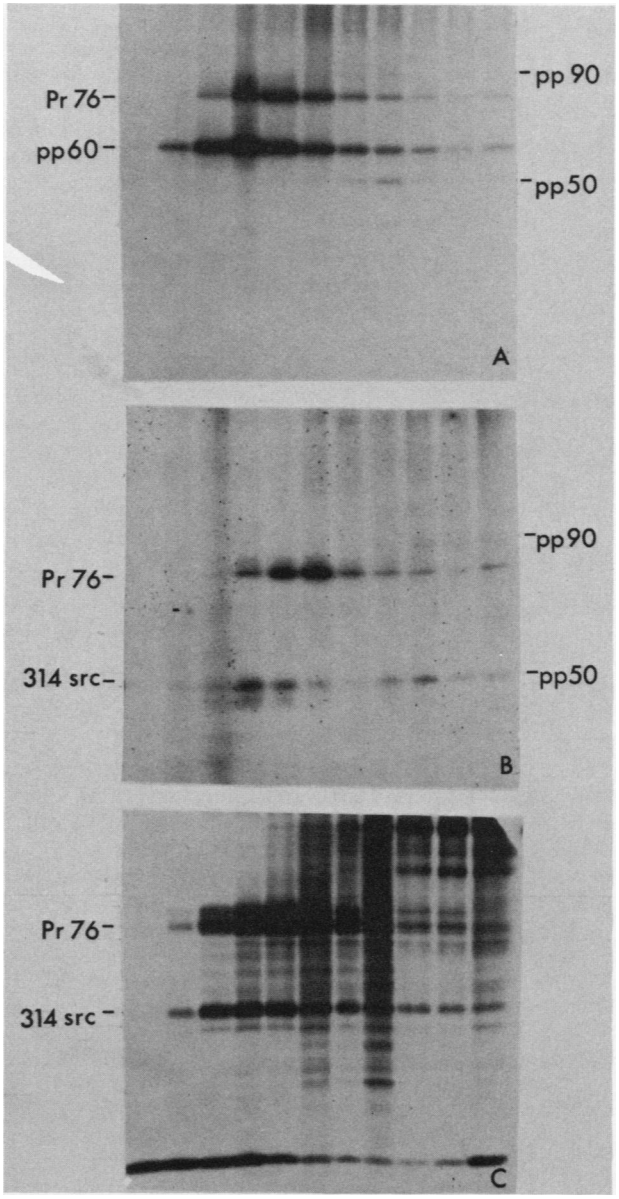

FIG. 4. Complex formation by *src* proteins of wt SRA and NY314. Cells infected with SRA (A) or NY314 (B and C) were labeled with $^{32}P_i$ (A and B) or [^{3}H]leucine (C), and extracts were fractionated on glycerol gradients (3). Alternate fractions were immunoprecipitated with TBR serum. Sedimentation was from left to right. The positions of Pr76, a viral structural protein precursor, pp60^{src}, and the cellular proteins pp90 and pp50 are indicated. The *src* protein of NY314 almost comigrates with pp50.

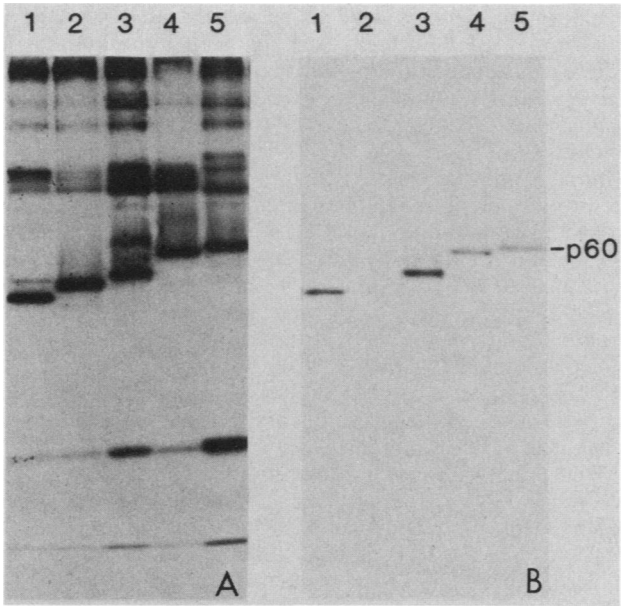

FIG. 5. Myristylation of mutant *src* proteins. Cells infected with various viruses were labeled with [^{3}H]leucine (A) or [^{3}H]myristate (B), and *src* proteins were immunoprecipitated with TBR serum. Cells were infected with the following viruses: lanes 1, NY18-3, a transformation-defective virus with a deletion of amino acids 169 to 264 (unpublished data); lanes 2, NY314; lanes 3, NY309; lanes 4, NY307; lanes 5, wt SRA. The position of wt p60^{src} is indicated.

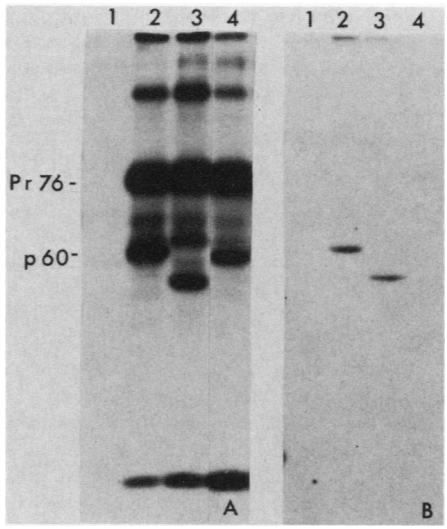

FIG. 6. Lack of myristylation of NY315 *src* protein. Uninfected cells (lanes 1) or cells infected with SRA (lanes 2), NY309 (lanes 3), or NY315 (lanes 4) were labeled with [^{3}H]leucine (A) or with [^{3}H]myristic acid (B), and *src* proteins were immunoprecipitated with TBR serum. The positions of wt p60^{src} and the viral structural protein precursor Pr76 are indicated.

brane association and myristylation of the protein and cell transformation by the protein. The NY309 deletion, with Pro-Asp-Leu substituted for amino acids 15 to 81, was positive for all three. The NY314 deletion, with Asp-Leu substituted for amino acids 2 to 81, was negative, as was the NY315 deletion, with Asp-Leu-Gly substituted for amino acids 2 to 15. This suggested that amino acids 2 to 15 are required for membrane association, myristylation, and cell transformation. We believe that a specific inhibitory effect of the Asp-Leu sequence is unlikely for the following reasons. (i) Asp-Leu is also found in the NY309 sequence. NY314 is identical to NY309 except for the deletion of amino acids 2 to 14 plus the proline residue derived from the linker insertion in NY309 (Fig. 1 and 2). (ii) We have reproduced the behavior of NY314 and NY315 with a mutant with the sequence Asn-Arg-Ser-Gly replacing amino acids 2 to 4 (unpublished data). Therefore, we believe that the lesion in NY314 and NY315 is due to the loss of functional sequences at the extreme N terminus (amino acids 2 to 15) and not to a specific inhibitory effect of the inserted Asp-Leu sequence.

Myristylation, membrane association, and cell transformation require only amino acids 1 to 14 of the N-terminal 9 kd. Our speculation is that myristylation is required for membrane association, which is required for cell transformation. Obviously, we cannot prove this causal model with correlational evidence. However, we discuss below how these three phenomena might interrelate.

Sequence requirements for myristylation. The site of palmitic acid attachment to p60^{src} is in the N-terminal 16 kd (45), and we have found the same to be true of the site of myristic acid attachment (data not shown). We have found that a mutant protein with amino acids 15 to 169 deleted can be labeled with myristic acid, and this deletion probably covers the entire N-terminal 16 kd (unpublished data). Combined with the result that amino acids 169 to 264 can be deleted without preventing myristylation (Fig. 5, lane 1), this shows that the only sequence in the N-terminal half of the protein that is required for myristylation is amino acids 1 to 14. Probably amino acids 1 to 14 contain the site of myristic acid attachment, and this may explain the lack of myristylation of the *src* proteins of NY314 and NY315.

It is interesting to draw an analogy between myristylation of murine leukemia virus p15 and the catalytic subunit of cyclic AMP-dependent protein kinase and myristylation of *src* protein. Myristic acid has been shown to be attached in an amide linkage to the glycine residue at position 2 of

TABLE 5. Summary of biological and biochemical characterization of mutant viruses[a]

Virus	Amino acids deleted	Amino acids substituted	Morphology	Anchorage independence	Kinase (IgG)	Cell P-Tyr.	Membrane association	Myristylation
SRA			Round	+	1.0	1.0	+	+
NY307	15–27	PQIW	Round	+	1.0	0.8	+	+
NY308	15–49	PRSG	Round	+	1.1	ND	+	+
NY309	15–81	PDL	Fusif	+	0.8	1.1	+	+
NY314	2–81	DL	Flat	−	0.6	0.5	−	−
NY315	2–15	DLG	Flat	−	1.0	ND	−	−

[a] Abbreviations: Kinase (IgG), *src* tyrosine kinase assay measured by TBR IgG phosphorylation (fraction of SRA value); cell P-Tyr., level of cellular phosphotyrosine (fractions of SRA value); Fusif, fusiform morphology; P, proline; Q, glutamine; I, isoleucine; W, tryptophan; R, arginine; S, serine; G, glycine; D, aspartic acid; L, leucine; ND, not done.

murine leukemia virus p15 (the initiator methionine residue is removed, and the myristylation blocks the N terminus of the mature p15) and to the N-terminal glycine of the catalytic subunit (6, 21, 41). $p60^{src}$ has a glycine at position 2 of its deduced primary sequence (42, 47). In addition, its N terminus is blocked (S. Oroszlan, personal communication). If the linkage of myristic acid to *src* protein is also by an amide linkage to Gly 2, then the lack of myristic acid attachment to the NY314 and NY315 *src* proteins might be a direct consequence of the substitution of aspartic acid for Gly 2 in these proteins. The *src* proteins of rASV1702 and rASV157, which are not myristylated (unpublished data), contain deletions or insertions or both in the N-terminal 8 kd of *src* (23). It will be interesting to learn if these alterations change the second amino acid of the sequence.

Myristic acid attachment and membrane association. The *src* protein of tsNY68 is not membrane associated at nonpermissive temperature (5, 12, 18). However, at this temperature much of this *src* protein is bound in a complex with pp50 and pp90 (5, 12), and this might affect its distribution in cell fractionation experiments. In contrast, little of the NY314 and NY315 *src* proteins are in the complex. Therefore, although increased complex binding may account for the solubility of the *src* protein of tsNY68, it cannot account for the solubility of the NY314 and NY315 *src* proteins.

The solubility of the NY314 and NY315 *src* proteins could be explained by a block in myristylation of these proteins. Consistent with this possibility, we have found that myristylation of wt *src* protein precedes its association with the membrane (unpublished data).

Previous results with the rASVs 157 and 1702, whose *src* proteins are not labeled with palmitic acid (7) or myristic acid (unpublished data) and which are reduced in their membrane association (26), are also consistent with this idea.

$p60^{src}$ membrane association and transformation. NY307, NY308, and NY309 all encode *src* proteins that associate with the plasma membrane, and the viruses transform CEF with the same efficiency as wt SRA. NY309 is somewhat defective in tumor induction (data not shown), and there is also an effect of the mutation in this virus on the morphology of transformed cells. These changes do not correlate with any alterations in the biochemical behavior of the NY309 *src* protein that we have been able to detect, and this indicates that the N-terminal 9 kd of $p60^{src}$ may have a more complex functional role than that of a membrane anchor. However, NY309-infected cells are fully transformed by the criteria of anchorage-independent growth and saturation density in monolayer culture.

By contrast, NY314 and NY315 are transformation defective by the criteria of focus formation, infected cell morphology, and induction of anchorage-independent growth. The NY314 and NY315 *src* proteins behave like soluble proteins in cell fractionation experiments. Their tyrosine kinase activities are close to normal both in vitro and in vivo. The transformation-defective nature of these viruses may be caused in part by the abnormal subcellular location of their *src* proteins. For example, it may be that some critical target for the *src* tyrosine kinase activity is located at or near the cell membrane and that *src* membrane association is required for access to this target.

The *src* proteins of rASV1702 and rASV157 fractionate as soluble proteins in isotonic salt (26), as does the transforming protein of Fujinami sarcoma virus, which is also a tyrosine kinase (15). Consideration of the behavior of these viruses leads to the conclusion that not all tyrosine kinase transforming proteins need to be membrane associated to transform CEF. Further work is needed to clarify the differences between the behavior of NY314 and NY315 and these viruses. It may be relevant that recent work with immunofluorescence staining (25) has shown that the *src* proteins of rASV157 and rASV1702 appear to be associated with adhesion plaques. Also, the rASV157 and rASV1702 *src* proteins are found in the membrane pellet when cells are extracted in low-salt buffer (26), whereas the *src* proteins of NY314 and NY315 show much less salt dependence in their fractionation behavior (data not shown). Sequence differences between the rASVs and NY314 and NY315 *src* proteins either at the very N terminus or elsewhere in the molecule could allow the rASV *src* proteins to associate with the cell periphery without the wt N-terminal sequence or myristic acid addition, and this association might be important for cell transformation.

We cannot rule out an alteration in the affinity of the NY314 and NY315 *src* proteins for some relevant target, independent of subcellular location. For example, it is possible that the lack of myristic acid addition alters the substrate specificity of the protein. Even if this is so, these viruses are unique in that they induce levels of *src* tyrosine kinase activity comparable to the level induced by wt RSV but do not transform infected cells. They may therefore be useful in determining the relevance to transformation of the various cellular proteins known to be phosphorylated on tyrosine in wt RSV-transformed cells (10).

Domain structure of $p60^{src}$. On the basis of proteolytic dissection of $p60^{src}$, Levinson et al. (29) proposed two domains of the protein. The C-terminal 30 kd was shown to be isolatable as a proteolytic fragment which retained tyrosine kinase activity (2, 29). The N-terminal 13 kd was proposed to function as a membrane anchor, based on the release of a 47-kd C-terminal fragment from trypsin-treated membrane vesicles (29). Krueger et al. (26, 27) also demonstrated a role for the N-terminal 8 kd in membrane association of *src* protein.

The experiments we describe support this two-domain model of $p60^{src}$. The high tyrosine kinase activity of the NY309, NY314, and NY315 *src* proteins shows that the tyrosine kinase domain does not require the N-terminal 9 kd for its function. The subcellular fractionation of these *src* proteins confirms the involvement of the N-terminal 9 kd in membrane association and sharpens the mapping of the membrane association domain to the N-terminal 15 amino acids. Our results show a coincidence between this membrane association domain and a critical sequence required for $p60^{src}$ myristylation, and a critical sequence for cell transformation. Current experiments are aimed at further exploring the relationship between $p60^{src}$ myristylation, membrane association, and transformation, both by testing additional constructs and by examining the behavior of the wt protein.

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