Lysine Residues Form an Integral Component of a Novel NH_2-terminal Membrane Targeting Motif for Myristylated $pp60^{v\text{-}src}$

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Abstract. Association of $pp60^{v\text{-}src}$ with the plasma membrane is fundamental to generation of the transformed phenotype. Although myristylation of $pp60^{v\text{-}src}$ is required for interaction with a membrane-bound receptor, the importance of NH_2-terminal amino acids in receptor binding has not yet been uncoupled from their role in signaling myristylation. Using chimeric src proteins, peptides identical or related to the NH_2 terminus of src, and site-directed mutagenesis, we demonstrate that NH_2-terminal lysines in conjunction with myristate are essential for membrane localization. Subsequent to NH_2-terminal interaction with the "src receptor," internal regions of the src protein also participate in membrane binding. This novel NH_2-terminal motif and internal contact mechanism may direct other members of the src family of tyrosine kinases to their membrane receptors.

INTERACTION of $pp60^{v\text{-}src}$ with cellular membranes plays a pivotal role in viral transformation. $pp60^{v\text{-}src}$, the protein product of the v-src gene of Rous sarcoma virus, transforms cells as a consequence of its tyrosine kinase activity (Brugge and Erikson, 1977; Collett et al., 1980; Hunter and Sefton, 1980). Like other members of the family of non-receptor protein tyrosine kinases, $pp60^{v\text{-}src}$ is predominantly membrane bound in vivo (Courtneidge et al., 1980). Membrane association has been shown to be mediated by interaction with a membrane-bound src receptor (Resh, 1989). Mutant v-src proteins that fail to associate with the plasma membrane are unable to induce transformation despite the fact that they retain normal kinase activity (Cross et al., 1984; Kamps et al., 1985).

Unlike many membrane-bound proteins, $pp60^{v\text{-}src}$ is synthesized on free ribosomes (Lee et al., 1979) and binds to cellular membranes posttranslationally. $pp60^{v\text{-}src}$ lacks any distinct stretches of hydrophobic amino acids that could serve as a signal sequence or membrane-anchoring domain (Takeya et al., 1982). Rather, the principal component of hydrophobicity is provided by the presence of the 14-carbon fatty acid myristate, at the amino terminus of the polypeptide. Myristate is cotranslationally coupled to $pp60^{v\text{-}src}$ by amide linkage to the amino-terminal glycine residue (Buss and Sefton, 1985; Deichaite et al., 1988; Wilcox et al., 1987). This modification occurs by the action of myristyl transferase after removal of the initiating methionine by aminopeptidase (Wilcox et al., 1987). Modification by myristate is required for the association of $pp60^{v\text{-}src}$ with the plasma membrane (Cross et al., 1984; Kamps et al., 1985; Garber and Hanafusa, 1987). However, although myristylation is necessary for the interaction of $pp60^{v\text{-}src}$ with the plasma membrane, it is not sufficient, as there are myristylated variants of $pp60^{v\text{-}src}$ that fail to associate with the plasma membrane (Buss et al., 1984; Cross et al., 1985; Garber et al., 1985). Furthermore, the addition of a myristate moiety per se does not promote membrane binding, as only a subset of other myristylated viral and cellular proteins is membrane associated (Towler et al., 1988).

Amino-terminal amino acids are required for myristylation (Cross et al., 1984; Cross et al., 1985; Pellman et al., 1985; Kaplan et al., 1988; Brooks-Wilson et al., 1989), thus making it impossible to distinguish between the role of these amino acids in directing myristylation and a potential role in providing a membrane recognition motif. Here, we demonstrate that a cluster of amino-terminal lysines, together with the myristate moiety, form a binding motif essential for interaction of $pp60^{v\text{-}src}$ with the plasma membrane. Similar motifs are also found in several other non-receptor protein tyrosine kinases, suggesting a general mechanism for membrane targeting of this family of proteins.

Materials and Methods

Cell Culture, Cell Fractionation, and Immunoprecipitation

RSV-transformed field vole cells (clone 1T) and uninfected, normal vole cells were maintained in tissue culture as previously described (Resh and Erikson, 1985). Membrane fractions were prepared by hypotonic lysis, homogenization, and ultracentrifugation of cells as described (Resh and Erikson, 1985) and resuspended to a protein concentration of 0.5 mg/ml in NTE buffer (100 mM NaCl/10 mM Tris [pH 7.4]/1 mM EDTA). The plasma membrane-enriched fraction is designated P100, and the intracellular and nuclear membrane-enriched fraction is designated P1. The non-membrane associated fraction is designated S100. Fractions were adjusted to RIPA buffer (Brugge and Erikson, 1977) and clarified at 40,000 *g* for 30 min at 4°C. Clarified lysates were immunoprecipitated with α p60 serum (Resh and Erikson, 1985). The in vitro kinase assay was performed as described (Resh and Erikson, 1985) after adjusting to kinase precipitation buffer.

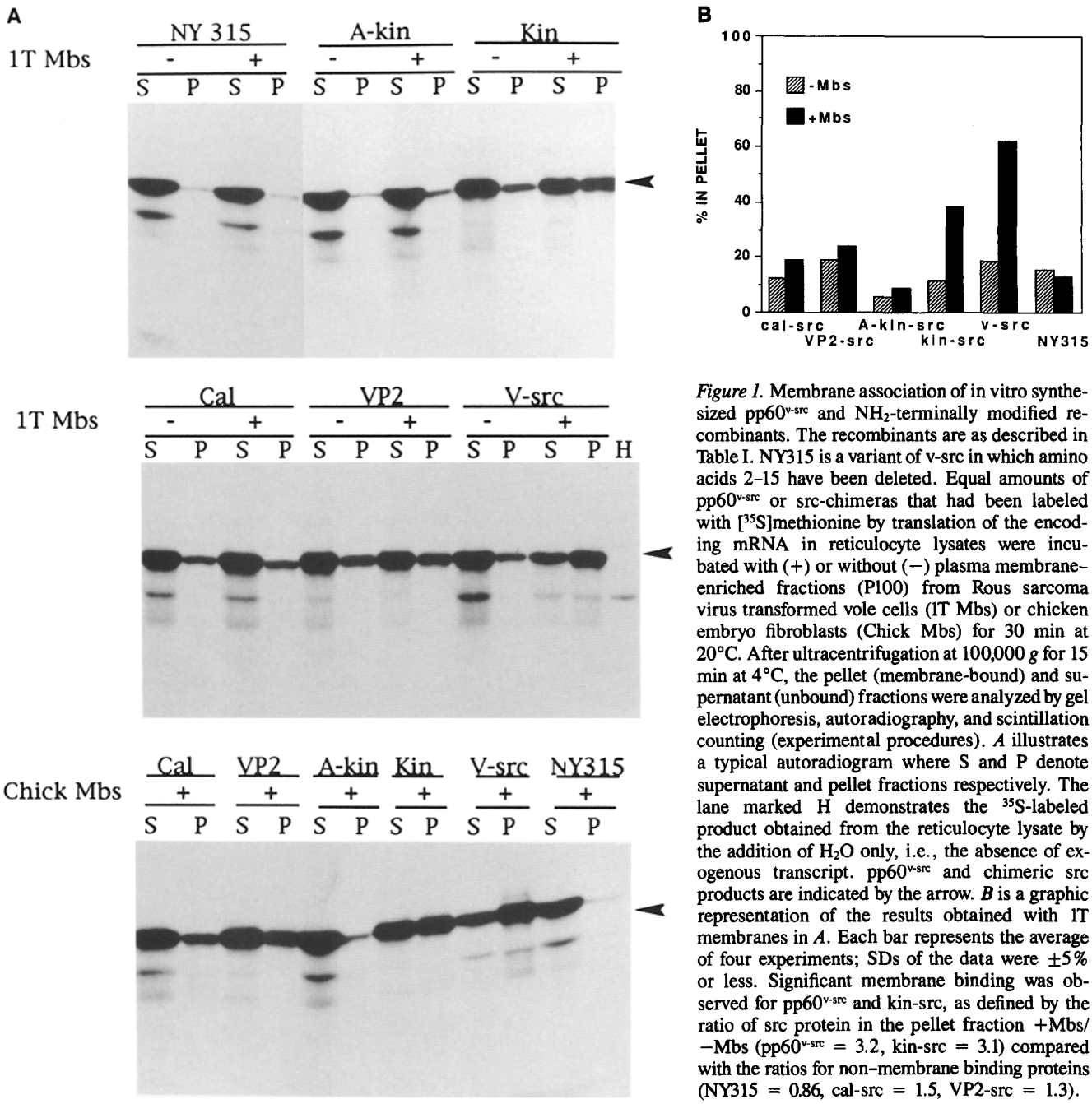

Figure 1. Membrane association of in vitro synthesized pp60$^{v\text{-}src}$ and NH_2-terminally modified recombinants. The recombinants are as described in Table I. NY315 is a variant of v-src in which amino acids 2–15 have been deleted. Equal amounts of pp60$^{v\text{-}src}$ or src-chimeras that had been labeled with [^{35}S]methionine by translation of the encoding mRNA in reticulocyte lysates were incubated with (+) or without (−) plasma membrane-enriched fractions (P100) from Rous sarcoma virus transformed vole cells (1T Mbs) or chicken embryo fibroblasts (Chick Mbs) for 30 min at 20°C. After ultracentrifugation at 100,000 *g* for 15 min at 4°C, the pellet (membrane-bound) and supernatant (unbound) fractions were analyzed by gel electrophoresis, autoradiography, and scintillation counting (experimental procedures). *A* illustrates a typical autoradiogram where S and P denote supernatant and pellet fractions respectively. The lane marked H demonstrates the ^{35}S-labeled product obtained from the reticulocyte lysate by the addition of H_2O only, i.e., the absence of exogenous transcript. pp60$^{v\text{-}src}$ and chimeric src products are indicated by the arrow. *B* is a graphic representation of the results obtained with 1T membranes in *A*. Each bar represents the average of four experiments; SDs of the data were ±5% or less. Significant membrane binding was observed for pp60$^{v\text{-}src}$ and kin-src, as defined by the ratio of src protein in the pellet fraction +Mbs/−Mbs (pp60$^{v\text{-}src}$ = 3.2, kin-src = 3.1) compared with the ratios for non-membrane binding proteins (NY315 = 0.86, cal-src = 1.5, VP2-src = 1.3).

Plasmid Construction, Transfection, and G418 Selection

The plasmid pGEM-src (Deichaite et al., 1988) was used to construct the chimeric plasmids kin-src, A-kin-src, cal-src, VP2-src, v-src N5, v-src N9, v-src N5+N9, kin-src N8, kin-src N9, kin-src N8+N9. pGEM-src was digested with NcoI and NaeI, resulting in the deletion of 42 nucleotides that encode the 14 amino-terminal amino acids of pp60$^{v\text{-}src}$. Oligonucleotides encoding the first 14 amino acids of cAMP-dependent protein kinase (kin) (Uhler et al., 1986); mutants of cAMP-dependent protein kinase in which the amino-terminal glycine has been replaced with alanine (A-kin), lysine 8 changed to asparagine (kin-src N8), lysine 9 changed to asparagine (kin-src N9), and lysines 8 and 9 changed to asparagine simultaneously (kin-src N8 + 9); the B subunit of calcineurin (cal) (Guerini et al., 1989), the VP2 capsid protein of SV40 (VP2) (Fiers et al., 1978), mutants of v-src in which lysine 5 has been converted to asparagine (v-src N5), lysine 9 converted to asparagine (v-src N9), and both lysines 5 and 9 converted to asparagine (v-src N5 + 9); and the complementary noncoding strands were synthesized (M. Flocco, Princeton University), hybridized, and inserted at the site of the pGEM-src deletion. All 10 constructs were confirmed by sequencing. The plasmids PLJ-v-src and PLJ-kin-src were prepared by excising v-src and kin-src from pGEM by digestion with AccI and EcoRI, made blunt with T4 DNA polymerase, and inserting the 2.2-kb fragment into the retroviral vector PLJ (a kind gift of J. Schwarzbauer, Princeton University), which had previously been linearized by digestion with BamHI and similarly made blunt ended. Transfection and G418 selection was as reported previously (Cone and Mulligan, 1984). Growth in soft agar was determined as described (Jakobovits et al., 1984). The plasmid pGEM-src was used to construct del 15-263. pGEM-src was digested with NaeI and SmaI, resulting in deletion of the nucleotides encoding amino acids 15-263 before religation of the plasmid.

In Vitro Synthesis and Membrane Binding of pp60$^{v\text{-}src}$, Chimeric-src, and Deletion src Proteins

src mRNA synthesized by in vitro transcription of pGEM clones (Deichaite

et al., 1988) was translated in rabbit reticulocyte lysates as described (Deichaite et al., 1988; Resh, 1989) in the presence of 0.4 μCi/μl [^{35}S]methionine or 0.7 μCi/μl [^{3}H]myristic acid for 90 min at 30°C. Triton X-100 was then added to a final concentration of 0.075%. A 20-μl aliquot of the reaction was incubated with 30 μl of NTE buffer or with 30 μl of a plasma membrane-enriched fraction (P100) in NTE for 30 min at 20°C. Following ultracentrifugation at 100,000 *g*, the pellet and supernatant fractions were analyzed by gel electrophoresis (Laemmli, 1970) and autoradiography. The total amount of src in each gel fraction was quantitated by excising the radioactivity from the dried gel and analyzing radioactivity in the gel slices by liquid scintillation counting. Nonmyristylated pp60^{v-src} protein was derived from mRNA produced from a recombinant plasmid containing pSR-XD315 src DNA (Cross et al., 1984) (a kind gift from H. Hanafusa, Rockefeller University), which encodes a mutant src molecule with amino acids 2–15 deleted.

Immunofluorescence

G418-resistant cells were grown on 25-mm glass coverslips. All subsequent steps were performed at room temperature. The coverslips were rinsed with PBS, and cells were fixed with 3.7% formaldehyde in PBS for 15 min. After washing with PBS, the cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min and then washed with PBS. Reaction with the primary antibody was in PBS containing 10% FCS and a 1:200 dilution of rabbit αp60 serum, using 125 μl per coverslip for 45 min. The coverslips were washed four times with PBS, and then incubated with PBS containing 10% FCS and a 1:75 dilution of fluorescein-conjugated goat anti-rabbit IgG (affinity purified, 900 μg/ml final concentration; Boehringer Mannheim Biochemicals, Indianapolis, IN), 300 μl per coverslip, for 30 min. The coverslips were washed four times with PBS, mounted in 90% glycerol/0.1% phenylene diamine in PBS pH 9, on 25 × 75 microscope slides, and sealed with nail polish. Cells were observed through a ×63 oil-immersion objective on a Zeiss microscope (Carl Zeiss, Inc., Oberkochen, Germany), and cells were photographed with Kodak TMAX 400 film (Eastman Kodak Co., Rochester, NY) using an automatic exposure meter.

Peptides

A src dodecapeptide containing the amino acid sequence GSSKSKPKDPSY or the src-related sequences GSSKSNPNDPSY, GSSNSKPNDPSY, GSSNSNPKDPSY, GSSNSNPNDPSY, and GSSRSRPRDPSY were synthesized, to which myristic acid was covalently linked via an amide bond to the amino-terminal glycine residue (Multiple Peptide Systems, San Diego, CA). The amino acid sequence corresponding to wild-type src was also prepared in a nonmyristylated form. Similarly, a myristylated peptide and nonmyristylated peptide corresponding to the amino-terminal sequences of the gag protein of murine leukemia virus (GQTVTTPLY) and the amino-terminal sequence of the catalytic subunit of cAMP-dependent protein kinase (GNAAAAKKY) were prepared as previously described (Resh, 1989). An additional, longer peptide corresponding to the amino terminus of the catalytic subunit of cAMP-dependent protein kinase (GNAAAAKKGSEQESY) was a generous gift from D. Feder and J. M. Bishop (University of California, San Francisco).

Crosslinking

Crosslinking was performed as described previously (Resh and Ling, 1990). Briefly, vole fibrolast membranes were resuspended in 10 mM Hepes (pH 7.4), 100 mM NaCl, 1 mM EDTA. Iodinated MGYsrc peptide was incubated with membranes for 15 min at 20°C, followed by addition of BS^{3} crosslinker (Pierce Chemical Co., Rockford, IL) for 15 min at 20°C. Membranes were reisolated by ultracentrifugation at 100,000 *g*, and the resulting pellet was solubilized by sonication and boiling in SDS-PAGE sample buffer. The released material was analyzed on a 10% acrylamide gel and autoradiography. The intensity of the resulting radiolabeled band was determined by excising the corresponding region of the gel and counting in a gamma counter.

Results

Amino-Terminal Myristylation Sequences of Heterologous Proteins Cannot Confer Binding of pp60^{v-src} to the Plasma Membrane

Binding of pp60^{v-src} to the plasma membrane is mediated by interaction with a membrane-bound src receptor (Resh, 1989; Resh and Ling, 1990). It has previously been shown that this binding can be competed by a myristylated peptide identical in sequence to the amino terminus of pp60^{v-src} (Resh, 1989). The binding assay takes advantage of the ability of in vitro translated pp60^{v-src} to bind to plasma membranes in a saturable and specific manner. This model system was used to investigate the molecular nature of the amino-terminal motif mediating membrane interaction of pp60^{v-src}.

Table I. Sequences of Amino-Terminal Chimeric Src Constructs

Construct	Sequences
pp60^{v-src}	M G S S K S K P K D P S Q R
kin-src	M G N A A A A K K G S E Q E
A-kin-src	M A N A A A A K K G S E Q E
cal-src	M G N E A S Y P L E M M S H
VP2-src	M G A A L T L L G D L I A T

Chimeric src genes were prepared by deletion of the nucleotides encoding the 14 amino-terminal amino acids of pp60^{v-src}. These nucleotides were replaced by 42 nucleotides that encode the 14 amino-terminal amino acids of several proteins, culminating in the construction of four chimeric genes as described in experimental procedures. v-src was fused to the following: cAMP-dependent protein kinase, kin-src; a myristylation negative mutant of cAMP-dependent protein kinase, A-kin-src; the β subunit of calcineurin, cal-src; VP2 capsid protein of SV40, VP2-src.

To uncouple the role of amino-terminal amino acids in membrane binding from their role in directing myristylation, we have taken advantage of the broad substrate specificity of myristyl transferase. Cassette mutagenesis was used to replace the NH_2-terminal amino acids of pp60^{v-src} with heterologous sequences. This was accomplished by deletion of the nucleotides encoding the first 14 amino acids of pp60^{v-src} and substitution with a cassette encoding the amino-terminal 14 amino acids of each of three other myristylated soluble proteins: the catalytic subunit of cAMP-dependent protein kinase, kin-src (Carr et al., 1982; Uhler et al., 1986), the β subunit of calcineurin, cal-src (Aitken et al., 1984, Guerini et al., 1989), and the VP2 capsid protein of SV40, VP2-src (Fiers et al., 1978; Streuli and Griffin, 1987). Additionally, the amino terminus of pp60^{v-src} was replaced by that of a nonmyristylated variant of cAMP-dependent protein kinase in which the amino-terminal glycine has been substituted by alanine, A-kin-src (Table I) (Clegg et al., 1989). Unmodified v-src and chimeric-src constructs were then tested for in vitro membrane binding (Resh, 1989).

Synthesis of pp60^{v-src} and the chimeric proteins was achieved by in vitro transcription, followed by translation of the corresponding mRNA in a reticulocyte lysate. Binding was compared in the presence and absence of membranes, the latter representing the background of intrinsic aggregation of the various src proteins. The extent of aggregation varied with different lots of reticulocyte lysate, and was dependent on the presence of the myristate moiety and the composition of the adjacent NH_2-terminal residues.

Upon addition of vole fibroblast plasma membranes, ~65% of pp60^{v-src} became membrane associated, based upon redistribution of the [^{35}S]methionine-labeled protein to the pellet fraction following ultracentrifugation (Fig. 1 *A* and *B*). Myristylation was required for membrane attachment of pp60^{v-src}, because a nonmyristylated variant,

Table II. src Mutants Are Efficiently Myristylated

src Construct	Relative incorporation [^{3}H]myristate/[^{35}S]methionine
v-src	1.00
kin-src	1.11
cal-src	1.23
VP2-src	1.18
v-src N5	0.99
v-src N9	0.98
v-src N5+9	0.93
kin-src N8	0.88
kin-src N9	1.00
kin-src N8+9	0.98

Chimeric src constructs were tested for efficiency of myristylation: mRNA synthesized by in vitro transcription of the encoding plasmids was translated in rabbit reticulocyte lysates in the presence of [^{3}H]myristic acid or [^{35}S]methionine, and the resulting products were analyzed by gel electrophoresis and autoradiography. Densitometry was used to determine incorporation of [^{3}H]-myristic acid relative to [^{35}S]methionine. All mutants were normalized to pp60$^{v\text{-}src}$ where the ratio of [^{3}H]/[^{35}S] incorporation was defined as 1.

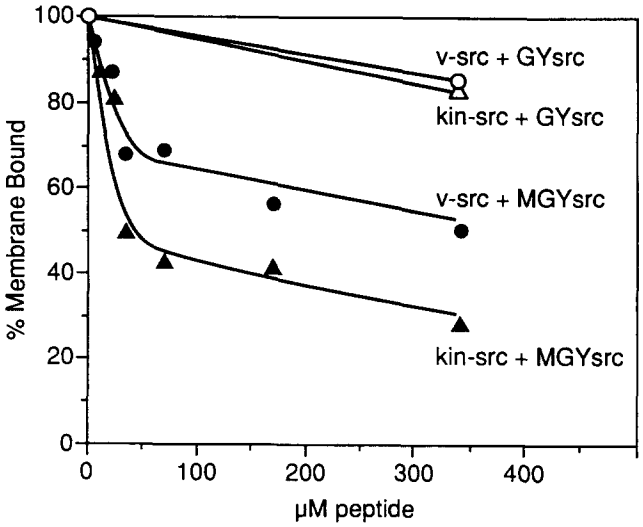

Figure 2. Inhibition of pp60$^{v\text{-}src}$ and kin-src membrane binding by increasing concentrations of src peptide. 1T cell plasma membrane–enriched fractions were incubated with in vitro translated pp60$^{v\text{-}src}$ and kin-src in the presence of increasing concentrations of the myristylated v-src peptide (MGYsrc) or with nonmyristylated v-src peptide (GYsrc) at 340 μM/ml. The amount of membrane bound material was quantitated as described in Fig. 1. Material pelleted in the absence of membranes was subtracted from the amount of material pelleted in the presence of membranes to derive the values for percent membrane bound. pp60$^{v\text{-}src}$ or kin-src bound to untreated membranes was normalized to 100%.

NY315 (Cross et al., 1984), did not exhibit binding. However, the chimeric myristylated proteins cal-src and VP2-src did not bind to membranes to any appreciable extent above background (compare pellet fractions in the presence and absence of membranes; Fig. 1). Identical results were obtained when binding to chicken embryo fibroblast membranes was studied (Fig. 1 *A*). We conclude that the mere presence of a myristate moiety within a heterologous amino acid sequence is not sufficient for binding of the src polypeptide to the membrane.

Amino Terminus of cAMP-Dependent Protein Kinase Can Partially Substitute for the Amino Terminus of pp60$^{v\text{-}src}$ in Conferring Membrane Binding

In sharp contrast to the cal-src and VP2-src chimeras, nearly 40% of the total kin-src protein became membrane bound (Fig. 1). Varying binding capacities were not due to differential myristylation: all three of the fusion proteins were myristylated as efficiently as pp60$^{v\text{-}src}$ (as determined by incorporation of [^{3}H]myristate with normalization to labeling by [^{35}S]methionine; Table II). Binding by kin-src was dependent upon the presence of the myristate moiety, as the nonmyristylated variant A-kin-src was vastly reduced in its membrane association. Myristylation of the kin-src chimera is therefore necessary for membrane binding.

Membrane interaction of kin-src and pp60$^{v\text{-}src}$ was characterized in more detail to determine whether these two proteins shared similar binding properties. Binding of both proteins was equally stable to treatment of membranes with 0.5 M NaCl and 50 mM EDTA, and detergent extraction of membranes abolished subsequent binding (data not shown). These properties are typical of integrally associated membrane proteins.

If kin-src interacts specifically with the src receptor, it should be possible to displace kin-src from the plasma membrane with a myristylated peptide corresponding to the amino-terminal sequences of pp60$^{v\text{-}src}$ (termed MGYsrc; Resh, 1989). Increasing amounts of competing MGYsrc peptide were added to a binding assay containing either kin-src or pp60$^{v\text{-}src}$. As depicted in Fig. 2, both proteins were displaced from the plasma membrane fraction as the concentration of MGYsrc peptide was increased. Analysis of the competition data with MGYsrc revealed a K_i of 48 μM peptide for inhibition of kin-src binding and 60 μM for pp60$^{v\text{-}src}$ binding. GYsrc, the nonmyristylated version of the MGYsrc peptide inhibited binding of both kin-src and pp60$^{v\text{-}src}$ by only 10–20%, thereby emphasizing the role of myristate. When polypeptide ligands were used as inhibitors, we observed that radiolabeled kin-src binding was readily competed by addition of equimolar or submolar concentrations of in vitro translated pp60$^{v\text{-}src}$. Likewise, pp60$^{v\text{-}src}$ binding was diminished when the kin-src polypeptide was added to the binding assay, albeit to a lesser extent (data not shown). The striking similarity in binding parameters between kin-src and pp60$^{v\text{-}src}$ strongly suggests that a specific interaction occurs between kin-src and the src receptor.

The ability of other myristylated peptide sequences to inhibit kin-src binding was also tested. As had previously been observed with pp60$^{v\text{-}src}$, a myristylated peptide derived from the NH_2-terminal region of the gag polyprotein of murine leukemia virus did not have a significant effect on the binding of kin-src (data not shown). Of greater interest, however, was the effect of a myristylated peptide containing the same NH_2-terminal sequence as kin-src (i.e., derived from catalytic subunit of A kinase). Previous experiments (Resh, 1989) had failed to detect inhibition of pp60$^{v\text{-}src}$ binding by a myristylated peptide containing the first eight amino acids of the mature A kinase sequence (GNAAA-AKK). This peptide, as well as a longer version (GNAAAA-KKGSEQESY), were reexamined for their ability to affect kin-src as well as pp60$^{v\text{-}src}$ binding. Each peptide, at a con-

centration of 92 μM (8 mer) and 58 μM (15 mer), inhibited membrane binding of kin-src by 35%. However, as the concentration of these kin peptides was increased, aggregation of both kin-src and pp60$^{v\text{-}src}$ occurred, resulting in redistribution of these proteins into the pellet fraction even in the absence of membranes. Unfortunately, aggregation of pp60$^{v\text{-}src}$ obscured the determination of any potential effect upon membrane binding and explains a previous inability to observe competition with the kin peptide. It was therefore not possible to obtain a reliable K_i for the kin peptides.

Kin-src Is Membrane Associated In Vivo

The binding results obtained in vitro predict that the amino terminus of cAMP-dependent protein kinase should direct src to the plasma membrane in vivo, though possibly with a lower efficiency than the authentic amino terminus. To test this hypothesis, both pp60$^{v\text{-}src}$ and kin-src were introduced into NIH3T3 cells by retroviral mediated gene transfer, utilizing resistance to the aminoglycoside G418 as the selectable marker (Cone and Mulligan, 1984). The morphology of G418-resistant clonal populations expressing either kin-src, pp60$^{v\text{-}src}$, or the retroviral vector PLJ alone is illustrated in Fig. 3. Control cells under G418 selection maintained a flat phenotype, whereas pp60$^{v\text{-}src}$-expressing cells became fully transformed, with extensive rounding. Although some kin-src–expressing cells also became rounded, the majority took on a fusiform appearance, indicating partial transformation. This difference in appearance was not due to differences in the level of expression of nor the extent of myristylation or phosphorylation of these src proteins (data not shown). Furthermore, both types of transformed cells were capable of growth in soft agar (Table III).

Appearance of a transformed phenotype implies that the kin-src chimera is capable of membrane association. The subcellular distribution of kin-src and pp60$^{v\text{-}src}$ was therefore confirmed by subcellular fractionation, using hypotonic lysis and differential centrifugation. As outlined in Table III, 80–90% of the kin-src and pp60$^{v\text{-}src}$ polypeptides were membrane associated. Moreover, localization by indirect immunofluorescence microscopy revealed characteristic punctate membrane staining, indicative of localization to adhesion plaques or podosomes, for both pp60$^{v\text{-}src}$ and kin-src (Fig. 3). Furthermore, diffuse cytoplasmic staining together with cellular fractionation studies confirmed that cal-src and VP2-src were cytosolic, non-membrane bound proteins (data not shown). Thus data obtained from in vitro and in vivo studies are in agreement, confirming that some feature(s) of the amino terminus of cAMP-dependent protein kinase sequence contains targeting information sufficient to localize src to the plasma membrane in vivo and in vitro. This association appears to be by a mechanism and binding site analogous to that used for recognition of wild-type v-src.

Essential Role for Lysine Residues in Membrane Targeting

We have shown above that the amino-terminal 14–amino acid residues of cAMP-dependent protein kinase mimics the amino terminus of wild-type pp60$^{v\text{-}src}$ with respect to protein localization. Comparison of the amino-terminal residues of wild-type pp60$^{v\text{-}src}$ and kin-src revealed little conservation of amino acid sequence, hydrophobicity, or charge, with the exception of two lysine residues in similar positions in both sequences and an additional lysine in the pp60$^{v\text{-}src}$ sequence (Table I). We speculated that the two adjacent lysines at positions 7 and 8 in kin-src might mimic the lys-pro-lys sequence of v-src at positions 7–9 and/or the lys-ser-lys at positions 5–7. To test the importance of lysine residues in membrane binding of pp60$^{v\text{-}src}$, a series of myristylated peptides, corresponding to the amino terminus of pp60$^{v\text{-}src}$, was synthesized in which pairs of lysine residues or all three lysines were replaced by asparagine. These peptides were tested to determine their capacity to inhibit pp60$^{v\text{-}src}$ binding in vitro (Fig. 4). As previously shown, a myristyl peptide corresponding to the amino terminus of pp60$^{v\text{-}src}$ inhibited membrane binding of pp60$^{v\text{-}src}$ (KKK-src). However, a peptide in which all three lysines were replaced was ineffective (NNN-src), indicating an essential role for lysine residues in membrane binding. Restoration of a single lysine at position 5 partially reestablished the ability of the peptide to compete with wild-type pp60$^{v\text{-}src}$ (KNN). A more dramatic effect was observed when lysine 7 or 9 was included in the src-like peptide sequence (NKN, NNK). A similar effect was observed for inhibition of kin-src binding (data not shown).

The enhanced binding imparted by the presence of lysines could be due to the presentation of a positively charged amino acid, or to a specific requirement for a lysine residue(s). To distinguish between these two alternatives, an additional src-related peptide was synthesized in which all three lysines were replaced with arginine. Fig. 4 shows that the arginine-containing myristylated peptide was unable to inhibit pp60$^{v\text{-}src}$ binding. Thus, lysines, in conjunction with myristate, appear to be specifically required for interaction of pp60$^{v\text{-}src}$ with its membrane-bound receptor.

To further establish the importance of the amino-terminal lysine residues in the context of the pp60$^{v\text{-}src}$ and kin-src polypeptides, site-directed mutagenesis of these residues was performed. pp60$^{v\text{-}src}$ contains three NH_2-terminal lysines at positions 5, 7, and 9. Lysine 7 is essential for recognition of pp60$^{v\text{-}src}$ by myristyl transferase (Kaplan et al., 1988) and therefore was not altered. However, three other mutants of pp60$^{v\text{-}src}$ were created in which Lys 5, Lys 9, and both lysines 5 + 9 were converted to asparagine to create v-src N5, N9, and N5 + 9. Mutagenesis was also performed on the corresponding lysine residues in kin-src wherein Lys 8, Lys 9, and both lysines 8 + 9 were changed to asparagine to create kin-src N8, N9, and N8 + 9. The proteins encoded by these mutant constructs were then compared with wild-type pp60$^{v\text{-}src}$ and kin-src for membrane-binding capacity in the in vitro binding assay.

Fig. 5 illustrates that replacement of Lys 5 decreased membrane-binding capacity of pp60$^{v\text{-}src}$ by 30%, Lys 9 by 45%, and the double mutant was impaired by ~60%. Interestingly, removal of Lys 9 had a somewhat greater effect, consistent with the peptide inhibition data. It is important to note that even when both of these lysine residues were removed, a significant proportion of N5 + 9 still became membrane associated. This could have been due to the necessary retention of Lys 7 or to the presence of additional NH_2-terminal pp60$^{v\text{-}src}$ sequences. We therefore tested the effect of removing both lysines from kin-src. Mutation of Lys 8 or Lys 9 within kin-src decreased binding by 60–75%, and the double mutant was nearly totally non–membrane bound (Fig. 5). These results were not due to inefficient myris-

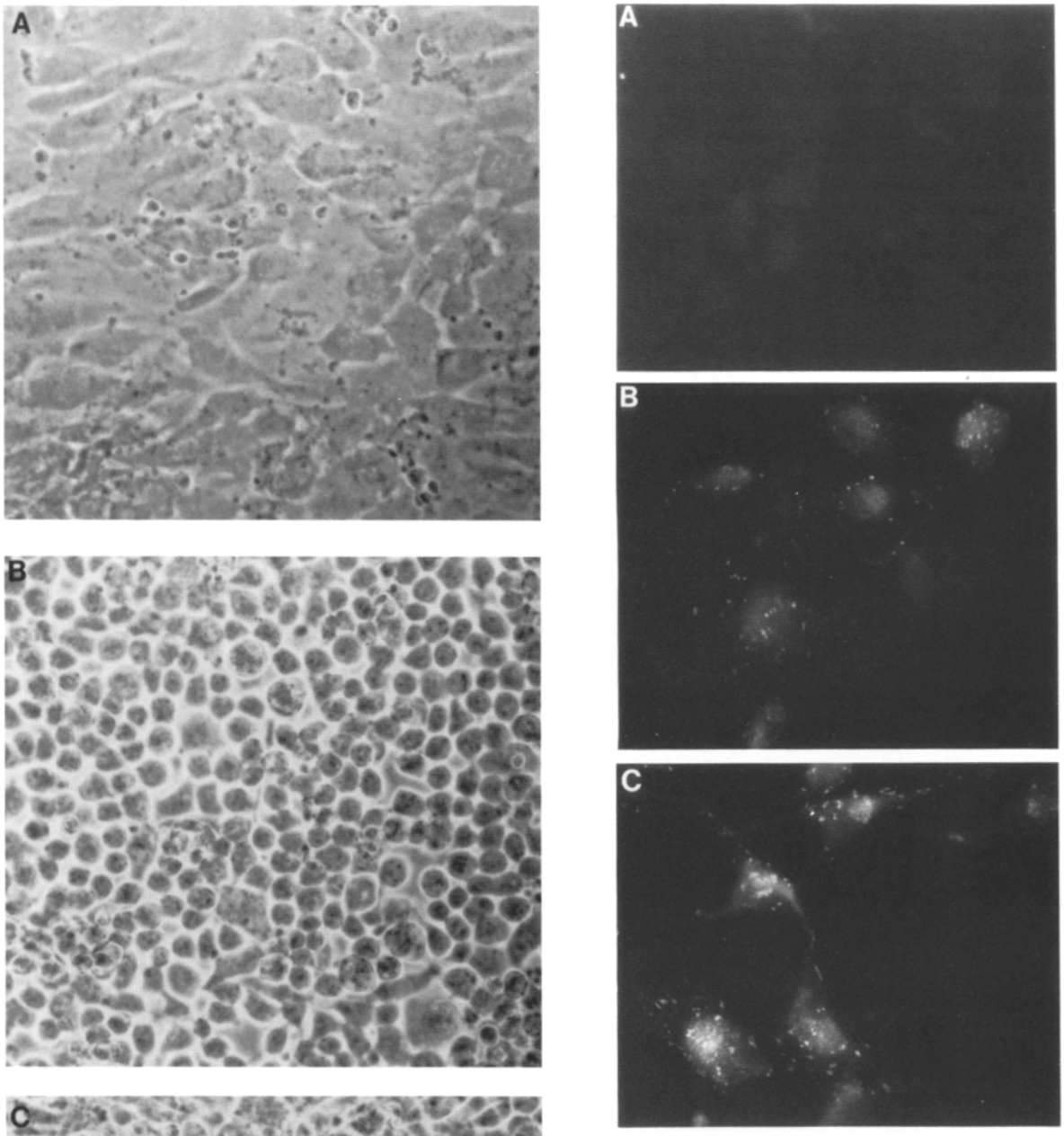

Figure 3. Characteristics of pp60$^{v\text{-}src}$ and kin-src in vivo. pp60$^{v\text{-}src}$ and kin-src were introduced into NIH-3T3 cells by retroviral mediated gene transfer as described in Experimental Procedures. Clones of G418-resistant cells were expanded for analysis. (*Left*) Phase contrast microscopy. (*A*) PLJ: G418-resistant cells containing vector alone. (*B*) PLJ-v-src: G418-resistant cells that express pp60$^{v\text{-}src}$. (*C*) PLJ-kin-src: G418-resistant cells that express the kin-src construct. (*Right*) Indirect immunofluorescent localization of pp60$^{v\text{-}src}$ and kin-src in G418-resistant cells. All cells were fixed with 3.7% formaldehyde, permeabilized with 0.2% Triton X-100, reacted with primary αp60 rabbit antibody followed by reaction with fluorescein-conjugated goat anti-rabbit IgG second antibody. All cells were viewed through a ×63 oil-immersion objective and photographed on Kodak TMAX 400 film. (*A*) PLJ. (*B*) PLJ-v-src. (*C*) PLJ-kin-src.

Table III. pp60v-src and kin-src Associate with Membrane Fractions In Vivo

Cell line	Phenotype	Growth in soft agar	Percent association with cell fraction		
			P1	S100	P100
PLJ	Flat	–	–	–	–
kin-src-1	Elongated ropy arrays and rounded cells	+	45	11	44
kin-src-2	Elongated ropy arrays and rounded cells	+	33	20	47
kin-src-3	Elongated ropy arrays and rounded cells	+	40	20	40
kin-src-4	Elongated ropy arrays and rounded cells	+	23	29	48
v-src-1	Rounded	+	43	10	47
v-src-2	Rounded	+	32	7	61
v-src-3	Rounded	+	17	16	67
v-src-4	Rounded	+	34	9	57

G418-resistant clones of NIH3T3 cells expressing pp60$^{v\text{-}src}$ and kin-src were labeled with 50 μCi/ml [^{35}S]methionine for 1 h. At the end of the labeling period, cells were lysed by Dounce homogenization in hypotonic buffer and fractionated by differential centrifugation (Resh and Erikson, 1985) into a 1,000-*g* pellet (P1) and a 100,000-*g* pellet (P100), and supernatant (S100). Each fraction was adjusted to kinase buffer, immunoprecipitated with α p60 serum, subjected to an in vitro kinase assay (Resh and Erikson, 1985), and analyzed by SDS-PAGE, autoradiography, and liquid scintillation counting. The values listed in each column refer to the percentage of total src kinase activity found in each fraction. Similar results were obtained by adjusting to RIPA buffer and immunoprecipitation with α p60 serum without proceeding to the in vitro kinase assay (data not shown). The ability to grow in soft agar was determined as previously described (Jakobovits et al., 1984).

tylation, as all mutant proteins incorporated [^{3}H]myristic acid with an efficiency comparable to wild-type pp60$^{v\text{-}src}$ (Table II).

Given that removal of both lysines from kin-src virtually eliminated membrane binding, we suspect that Lys 7 contributes to the residual membrane binding of v-src N5 + 9. Furthermore, these results are in excellent agreement with the conclusions drawn from the peptide inhibition experiments (Fig. 4). Taken together, these data strongly suggest that NH_2-terminal lysine residues are critical determinants in mediating the membrane binding of both kin-src and pp60$^{v\text{-}src}$.

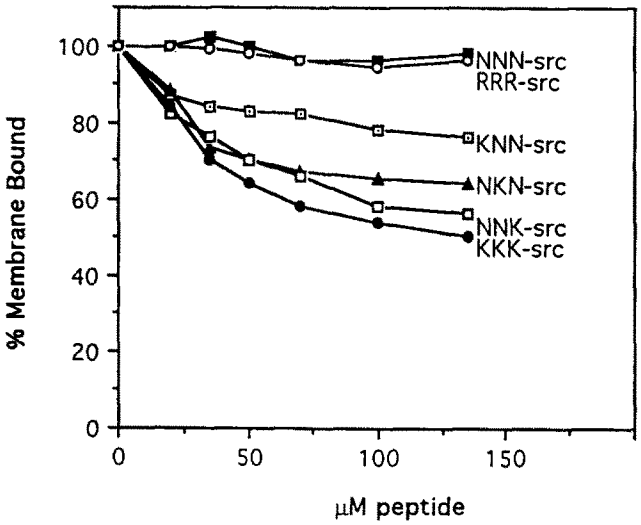

Figure 4. Inhibition of pp60$^{v\text{-}src}$ membrane binding by myristylated src-related peptides requires the presence of lysine. Plasma membranes (P100) isolated from 1T cells were incubated with in vitro translated pp60$^{v\text{-}src}$ in the presence of increasing concentrations of myristylated peptides corresponding to the amino-terminal sequences of pp60$^{v\text{-}src}$, KKK (GSSKSKPKDPSY) previously referred to as MGYsrc, or myristylated peptides corresponding to the amino terminus of pp60$^{v\text{-}src}$ in which pairs of lysines were replaced by asparagine, KNN (GSSKSNPNDPSY), NKN (GSSNSKPNDPSY), NNK (GSSNSNPKDPSY), or in which all three lysines were replaced by asparagine NNN (GSSNSNPNDPSY) or arginine RRR (GSSRSRPRDPSY). The amount of membrane-bound material was quantitated as described in Fig. 1. Material pelleted in the absence of membranes was subtracted from the amount of material pelleted in the presence of membranes to derive the values for percent membrane bound. The amount of pp60$^{v\text{-}src}$ bound to untreated membranes was normalized to 100%.

Role of Internal Membrane Binding Domains

Throughout these studies, it had become apparent that competition of pp60$^{v\text{-}src}$ binding with MGYsrc peptide was never complete, reaching a maximum of 50–60% inhibition on average (Figs. 2 and 4). Several observations had suggested that additional regions of pp60$^{v\text{-}src}$, beyond the NH_2-terminal domain, were contributing to membrane binding (Resh, 1989; Kaplan et al., 1990). We therefore constructed a deletion mutant lacking the putative internal binding domains by deletion of nucleotides encoding amino acids 15–263. Del 15–263 mRNA and protein were produced by in vitro transcription and translation, and the interaction of this mutant protein with membranes was compared to that of wild-type pp60$^{v\text{-}src}$. Fig. 6 demonstrates that nearly 80–90% of the binding of the del 15–263 polypeptide was inhibited by MGYsrc peptide. These results imply that the NH_2-terminal domain contributes most of the binding capacity of this mutant while additional domain(s) between amino acids 15–263 contribute to the MGYsrc peptide insensitive binding of pp60$^{v\text{-}src}$.

If the interaction of the del 15–263 polypeptide with membranes is mediated predominantly by its NH_2-terminal domain, one would predict the membrane binding parameters to closely mimic those exhibited by a 32-K NH_2-terminal src peptide binding protein previously identified by this laboratory by chemical crosslinking of ^{125}I-MGYsrc peptide (Resh and Ling, 1990). Parallel sets of membranes were therefore employed to test the effects of increasing amounts of MGYsrc peptide upon del 15-263 binding, and upon labeling of the 32-K protein, by crosslinking of ^{125}I-MGYsrc peptide. The competition curves for membrane binding of del 15-263 and crosslinking of ^{125}I MGYsrc peptide to the

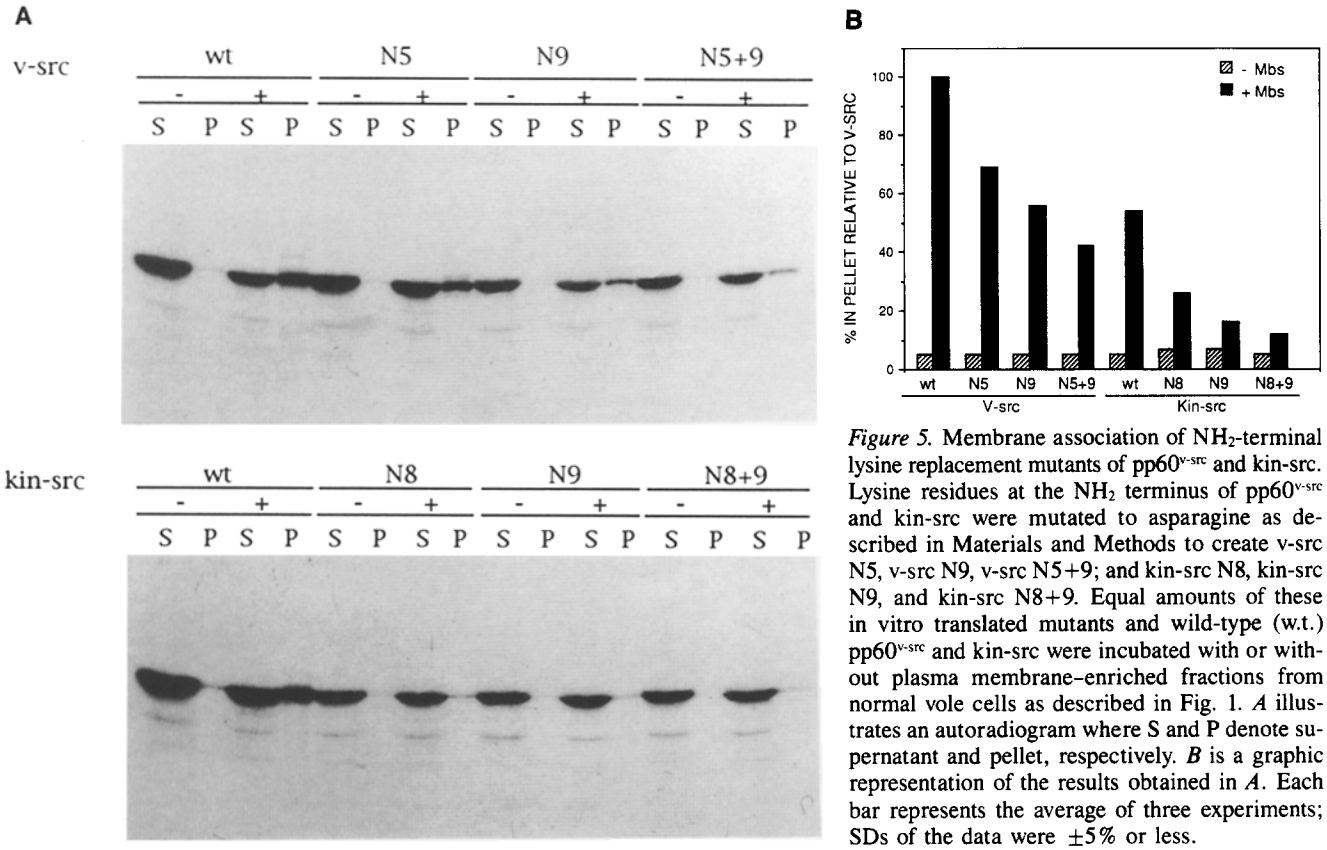

Figure 5. Membrane association of NH_2-terminal lysine replacement mutants of pp60$^{v\text{-}src}$ and kin-src. Lysine residues at the NH_2 terminus of pp60$^{v\text{-}src}$ and kin-src were mutated to asparagine as described in Materials and Methods to create v-src N5, v-src N9, v-src N5+9; and kin-src N8, kin-src N9, and kin-src N8+9. Equal amounts of these in vitro translated mutants and wild-type (w.t.) pp60$^{v\text{-}src}$ and kin-src were incubated with or without plasma membrane-enriched fractions from normal vole cells as described in Fig. 1. *A* illustrates an autoradiogram where S and P denote supernatant and pellet, respectively. *B* is a graphic representation of the results obtained in *A*. Each bar represents the average of three experiments; SDs of the data were ±5% or less.

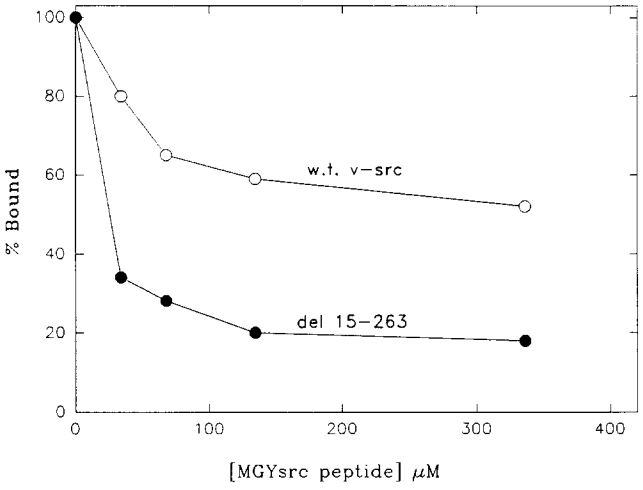

Figure 6. Inhibition of pp60$^{v\text{-}src}$ and del 15-263 membrane binding by increasing concentrations of myristylated src peptide. Vole cell membranes (P100) were incubated with in vitro translated pp60$^{v\text{-}src}$ and del 15-263 in the presence of increasing concentrations of the myristylated v-src peptide MGYsrc. The amount of membrane-bound material was quantitated as described in Fig. 1. Material pelleted in the absence of membranes was subtracted from the amount of material pelleted in the presence of membranes to derive values for percent membrane bound. The translational efficiency of del 15-263 was extremely high, reflected in a lower binding efficiency (bound/total = 20%). The amount of pp60$^{v\text{-}src}$ or del 15-263 bound to membranes in the absence of peptide was normalized to 100%.

32-K protein were nearly identical (Fig. 7), both exhibiting a Ki of 35 μM MGYsrc. These data, as well as experiments with other peptides (not shown), strongly suggest that in the absence of internal domains, interaction of pp60$^{v\text{-}src}$ with the membrane is mediated primarily by binding to the 32-K protein.

Discussion

In this paper, we have used in vitro membrane-binding studies of chimeric src molecules to demonstrate that the nature of the amino acid residues within the pp60$^{v\text{-}src}$ NH_2 terminus is important in determining binding of the protein to the plasma membrane. Despite the presence of a myristate moiety, amino-termini of soluble myristylated proteins were unable to direct the v-src protein to the plasma membrane. One exception was provided by a chimera containing the NH_2 terminus of cAMP-dependent protein kinase. Further investigation, utilizing peptides corresponding to the amino terminus of src, revealed that amino-terminal lysines function together with myristate to target pp60$^{v\text{-}src}$ to the plasma membrane. As the NH_2-terminal seven amino acids of pp60$^{v\text{-}src}$ also provide a recognition sequence for myristyl transferase (Kaplan et al., 1988), the signals for myristylation and membrane binding apparently overlap.

Several lines of evidence support the conclusion that the kin-src chimera is binding to the plasma membrane in a manner similar to that of authentic pp60$^{v\text{-}src}$. First, both kin-src as well as pp60$^{v\text{-}src}$ polypeptide binding to mem-

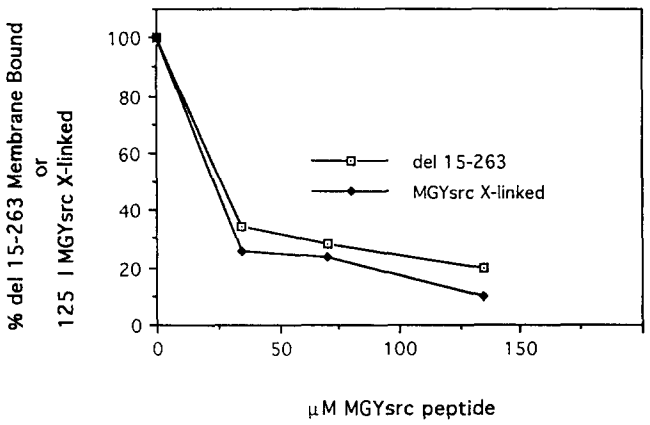

Figure 7. Inhibition of del 15-263 binding and ^{125}I MGYsrc crosslinking by increasing concentrations of MGYsrc peptide. Vole membranes (P100) were incubated with in vitro translated del 15-263 in the presence of increasing concentrations of MGYsrc peptide as described in Fig. 5. Parallel sets of membranes were incubated in the presence of 4×10^5 c.p.m. ^{125}I-labeled MGYsrc peptide and BS^3 crosslinker (experimental procedures) in the absence or presence of increasing concentrations of nonradioactive MGYsrc peptide. This crosslinking reaction results in the specific labeling of a protein of 32 K as described previously (Resh and Ling, 1990). After quenching of the crosslinking reactions, membranes were reisolated and analyzed by gel electrophoresis, autoradiography, and analysis of γ emission. Crosslinking of ^{125}I MGYsrc in the absence of competitor was normalized to 100%.

branes is dependent on myristylation of their respective NH_2 termini (Fig. 1). Second, both polypeptides exhibit tight binding to the membrane pellet that is resistant to extraction with salt or cation chelators. Third, MGYsrc, a myristylated peptide containing the NH_2-terminal pp60$^{v\text{-}src}$ sequence, inhibits binding of both kin-src and pp60$^{v\text{-}src}$ with a similar Ki (Fig. 2). Fourth, pp60$^{v\text{-}src}$ polypeptide inhibits binding of kin-src polypeptide and vice versa. Finally, in vivo expression of kin-src in NIH3T3 cells results in membrane association of the chimeric protein, and in a striking partially transformed phenotype. The latter phenotype may reflect the inability of kin-src to completely mimic the NH_2 terminus of pp60$^{v\text{-}src}$, due to differences in amino acid sequence. Taken together, these data strongly suggest that the amino acid sequence of cAMP-dependent protein kinase contains residues, not present in calcineurin or VP2, that functionally substitute for those present in pp60$^{v\text{-}src}$.

The most likely explanation to account for membrane binding of pp60$^{v\text{-}src}$ and kin-src is the presence of NH_2-terminal lysine residues: three lysines are present in the pp60$^{v\text{-}src}$ sequence, two in cAMP-dependent protein kinase, and none in calcineurin or VP2. Initially, myristylated src-related peptides were utilized as inhibitors of membrane binding. The data in Fig. 4 clearly illustrate that substitution of lysine with asparagine or arginine severely impairs the ability of myristylated src peptides to compete with pp60$^{v\text{-}src}$ for membrane binding. Subsequently, site-directed mutagenesis was performed to verify that NH_2-terminal lysine residues in the pp60$^{v\text{-}src}$ and kin-src polypeptides were responsible for mediating membrane binding. The results in Fig. 5 indicate that replacement of lysine residues with asparagine greatly impairs membrane binding. The effect appears to be more dramatic in the kin-src chimera. However, it must be emphasized that removal of lysine-7 was not technically feasible for pp60$^{v\text{-}src}$, as mutation of lysine-7 has been shown to abrogate recognition by myristyl transferase (Kaplan et al., 1988). Thus, it was not possible to remove all three lysines simultaneously. The results obtained by both peptide inhibition and site-directed mutagenesis indicate that the myristate + lysine motif is critical. Nonetheless, we cannot preclude a role for other NH_2-terminal src amino acids in membrane recognition.

The use of the first 14 amino acids for both acylation and membrane association creates a compact NH_2-terminal domain, thereby leaving internal sequences available for other protein–protein interactions. Myristate might function by initiating and mediating attachment to the phospholipid bilayer and simultaneously delivering the adjacent amino acids to the membrane-bound receptor. Alternatively, the myristate moiety may be involved in direct protein–protein interaction as has been observed for the myristylated VP4 capsid protein of poliovirus (Chow et al., 1987). By juxtaposing highly charged lysine residues next to myristate, the hydrophobic fatty acid may be prevented from being deeply buried as the src molecule is folded, maintaining the NH_2 terminus is a more "flexible" conformation for subsequent efficient interaction with its receptor. Upon receptor recognition, lysines again become critical for imparting specificity to the interaction. This interaction is not solely electrostatic in nature as evidenced by the inability of arginine residues to substitute.

Interaction of the NH_2 terminus of cAMP-dependent protein kinase with the plasma membrane can be explained by two models. It is possible that the two adjacent lysines in the kin-src amino terminus are sufficiently similar in orientation to the alignment achieved by the lys-pro-lys sequence in pp60$^{v\text{-}src}$. Alternatively, contact by the receptor with a single lysine (at any one time) might be sufficient for limited interaction. In either case, the inability to make an optimal initial contact may lead to the lower transformation efficiency observed with kin-src.

Apparently, the ability of the NH_2-terminal cAMP-dependent protein kinase sequence to confer membrane binding is dependent upon context, as myristylated catalytic subunit behaves as a soluble protein (Carr et al., 1982; Towler et al., 1988). Furthermore, nonmyristylated kin proteins are unaffected in their activity and/or regulation by the regulatory subunit (Clegg et al., 1989). Solubility could be due to lack of one or more essential src domains. Alternatively, cAMP-dependent protein kinase may possess an inhibitory determinant rendering its NH_2 terminus inaccessible to membrane interaction.

It is interesting to compare our results with those obtained by Hancock et al. (1990) for the family of ras proteins, which exhibit a dual requirement for isoprenylation/methylesterification and palmitoylation for localization to the plasma membrane. One variant, p21$^{K\text{-}ras(B)}$ is not palmitoylated and instead contains a polybasic domain of six consecutive lysine residues, which is required for membrane binding. Unlike pp60$^{v\text{-}src}$, these signals are located at the COOH terminus of the protein and do not overlap with the CAAX box, which signals isoprenylation. Furthermore, neither palmitoylation nor the polybasic domain are required for transformation by

Table IV. Src Family Members

src	GLY-SER-SER-LYS-SER-LYS-PRO-LYS-ASP-PRO
lyn	GLY-CYS-ILE-LYS-SER-LYS-GLY-LYS-ASP-SER
yes	GLY-CYS-ILE-LYS-SER-LYS-GLU-ASP-LYS-GLY
syn/fyn	GLY-CYS-VAL-GLN-CYS-LYS-ASP-LYS-GLU-ALA
hck	GLY-CYS-MET-LYS-SER-LYS-PHE-LEU-GLN-VAL
blk	GLY-LEU-LEU-SER-SER-LYS-ARG-GLN-VAL-SER
lck	GLY-CYS-VAL-CYS-SER-SER-ASN-PRO-GLU-ASP

The amino-terminal residues of src family members are as indicated. Lysine residues have been underlined to denote their conservation.

activated forms of the protein, although they do increase its efficiency.

Like $pp60^{v\text{-}src}$, some α subunits of heterotrimeric G proteins are also modified by myristic acid on the amino-terminal glycine. Mutagenesis of the amino-terminus of the α-subunit of Go indicates that while this modification is essential for stable trimer formation, nonmyristylated α is capable of transient interaction with $\beta\gamma$ subunits. Furthermore, amino-terminal amino acids 7–10 appear to contribute to heterotrimer formation in both the presence or absence of myristylation (Denker et al., 1992).

Another member of the src family, $p56^{lck}$, has been shown to utilize amino-terminal amino acids for interaction with the cytoplasmic domain of the transmembrane proteins CD4 and CD8 (Shaw et al., 1989; Turner et al., 1990). Interaction of $p56^{lck}$ with these T lymphocyte proteins is dependent upon the presence of cysteine residues not present in $pp60^{v\text{-}src}$. The utilization of cysteine residues for membrane protein interaction may prove to be unique among the src-related tyrosine kinases, but is consistent with the model that the amino terminus of this group of proteins is critical for subcellular localization.

The src family of non-receptor protein tyrosine kinases share extensive homology save for a unique region of 40–80 residues at the amino terminus. This variant domain is proposed to mediate specific interactions unique to each kinase. Despite this divergence, homology has been preserved among several members, as illustrated in Table IV. In addition to myristylation, there is strong conservation of lysine residues among members of this family. We propose a general mechanism of membrane binding wherein the hydrophobicity imparted by myristate acts in concert with lysine residues as a targeting motif. In addition to this extreme amino-terminal domain, other unique regions of each kinase may provide specificity, thus directing each kinase to its appropriate site of action.

The NH_2-terminal 12 amino acids of the cellular homolog, $pp60^{c\text{-}src}$ are identical to that of $pp60^{v\text{-}src}$. Recently, Soriano et al. (1991) created a homozygous null mutation of the c-src gene of mice. Their results demonstrated that c-src is not absolutely essential for growth and differentiation. The ability of these mice to develop and exhibit disease secondary to their genetic alteration has been attributed to the functional redundancy among the other members of the src family. Perhaps the sequence similarities noted in Table IV may permit this substitution by utilization of similar membrane binding motifs by the various members of the src kinase family.

It is clear, however, from our binding data (Figs. 2 and 4; Resh, 1989) as well as the data of others (Kaplan et al., 1990) that additional downstream domains of the src protein contribute to membrane binding. We propose that binding of $pp60^{v\text{-}src}$ to the membrane is mediated by at least two mechanisms that may be independent or sequential. One binding modality involves the interaction of the myristylated NH_2-terminal domain with the 32-K NH_2-terminal src peptide binding protein, recently identified by this lab (Resh and Ling, 1990). The myristate moiety is essential for initiation (and potentially maintenance) of this interaction. In addition to $pp60^{v\text{-}src}$, $pp60^{c\text{-}src}$ and other src-related kinases may also utilize this or a similar membrane protein as part of their plasma membrane anchoring apparatus. A second (and potentially subsequent) membrane binding would occur in which internal domains of the $pp60^{v\text{-}src}$ polypeptide chain make additional contacts.

The two types of binding can be physically separated by deletion of internal domains (del 15-263), thus producing a molecule in which binding is apparently mediated entirely by 32-K interactions. In vitro, at least 50% of the wild-type $pp60^{v\text{-}src}$ appears to utilize internal binding, which is resistant to competition with MGYsrc. Based on the shape of the binding curve in Fig. 2, it appears that the kin-src chimera is less able to utilize internal domains because nearly 75% of the polypeptide can be released from the membrane by MGYsrc. It remains to be determined what percentage of $pp60^{v\text{-}src}$ in vivo remains bound to the 32-K protein and whether the 32-K protein functions catalytically to facilitate insertion of $pp60^{v\text{-}src}$ into the membrane. Future experiments will be directed toward answering these questions.

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