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Processing of p60$^{v\text{-}src}$ to Its Myristylated Membrane-Bound Form

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p60^{src} of wild-type Rous sarcoma virus is myristylated at its N-terminal glycine residue. We have shown previously that this myristylation is necessary for p60^{src} membrane association and for cell transformation by using *src* mutants with alterations within the N-terminal 30 kilodaltons of p60^{src}. In this study we analyzed the process of p60^{src} myristylation in wild type- and mutant-infected cells. All myristylated *src* proteins examined lack the initiator methionine, but two mutant *src* proteins lacking the initiator methionine are not myristylated, indicating that removal of the initiator methionine and myristylation are not obligatorily coupled. Analysis of the kinetics of myristylation and the association of p60^{src} with cellular proteins p50 and p90 indicated that myristylation occurs before p60^{src} becomes membrane associated and that transient association with p50 and p90 occurs regardless of myristylation. Myristylation is required for stable association of p60^{src} with the plasma membrane but is not sufficient for membrane association. A mutant with an *src* deletion of amino acids 169 through 264 has an *src* protein that is myristylated but not membrane bound, remaining stably associated with p50 and p90. This mutant is transformation defective. Several N-terminal deletion mutants possessing tyrosine kinase activity have myristylated and membrane-bound *src* proteins but are not fully active in cell transformation, suggesting that additional N-terminal functional domains exist.

The transforming protein p60^{src} of Rous sarcoma virus (RSV) (4) is a tyrosine protein kinase (12, 13, 21, 31, 32) that associates with the inner surface of the plasma membrane (for review, see reference 27). p60^{src} is acylated (7, 19, 43) at its amino terminus with the 14-carbon saturated fatty acid, myristic acid (8, 39). The myristyl moiety is in an amide linkage with the N-terminal glycine residue of the modified p60^{src} protein (39). Other proteins known to be modified precisely in this way (N-myristylated on glycine) are the catalytic subunit of the cyclic AMP-dependent protein kinase (9), the calcium-binding B subunit of the protein phosphatase calcineurin (1), the p15^{gag} structural protein of Moloney murine leukemia virus (20), and NADH-cytochrome b_5 reductase (35). Other myristylated proteins include the *src* cellular homolog p60$^{c\text{-}src}$ (8, 22), nonglycosylated forms of proteins containing N-terminal p15^{gag} protein (such as mammalian retroviral *gag* precursor polyproteins [40] and *gag-onc* fusion proteins such as Snyder-Theilen and Gardner-Arnstein feline sarcoma virus P85$^{gag\text{-}fes}$ and P110$^{gag\text{-}fes}$, respectively, Abelson murine leukemia virus P120$^{gag\text{-}abl}$, and rat sarcoma virus P29$^{gag\text{-}ras}$ [41]) and the murine lymphoma cell line LSTRA p56 tyrosine protein kinase (34, 49). The precise physiological role of N-terminal myristylation is as yet unknown, but it has been suggested that it is involved in interaction with membranes, enzyme-substrate interactions, or protein subunit interactions (1, 7–9, 16, 19, 20, 34–37, 39–41, 43).

We have previously shown by deletion mapping that amino acids 1 through 14 are required for p60^{src} N- myristylation, which may be required for membrane association and cell transformation (16, 37). The first 14 amino acids of p60^{src} contain a sequence which is sufficient for myristylation and which may direct proteins to the plasma membrane (36). In this study, we used RSV mutants with alterations within the N-terminal 30 kilodaltons of the p60^{src} coding sequence to examine how soon and where p60^{src} is myristylated and whether this modification is always coupled with the removal of the initiator methionine residue. We examined the membrane association of mutant *src* proteins and the association of mutant *src* proteins with cellular proteins p50 and p90 (thought to be involved in transport of wild-type [wt] p60^{src} to the plasma membrane [3, 5, 14]) to analyze the process of maturation and membrane binding of the transforming protein.

MATERIALS AND METHODS

Cell culture and viruses. Cultures of secondary chicken embryo fibroblasts (CEF) were maintained, transfected, or infected as described previously (16, 17). The construction of N-terminal *src* mutants of Schmidt-Ruppin RSV subgroup A (SRA) has been previously described (15a, 16, 37).

Protein biochemistry and cell fractionation. Labeling of cultures with [^{3}H]leucine, [^{35}S]methionine, or [^{3}H]myristic acid and immunoprecipitation of proteins with tumor-bearing rabbit (TBR) serum were essentially as described previously (16). Cell extracts were prepared in RIPA buffer containing 10 mM Tris hydrochloride (pH 7.4), 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride, and 100 kallikrein-inactivating units of Trasylol per ml.

Partial proteolytic mapping of p60^{src} was performed on excised gel bands with 25 to 500 ng of *S. aureus* V8 protease essentially as previously described (11). Cells were fractionated by differential centrifugation into particulate (P) and cytosolic (S) fractions as described previously (16, 36, 37). Protein kinase activity in cell fractions was determined by the TBR immunoglobulin G phosphorylation assay as previously described (16). Fractionation of cell extracts on glycerol gradients for analysis of the sedimentation behavior of *src* protein was as previously described (3).

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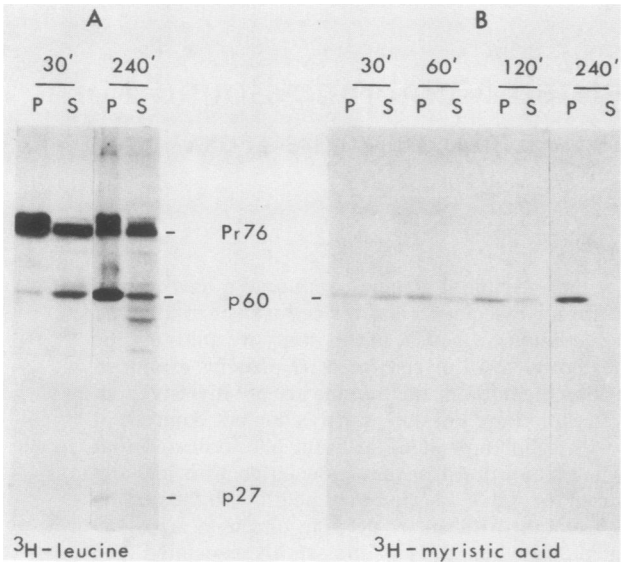

FIG. 1. Redistribution of p60^{src} from the S to the P fraction with increasing labeling times. SRA-infected CEF were labeled with [^{3}H]leucine (A) or [^{3}H]myristic acid (B) for the indicated times and were fractionated into P and S fractions. Fractions were solubilized with RIPA buffer and immunoprecipitated with TBR serum. This TBR serum, which was also used for the experiments described in the legends to Fig. 3 and 5, immunoprecipitated gPr92^{env}. For panel A, exposure time was 4 days, and for panel B it was 10 days.

RESULTS

Myristylation of p60^{src} precedes its association with membranes. Pulse-chase and cell fractionation studies indicate that p60^{src} is synthesized in the cytosol and becomes membrane associated within 15 to 20 min of synthesis (5, 14, 30). To determine whether myristylation of p60^{src} occurs while the protein is soluble, we fractionated CEF transformed with wt SRA into P and S fractions after various periods of labeling with [^{3}H]leucine or [^{3}H]myristic acid (Fig. 1). Continuous labeling was used, since too little is known about the turnover of labeled fatty acids in CEF to determine whether pulse-chase labeling would be effective over the short period of interest. Continuous labeling with [^{3}H]leucine (Fig. 1A) shows that the distribution of p60^{src} changes from mostly soluble (30-min label) to mostly particulate (240-min label). A parallel redistribution of p60^{src} from soluble to membrane bound occurred with [^{3}H]myristic acid labeling (Fig. 1B), suggesting that myristylation of p60^{src} occurs before the *src* protein associates with membranes. The incorporation of [^{3}H]myristic acid into p60^{src} immunoprecipitated from whole cell lysates was linear over the 4-h labeling period (data not shown).

Newly synthesized p60^{src} associates with two cellular proteins, p50 and p90, to form a soluble complex thought to be involved in the transport of p60^{src} to the plasma membrane (3, 5, 14). We detected [^{3}H]myristic acid-labeled p60^{src} in the fast-sedimenting p50:p60^{src}:p90 complex (data not shown), as has been reported by Buss et al. (7). p60^{src} can be labeled with a [^{3}H]myristic acid pulse as short as 5 min (data not shown), suggesting that the label is incorporated rapidly and that myristylation occurs rapidly.

Removal of initiator methionine and myristylation are not coupled. Mature p60^{src} of wt RSV lacks its initiator methionine residue and is N-myristylated on the glycine residue at position 2 (39). It has been suggested that myristylation of p60^{src} is cotranslational and that nascent chains are the only substrates of N-myristyltransferases (7). It also has been suggested that initiator methionine removal from nascent polypeptide chains occurs when the growing chain is sufficiently long (16 to 30 amino acid residues) for the N terminus to be exposed from the ribosome (24, 51, 52). To determine whether initiator methionine removal and myristylation are coupled processes, we assayed the presence of methionine 1 in a number of myristylated and nonmyristylated *src* proteins. This is easily assayed, since methionine 1 is the only methionine present within the N-terminal V8 protease fragments V3 and V4 (47, 48). Results of this analysis are shown in Fig. 2 and summarized in Table 1. Methionine 1 is excised

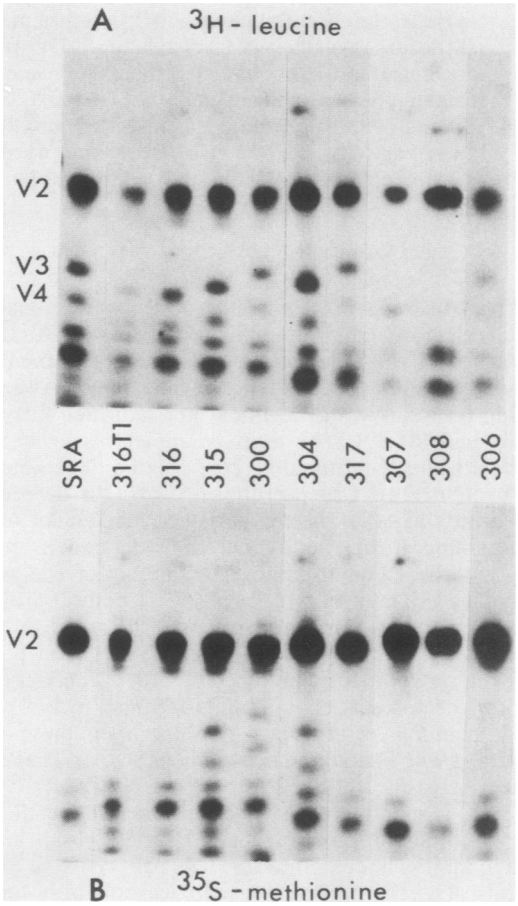

FIG. 2. Assay for the presence of methionine 1 in mutant *src* proteins by partial proteolytic mapping. Gel bands containing *src* protein immunoprecipitated with TBR serum from infected cells labeled for 4 h with [^{3}H]leucine (A) or [^{35}S]methionine (B) were excised and digested with 500 ng of V8 protease during reelectrophoresis on a 12.5% sodium dodecyl sulfate-polyacrylamide gel. Presence of initiator methionine was easily assayed, since methionine 1 is the only methionine present within the N-terminal V8 protease fragments V3 and V4 (47, 48), whose positions were determined by analysis of [^{3}H]leucine-labeled *src* protein. V8 protease fragments smaller than V3 and V4 were derived from the C-terminal fragment V2 and were the same size in all of the mutant *src* proteins. (The figure is a composite from several gels.) Viruses encoding mutant *src* proteins have been previously described (16, 37; Table 1). Designations between panels indicate viruses with which cells were infected.

from residues of glycine in the *src* proteins of SRA, NY306, NY307, NY308, NY316, and NY317, but not from aspartic acid in NY315 and NY304 or asparagine in NY300. All myristylated *src* proteins examined lack methionine 1, but two nonmyristylated *src* proteins (those encoded by NY306 and NY316) also lack methionine 1. This indicates that although myristylation and removal of the initiator methionine may be cotranslational, they are not obligatorily coupled processes. The fact that the removal of methionine 1 is not coupled with myristylation of the *src* proteins of NY306 and NY316 (see Table 1 for N-terminal *src* sequences) suggests that either the primary or secondary structure of the first 10 N-terminal residues is involved in recognition by or reaction with N-myristyltransferase(s). The cleavages of N-terminal methionine observed suggest that the methionine aminopeptidase in CEF has a specificity similar to that found in *Saccharomyces cerevisiae* by Sherman and Stewart (45).

Nonmyristylated mutants enter and leave the complex with cellular proteins p50 and p90. As described previously, newly synthesized wt $p60^{src}$ in the complex with cellular proteins p50 and p90 is myristylated. To examine the distribution of soluble nonmyristylated mutant *src* proteins between monomer and complex-bound forms, we carried out glycerol gradient analysis of extracts from 4-h [^{3}H]leucine-labeled cells on cells infected with NY316, NY300, NY304, or NY306 (see Table 1 for N-terminal *src* sequences). The behavior of these nonmyristylated *src* proteins (data not shown) was like that described for the *src* proteins of mutants NY314 and NY315 (16) or wt SRA (3, 5). To examine whether only myristylated *src* proteins destined for transport to the membrane enter and leave the complex, we examined the turnover of $p60^{src}$ in the complex in cells infected with a mutant encoding a nonmyristylated soluble *src* protein. NY316-infected cells were pulse-labeled with [^{3}H]leucine for 20 min (Fig. 3A) and chased with excess cold leucine for 210 min (Fig. 3B), and extracts were analyzed by sedimentation through glycerol gradients. About half of the $p60^{src}$ synthesized during the 20-min pulse sedimented as a complex (peak in fraction 8), which was released into monomer $p60^{src}$ (peak in fraction 4) during the 210-min chase period. Little p50 or p90 was detected under these labeling conditions, but fraction 8 corresponds to the region of the gradient which can be shown to contain the p50:$p60^{src}$:p90 complex with a long label (data not shown; 16). (The 52-kilodalton band cosedimenting with $p60^{src}$ [Fig. 3] was shown by partial proteolytic peptide mapping [data not shown] to be a breakdown product of $p60^{src}$ whose generation is likely to be an in vitro artifact [50].) Similar results were obtained on pulse-chase analysis of cells infected with NY300, NY304, NY306, NY314, and NY315 (data not shown), all of which encode soluble nonmyristylated *src* proteins (16, 37; Table 1), or cells infected with wt SRA or NY317, which encode membrane-bound myristylated *src* proteins (16, 37; Table 1). Therefore, nonmyristylated *src* proteins enter and leave the complex like wt $p60^{src}$.

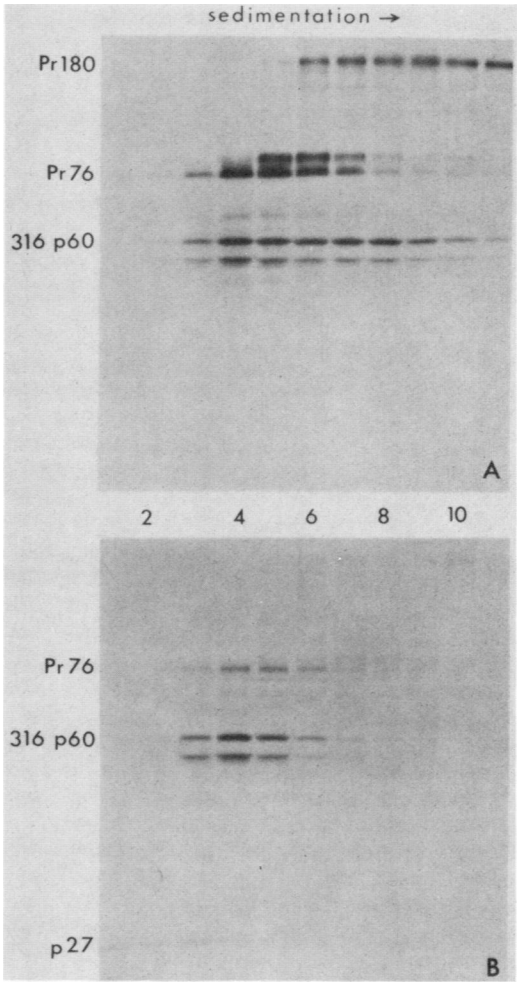

FIG. 3. Transient complex formation by NY316 *src* protein. NY316-infected cells starved of leucine for 1 h were labeled with [^{3}H]leucine for 20 min and harvested (A) or washed and incubated for 210 min (B) in medium containing 10× cold leucine. Cell lysates were fractionated on glycerol gradients, and fractions were immunoprecipitated with TBR serum. Sedimentation was from left to right. Exposure times for panels A and B were comparable. There was not quantitative recovery of the mutant *src* protein after the 210-min chase, since its half-life is shorter than that of wt $p60^{src}$ (16, 37). Numbers between panels indicate gradient fractions.

TABLE 1. Amino-terminal sequences of mutant viruses

Virus	Amino acids deleted	Amino acids substituted[a]	Amino-terminal sequence encoded[a]	Myr[b]	Met 1[c] in mature protein
SRA			MGSS	+	−
307	15–27	PQIW	MGSS	+	−
308	15–49	PRSG	MGSS	+	−
317	11–15	QICG	MGSS	+	−
315	2–15	DLG	MDLG	−	+
304		DL (between 1 and 2)	MDLG	−	+
300	2–4	NRSG	MNRS	−	+
306	3–4	RSG	MGRS	−	−
316	7–15	NRSG	MGSS	−	−
314	2–81	DL	MDLG	−	ND[d]
310	15–169	PRSG	MGSS	+	ND
311	15–149	PRSD	MGSS	+	ND
320	149–169	SQICG	MGSS	+	ND
18-3	169–264	PQICG	MGSS	+	ND
312	15–264	PRSG	MGSS	+	ND

[a] Abbreviations: C, cysteine; D, aspartic acid; G, glycine; I, isoleucine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; W, tryptophan.
[b] Myr, Myristylated *src* protein.
[c] Met 1, Methionine 1.
[d] ND, Not determined.

A myristylated mutant *src* protein that is restricted to the complex. We have recently described a transformation-

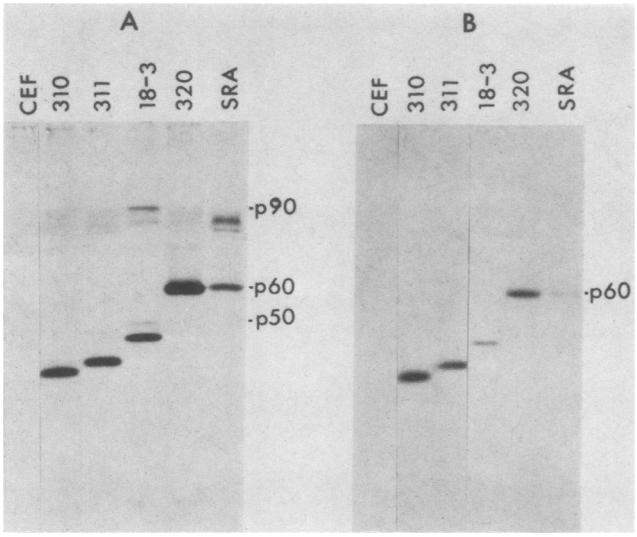

FIG. 4. Myristylation of mutant *src* proteins. Uninfected cells or cells infected with various viruses were labeled for 4 h at 39.5°C with [^{3}H]leucine (A) or [^{3}H]myristic acid (B), and *src* proteins were immunoprecipitated with TBR serum. For panel A, exposure time was 2 days, and for panel B it was 5 days.

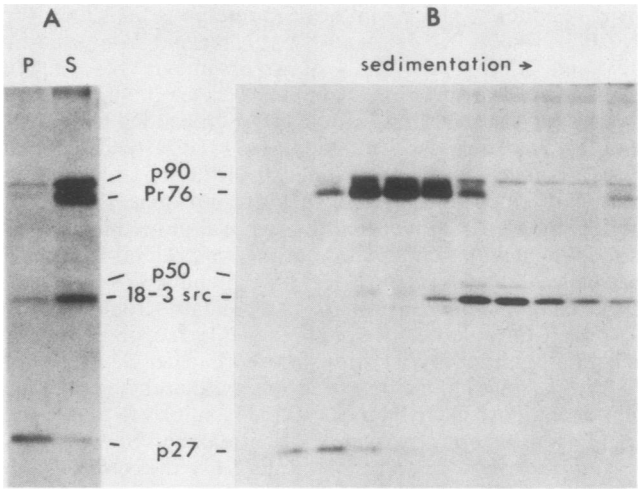

FIG. 5. Analysis of the NY18-3 *src* protein. NY18-3-infected cells were labeled with [^{3}H]leucine for 4 h at 39.5°C. (A) Cells were Dounce homogenized, and the postnuclear supernatant was fractionated into P and S fractions. Subcellular fractions were solubilized and immunoprecipitated with TBR serum. (B) A whole-cell RIPA buffer lysate was analyzed on a 10 to 30% glycerol gradient, and fractions were immunoprecipitated with TBR serum. Sedimentation was from left to right. Some of the protein comigrating with p90 in panel A was gPr92^{env}, which did not cosediment with the p50:p60^{src}:p90 complex in panel B.

defective mutant, NY18-3, with an in vitro-constructed *src* deletion of amino acids 169 through 264 (15a, 16) . NY18-3 encodes a myristylated *src* protein (Fig. 4; 16) with low tyrosine kinase activity (15a). Comparison of the number of leucine residues in the mutant and wt *src* sequences (47, 48) and of the relative incorporation of [^{3}H]leucine and [^{3}H]myristic acid (Fig. 4) suggests that the NY18-3 *src* protein is myristylated to the same extent as wt p60^{src}.

More p50 and p90 coprecipitate with the NY18-3 *src* protein than with the *src* proteins of wt SRA or other RSV mutants (Fig. 4A), suggesting that the NY18-3 *src* protein may behave like that of tsNY68, which is tightly bound in the soluble complex (3, 5, 14). To investigate this possibility, we fractionated cells infected with NY18-3 (Fig. 5A). Approximately 80% of the NY18-3 *src* protein was soluble. This subcellular distribution was neither salt nor temperature sensitive (data not shown). A lysate from NY18-3-infected cells was analyzed by sedimentation through a glycerol gradient (Fig. 5B). Virtually all of the NY18-3 *src* protein was found in a fast-sedimenting form complexed with p50 and p90. The NY18-3 *src* protein was found in the complex when either the P or S fraction was analyzed by glycerol gradient centrifugation (data not shown). This suggests that the *src* protein encoded by NY18-3 forms a stable complex with p50 and p90, like tsNY68 p60^{src} in infected cells at the nonpermissive temperature (5, 14).

We have previously reported that tsNY68 p60^{src} contains less fatty acid at the nonpermissive temperature than at the permissive temperature (19). This experiment had been performed with a 16-h label with [^{3}H]leucine and [^{3}H]palmitic acid, which has been shown to be incorporated into p60^{src} only after breakdown to myristic acid (8). On repeating this experiment with both SRA and tsNY68-infected cells at 33 or 42°C using [^{3}H]leucine, [^{3}H]myristic acid, and [^{3}H]palmitic acid for labeling times of 2, 4, and 16 h (data not shown), we found that the apparent decrease in the extent of acylation of tsNY68 p60^{src} at 42°C was not due to hypoacylation but was artifactually due to differences in [^{3}H]leucine incorporation specific to tsNY68-infected cells in the 16-h label. This effect is likely to be caused by differences in intracellular leucine pools between transformed and untransformed cells. With shorter labeling times, there was no difference in the extent of acylation of p60^{src}, in agreement with results reported by Buss et al. (7).

Partially transforming *src* deletion mutants that are myristylated and membrane associated. We examined the myristylation of *src* deletion mutants NY310, NY311, and NY320, whose *src* proteins have in vitro-constructed deletions of amino acids 15 through 169, 15 through 149, and 149 through 169, respectively (Table 1; 15a). All of these mutant *src* proteins were labeled well with [^{3}H]myristic acid (Fig. 4). Mutant NY312, whose *src* protein has a deletion of amino acids 15 through 264 (15a), is also myristylated (data not shown), indicating that only amino acids 1 through 14 of the N-terminal half of p60^{src} are required for myristylation.

TABLE 2. Fractionation of sedimentable *src* kinase activity on discontinuous sucrose gradients[a]

Virus	Sp act of immunoglobulin G kinase in membrane fractions at gradient of interfaces:		
	20%/35%	35%/40%	40%/50%
NY311	49.8	21.6	1.2
NY320	52.6	10.6	1.0
SRA	54.4	15.2	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 (by using RIPA buffer without sodium dodecyl sulfate). The 20%/35% interface fraction was enriched for plasma membranes, and the 40%/50% interface fraction was enriched for rough endoplasmic reticulum, Golgi, and mitochondrial membranes (15). The data were normalized to the specific activity of the SRA kinase activity found in the 40%/50% interface. Specific activity was defined as the ^{32}P counts per minute in immunoglobulin G in a kinase assay divided by the total amount of protein in the fraction as indicated by trichloroacetic acid-precipitable [^{3}H]leucine counts per minute.

To examine the membrane association of the *src* protein, we separated infected cells into P and S fractions and immunoprecipitated *src* protein from these fractions. We quantitated the distribution by determining the radioactivity associated with the *src* protein gel band or by kinase assay. By either method of quantitation, about 70 to 80% of the *src* proteins of NY310, NY311, and NY320 fractionated with the membrane pellet (data not shown). Crude membrane pellets from infected cells were further fractionated by flotation in discontinuous sucrose gradients (Table 2). The distributions of the SRA, NY311, and NY320 *src* proteins were similar, showing a large enrichment in the plasma membrane fraction (20%/35% interface).

On sedimentation of infected cell extracts through glycerol gradients, the *src* proteins of mutants NY311 and NY320 exhibited unusual behavior; they were fast sedimenting (Fig. 6). The identity of the fast-sedimenting mutant *src* proteins (peak in fraction 7 in Fig. 6C and D) was confirmed by V8 protease mapping (data not shown). The NY310 *src* protein also was fast sedimenting (data not shown). These *src* proteins sedimented at the same region of the gradient as the p50:p60^{src}:p90 complex. However, unlike the soluble, tightly complex-bound *src* proteins of NY18-3 or tsNY68 at the nonpermissive temperature (3, 5, 14), there was no detectable increase in coprecipitating p50 and p90 (Fig. 4–6). Using a number of different TBR sera, we could detect no coprecipitating proteins specific to NY310-, NY311-, or NY320-infected cells (data not shown) to account for the fast sedimentation behavior of these mutant *src* proteins. These results suggest either that a p50:*src* protein:p90 complex was formed but was unstable on immunoprecipitation or that these mutant *src* proteins self-aggregated (into 3- to 5-mers) to form a fast-sedimenting complex. The NY310, NY311, and NY320 *src* proteins were fast sedimenting irrespective of the time of labeling (e.g., 20- or 240-min [^{3}H]leucine label or 20-min [^{3}H]leucine pulse followed by a 210-min chase) or whether the P or S subcellular fractions were analyzed (data not shown). Fast sedimentation behavior was observed when solutions other than RIPA buffer [e.g., removal of sodium dodecyl sulfate and addition of ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] were used for cell lysis or glycerol gradient sedimentation (data not shown). These *src* proteins apparently have altered conformations producing this behavior observed in vitro. This may be related to the partially transforming phenotypes of these mutants (15a).

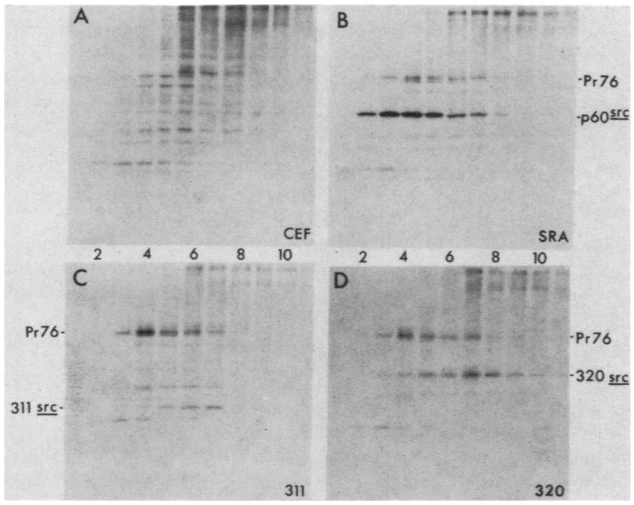

FIG. 6. Glycerol gradient sedimentation analysis of mutant *src* proteins. Uninfected cells (A) or cells infected with SRA (B), NY311 (C), or NY320 (D) were labeled for 4 h with [^{3}H]leucine, and RIPA buffer lysates were analyzed by sedimentation through 10 to 30% glycerol gradients. Fractions were immunoprecipitated with TBR serum. Sedimentation was from left to right.

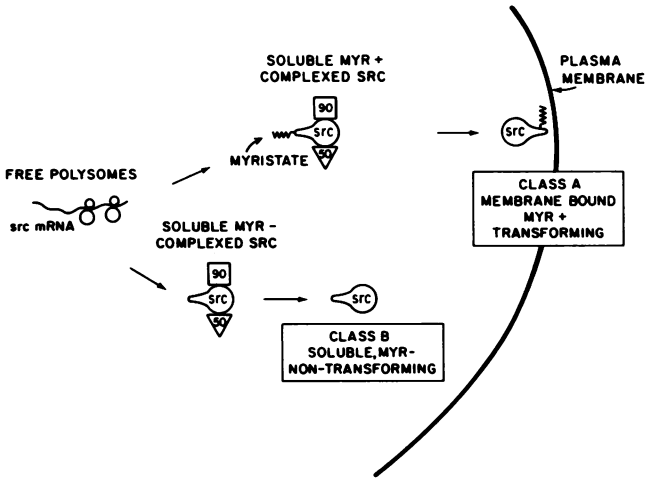

FIG. 7. Schematic model for the biogenesis of wt and mutant *src* proteins.

DISCUSSION

Sequence requirements for p60^{src} myristylation. By examining mutants with in vitro-constructed *src* deletions throughout the N-terminal half of p60^{src}, we have shown that only amino acids 1 through 14 are required for p60^{src} myristylation. The precise recognition requirements of N-myristyltransferases, other than an apparent requirement for an N-terminal glycine residue (16, 37), are not yet known. There is no obvious sequence homology between the N-terminal portions of p60^{src} and other N-myristylated proteins (1, 9, 20, 35, 39–41), except for the N-myristyl acceptor glycine and the presence of a potentially alpha-helix-disrupting proline or glycine residue (10) at a position between 7 and 9 residues from the N terminus of the modified protein. The absence of myristylation in the *src* proteins of mutants NY306 and NY316 despite the presence of an N-terminal glycine residue suggests that either the primary or secondary structure of the first 10 N-terminal residues is involved in recognition by N-myristyltransferase. A structure that can be recognized by myristyltransferase is restored (most likely by point mutation) in the generation of transforming revertants isolated from NY306 and NY316 (37).

Biogenesis of p60^{src} and cell transformation. We have previously found a correlation between p60^{src} myristylation, membrane association, and cell transformation (16, 37), suggesting that myristylation is required for membrane association, which in turn is required for transformation. Here we examined the kinetics of myristylation, membrane association, and the interaction of mutant *src* proteins with the cellular proteins p50 and p90 to test this hypothesis. Our findings are summarized schematically in Fig. 7.

src proteins containing the recognition signal for N-myristyltransferase(s) are acylated during or shortly after synthesis on free polysomes (7, 29, 38). Myristylation of

glycine 2 does not take place unless methionine 1 is removed. However, myristylation is not obligatorily coupled to cotranslational removal of the initiator methionine residue. Shortly after synthesis, regardless of its acylation, $p60^{src}$ associates with p50 and p90 in a short-lived complex found in the cytosol (5, 14). It has been suggested that p50 and p90 interact with the C terminus of $p60^{src}$ (44). p50 and p90 also form complexes with the transforming proteins of Fujinami and Y73 sarcoma viruses, which share carboxy-terminal kinase domain homologies with $p60^{src}$ (33). Therefore, it is not surprising that transient complex formation does not discriminate between wt $p60^{src}$ and mutant *src* proteins with altered N termini.

There are two major classes of *src* proteins possessing full tyrosine kinase activity, denoted classes A and B in Fig. 7, that differ chiefly in the myristylation and subcellular distribution of the *src* protein. These classes define a critical sequence for $p60^{src}$ myristylation and membrane association. *src* proteins of the first class are myristylated and membrane bound; its members are transforming and include wt SRA and in vitro-constructed deletion mutants NY307, NY308, NY309, and NY317 and transforming revertants isolated from mutants NY306 and NY316 (16, 37; Table 1). *src* proteins of the second class are not myristylated and fractionate as soluble proteins; its members are nontransforming in vitro-constructed mutants NY314, NY315, NY300, NY304, NY306, and NY316 (16, 37; Table 1). However, there are four groups of *src* proteins that do not belong to these major classes. (i) Viruses in which the v-*src* gene is replaced by the c-*src* gene have no transforming activity (23). $p60^{c\text{-}src}$ overproduced by these variants is myristylated and associated with the plasma membrane but is defective in tyrosine phosphorylation (22). This indicates that *src* protein myristylation is not sufficient for cell transformation. Elevated kinase activity is apparently also required for transformation (22, 42). (ii) *src* proteins that are restricted to the soluble p50:$p60^{src}$:p90 complex (such as NY18-3 or tsNY68 at 42°C) are not transforming. These mutant *src* proteins are myristylated, again indicating that myristylation is not in itself sufficient for membrane association and transformation. It is not yet clear whether the defect is in membrane binding or is due to increased binding of p50 and p90. However, these mutant *src* proteins are also defective in tyrosine phosphorylation (15a, 42). Other mutant viruses with temperature-sensitive defects in the *src* gene (such as tsLA27 and tsLA29 [5, 14]) have high levels of complex-bound $p60^{src}$, but this is not the case for the in vitro-constructed mutant tsCH119 (6). Its *src* protein, with a deletion of amino acids 173 through 227, is membrane associated at either temperature (6). (iii) The *src* proteins of recovered avian sarcoma viruses 1702 and 157 are not myristylated and fractionate as soluble tyrosine protein kinases in isotonic salt (19, 28). These *src* proteins appear to behave as peripheral membrane proteins that interact only with adhesion plaque-like structures in specialized regions of the plasma membrane (26). Recovered avian sarcoma viruses 1702 and 157 have decreased in vivo tumorigenicity (26, 28). Sequence differences appear to be present in the N-terminal region of these mutant *src* proteins (25), and this alteration may allow them to interact with specialized regions of the membrane. The substrate specificity of the *src* proteins of recovered avian sarcoma viruses 1702 and 157 may also be altered. (iv) Mutants NY310, NY311, and NY320 encode *src* proteins that are myristylated and plasma membrane associated, but the viruses are partially transformation defective (15a). Thus, $p60^{src}$ myristylation and membrane association, together with elevated tyrosine kinase activity, do not per se guarantee complete transformation. However, these proteins are exceptional in that they appear to exhibit conformational changes (as detected by glycerol gradient sedimentation), and their kinase activities are more susceptible to denaturation. It is possible that the amino acids deleted from these *src* proteins are part of a domain involved in determining substrate specificities required for complete transformation. This putative domain may also be affected in the N-terminal deletion mutant tsCH119 (6) or in the mutants tsLA32, ST529, WO101, WO201, and WO401, whose defects also map within the N-terminal half of $p60^{src}$ (18, 46).

Mutants in classes A and B define the first 14 amino acids of $p60^{src}$ as a structure required for $p60^{src}$ myristylation, membrane association, and cell transformation. The other mutants suggest that another N-terminal domain modulates additional functions required for complete transformation. These different varieties of *src* mutants suggest that there are multiple structural and functional domains, perhaps some relating to regulation of kinase activity or to substrate specificity, that are necessary for transformation.

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