Membrane Binding of Myristylated Peptides Corresponding to the NH_2 Terminus of Src†

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ABSTRACT: Membrane association is required for cell transformation by $pp60^{v\text{-}src}$ (v-Src), the product of the *v-src* oncogene of Rous sarcoma virus. Previous experiments have identified two NH_2-terminal membrane-binding motifs: a myristate (14-carbon acyl chain) attached to the NH_2-terminal glycine and three basic residues at positions 5, 7, and 9 of Src. We examined the membrane binding of each motif using myristylated (myr-src) and nonmyristylated (nonmyr-src) peptides corresponding to the NH_2 terminus of Src. All myristylated peptides partitioned equally well onto electrically neutral phosphatidylcholine vesicles (K_1 = 10^4 M^{-1}). Identical binding has been observed for simple myristylated peptides (e.g., myr-Gly) and arises from the hydrophobic insertion of the myristate into the bilayer. A nonmyristylated peptide corresponding to residues 2–16 of Src [nonmyr-src(2–16), net charge = +5] bound to vesicles containing 33% monovalent acidic phospholipids with K_1 = 10^3 M^{-1}. Penta(lysine) (+5 net charge) exhibits the same binding behavior, which is due to the electrostatic interaction between basic residues and acidic lipids. The corresponding myristylated peptide, myr-src(2–16), binds 3 orders of magnitude more strongly to vesicles containing 33% acidic lipids than to neutral vesicles. The resulting apparent association constant, K_1 = 10^7 M^{-1}, is approximately equal to the product of the partition coefficients for the two individual interactions. This 10^7 M^{-1} binding is sufficiently strong to anchor the Src protein to biological membranes. We propose a simple model that explains the observed synergism between the two peptide–membrane interactions.

$pp60^{v\text{-}src}$ (v-Src),[1] the product of the *v-src* gene of Rous sarcoma virus, transforms cells as a consequence of its tyrosine kinase activity [for reviews, see Parsons and Weber (1989) and Resh (1990b)]. Like many other nonreceptor tyrosine kinases, v-Src and its cellular counterpart, c-Src, are predominantly membrane-associated *in vivo* (Courtneidge et al., 1980). The interaction of v-Src with cellular membranes plays a fundamental role in viral transformation: v-Src mutants that fail to associate with membranes do not transform cells, even though they retain wild-type tyrosine kinase activity (Cross et al., 1984; Kamps et al., 1985). Two NH_2-terminal motifs are essential for the membrane localization of v-Src and c-Src (referred to collectively as Src): (i) a 14-carbon fatty acid, myristate, attached to the NH_2-terminal Gly residue through an amide bond (Cross et al., 1984; Kamps et al., 1985; Garber & Hanafusa, 1987) and (ii) three basic residues at positions 5, 7, and 9 (Silverman & Resh, 1992).

Experiments with proteins modified via site-directed mutagenesis (Cross et al., 1984, 1985; Pellman et al., 1985a,b; Kaplan et al., 1988) and with chimeric proteins (Kaplan et al., 1990) indicate that amino acids 2–7 direct myristylation. During the translation of Src on free ribosomes, the initiating methionine is removed by aminopeptidase, and myristate is coupled to Gly-2 by *N*-myristyltransferase (Cross et al., 1984; Garber et al., 1985). Myristylation itself is not sufficient to target and/or attach the protein to the membrane. While nonmyristylated (nonmyr) mutants of Src bind poorly to membranes, some myristylated (myr) variants of v-Src also fail to associate with the plasma membrane (Buss et al., 1984; Garber et al., 1985). Experiments with simple myristylated peptides (e.g., myr-Gly) indicate that the partition coefficient onto membranes (or apparent association constant of the peptide with monomeric lipid) is 10^4 M^{-1}, which "provides barely enough energy to attach a myristylated protein in the cytoplasm to the membrane" (Peitzsch & McLaughlin, 1993; Silvius & l'Heureux, 1994). Thus, membrane association of a myristylated protein will be exquisitely sensitive to factors that either enhance (e.g., electrostatic interactions between basic residues and acidic lipids) or detract [e.g., hydrophobic interaction between myristate and the covalently attached protein, such as occurs with cAMP-dependent protein kinase (Zheng et al., 1993) and recoverin (Zozulya & Stryer, 1992)] from the interaction of the myristate moiety and the membrane.

Experiments with chimeric proteins indicate that amino acids 2–14 of Src are also required for membrane association. For example, a chimeric protein containing only the first seven amino acids of Src is myristylated but remains soluble (Kaplan et al., 1990), while a chimeric protein containing residues 2–14 of Src is both myristylated and membrane-bound (Pellman et al., 1985b; Kaplan et al., 1990). The NH_2-terminal sequence of Src (myristate-Gly-Ser-Ser-Lys-Ser-

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[1] Abbreviations: DMSO, dimethyl sulfoxide; c-Src, $pp60^{c\text{-}src}$; CD, circular dichroism; LUVs, large unilamellar vesicles; MARCKS, myristylated alanine-rich C kinase substrate; MLVs, multilamellar vesicles; myr, myristylated; ^{3}H-myr, [9,10-$^3H_2$]myristylated; nonmyr, nonmyristylated; PC, phosphatidylcholine; PG, phosphatidylglycerol; PIP_2, phosphatidylinositol 4,5-biphosphate; PS, phosphatidylserine; SUVs, small unilamellar vesicles; v-Src, $pp60^{v\text{-}src}$.

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Lys-Pro-Lys-Asp-Pro-Ser-Gln-Arg-Arg-Arg) is hydrophilic, containing six basic, one acidic, and no hydrophobic residues; thus, it is unlikely that this region inserts into the phospholipid bilayer. Using a combination of site-directed mutagenesis and peptide competition experiments, Silverman and Resh (1992) demonstrated that membrane association of v-Src requires both the NH_2-terminal lysines (residues 5, 7, and 9) and myristate.

What binds the NH_2-terminal basic residues of Src? Earlier cross-linking experiments with a myristylated peptide corresponding to the NH_2 terminus of Src identified a 32 kDa binding protein (Resh & Ling, 1990), suggesting that this protein could be a Src receptor. However, the purified 32 kDa protein was later identified as the ADP/ATP carrier (Sigal & Resh, 1993), which is primarily localized to the inner mitochondrial membrane and is unlikely to be a membrane receptor protein for the Src polypeptide. To date, no specific protein receptor for the NH_2-terminal basic residues of Src has been identified [see Resh (1993)].

Detailed studies of model peptide–membrane systems show that basic peptides interact electrostatically with acidic lipids (Kim et al., 1991; Mosior & McLaughlin, 1992a,b; Montich et al., 1993). Eukaryotic cell membranes typically contain 10–20% acidic phospholipids (phosphatidylserine, phosphatidylinositols), most of which are localized at the inner leaflet of the bilayer (Op den Kamp, 1979; Devaux, 1992). Thus, the cytoplasmic surface of the plasma membrane contains about 30% negatively charged lipids. Several other proteins, e.g., myosin I (Pollard et al., 1991; Doberstein & Pollard, 1992), myristylated alanine-rich C kinase substrate (MARCKS; Taniguchi & Manenti, 1993; Kim et al., 1994), protein kinase C (Mosior & McLaughlin, 1991; Newton, 1993), and HIV gag (Zhou et al., 1994), contain clusters of basic amino acids that probably interact electrostatically with negatively charged phospholipids. Extensive studies of the membrane binding of penta(lysine), which has the same +5 net charge as residues 2–16 of Src, show that this peptide electrostatically binds to membranes containing 33% acidic lipids, with $K_1 = 10^3\ M^{-1}$ (Kim et al., 1991; Mosior & McLaughlin, 1992a). We propose that the NH_2-terminal region of Src could interact with the cytoplasmic surface of the plasma membrane in a similar manner.

While neither the hydrophobic insertion of the myristate alone nor the electrostatic interaction of basic residues and acidic lipids is sufficient to bind Src strongly to the plasma membrane, we propose that the two binding motifs act synergistically (with an apparent cooperativity) to anchor Src firmly to a membrane. In the work reported here, we use peptides related to the NH_2 terminus of Src to demonstrate that this synergism implies that the partition coefficients of the individual hydrophobic and electrostatic interactions multiply or the binding energies add. In parallel studies, we show that identical synergism between the two binding motifs results in strong binding of the intact c-Src and v-Src proteins to both phospholipid vesicles and biological membranes (Sigal et al., 1994).

MATERIALS AND METHODS

Materials. The zwitterionic lipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (PC) and the acidic lipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (PG) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (PS) were purchased from Avanti Polar Lipids (Birmingham, AL). Radiolabeled 1,2-di[1-^{14}C]oleoyl-L-3-phosphatidylcholine ([^{14}C]PC) was from Amersham (Arlington Heights, IL). The triammonium salt of the acidic lipid phosphatidylinositol 4,5-biphosphate (PIP_2) was obtained from Calbiochem (La Jolla, CA).

The NH_2-terminal sequence (residues 2–16) of myristylated Schmidt–Ruppin A v-Src or avian c-Src, myr-Gly-Ser-Ser-Lys-Ser-Lys-Pro-Lys-Asp-Pro-Ser-Gln-Arg-Arg-Arg, contains six basic and one acidic residues. Myristylated and nonmyristylated peptides corresponding to residues 2–12 [denoted myr-src(2–12) and nonmyr-src(2–12), respectively] and residues 2–16 [denoted myr-src(2–16) and nonmyr-src(2–16), respectively] were synthesized with an amide-blocked C terminus to >94% purity (determined by analytical HPLC, mass spectrographic, and amino acid analyses) by Multiple Peptide Systems (San Diego, CA) and used without further purification. Tritiated myristylated peptides [^{3}H-myr-src(2–12) and ^{3}H-myr-src(2–16)] were prepared enzymatically as described in Deichaite et al. (1988), using purified yeast *N*-myristyltransferase (Duronio et al., 1989). The ^{3}H-myr-src peptides were purified by HPLC using a C18 reverse-phase column.

Three other myristylated peptides with sequences related to the NH_2 terminus of Src were purchased from Multiple Peptide Systems. All contain a nonnative, unblocked C-terminal Tyr residue: (i) myr-src(2–12,Y^{COO-}) contains residues 2–12 of Src, (ii) myr-src(RRR,Y^{COO-}) has Lys-5, -7, and -9 replaced with Arg, and (iii) myr-src(NKN,Y^{COO-}) has Lys-5 and -9 replaced with Asn. Peptides i–iii were used previously under the notation MGY-src (or KKK-src), RRR-src, and NKN-src, respectively (Silverman & Resh, 1992). These peptides were initially radiolabeled with ^{125}I on the C-terminal Tyr residue (Resh, 1990a). Membrane-binding experiments with a similar iodinated peptide have been reported (Goddard et al., 1989). However, the iodine moiety changed the aggregation and binding properties of the peptides under our conditions, and this approach was abandoned. Nonradiolabeled peptides were initially dissolved to a concentration of 5 mg/mL in DMSO. Aqueous solutions were prepared with 18 MΩ H_2O (Super-Q, Millipore Corp., Bedford, MA) that was subsequently bidistilled in an all-quartz still.

Sucrose-Loaded Vesicle-Binding Assay. Initial lipid concentrations in $CHCl_3$ were measured on a Cahn electrobalance, a method that gives the same results as phosphate analysis (Kim et al., 1991; Peitzsch & McLaughlin, 1993). Multilamellar vesicles (MLVs) were formed by drying a $CHCl_3$ solution of lipids in a rotary evaporator and resuspending the lipids in a 176 mM sucrose solution (1 mM MOPS, pH 7). Using the method described by Hope et al. (1985), large unilamellar vesicles (LUVs) were prepared by taking MLVs through 5 cycles of freezing (77 K, liquid N_2) and thawing (40 °C, water bath), followed by 10 cycles of extrusion through a stack of two polycarbonate filters (0.1 μm diameter pore size) in a Lipex Biomembranes Extruder (Vancouver, BC, Canada). Vesicles of this size are unilamellar (Mui et al., 1993). The sucrose solution on the outside of the vesicles was removed by 5-fold dilution of the LUV solution into a salt buffer (typically 0.1 M KCl/1 mM MOPS at pH 7, which is isoosmotic with 176 mM sucrose) and centrifugation [1 h, 100000*g*, 25 °C; for details see Rebecchi et al. (1992)]. The supernatant was discarded, and the resuspended pellet was used for binding measurements. The relative lipid concentration throughout the experiment was monitored by the incorporation of trace amounts of [^{14}C]PC in the lipid mixture.

In the membrane-binding assay, peptide was mixed with sucrose-loaded LUVs; [peptide] ≪ [lipid], so that the peptide did not bind a significant fraction of the acidic lipid. After 15 min of equilibration at room temperature (22 °C), vesicle-

bound peptide was separated by centrifugation (1 h, 100000g, 25 °C). Ninety percent of the supernatant was retrieved immediately, and the concentration of peptide in the supernatant and pellet was measured by either a fluorescamine assay or scintillation counting (see following). Calculations of the percent of bound peptide were corrected for the 1–8% lipid that remained in the supernatant. Lipid loss was negligible for [lipid] > 10^{-7} M. However, at the lowest lipid concentrations (3×10^{-9} M), about 30% lipid was lost to the polyallomer tubes. We added 2% DMSO or 6.5×10^{-3}% Triton X-100 to the solution to prevent unbound myr-src-(2–16) from spinning down during the sucrose-loaded vesicle-binding assay; the same conditions were used in the binding assays of nonmyr-src(2–16). In control experiments, neither the solvent nor the detergent affected the membrane binding of the smaller myr-src(2–12) peptide, which did not spin down in the absence of these additions.

Non-Radiolabeled Peptides. The concentration of non-radiolabeled peptide in the supernatant and pellet was determined by a modified fluorescamine assay (Weigele et al., 1972): first, the pH of the sample (volume = 600 μL) was raised from 7 to 10 by the addition of KOH (0.1 N), and then 250 μL of fluorescamine (2.2 mM in dioxane) was added while vortex mixing. This assay provided reliable binding data for [src peptide] > 0.8 μM, where peptide loss was negligible during the binding assay. We used PC and mixtures of PC/PG (not PC/PS) for binding experiments with non-radiolabeled peptides, because fluorescamine reacts with primary amines. We measured the fluorescence intensity (I) of the supernatant and pellet and calculated the fraction of peptide bound to LUVs (f_b) from $f_b = 1 - [I(\text{supernatant})/(I(\text{supernatant}) + I(\text{pellet}))]$. Fluorescence intensity measurements were corrected for the percent lipid in the supernatant and the background light scattering of the lipids.

Radiolabeled Peptides. We used radiolabeled peptides in assays that required [peptide] < 0.8 μM; control experiments showed identical binding for both nonradiolabeled and radiolabeled myr-src peptides to PC LUVs (both sets of data are included in the PC curves of Figures 1 and 2). To minimize the loss of ^{3}H-myr-src(2–12) and ^{3}H-myr-src(2–16), we added the respective unlabeled peptide and treated the polyallomer tubes with Sigmacote from Sigma Chemical Co. (St. Louis, MO). Even with silanization, 40–60% [^{3}H]peptide was lost to the polyallomer centrifuge tubes during binding experiments at [lipid] < 10^{-6} M and initial peptide concentrations of 4–100 nM. Loss of peptide does not affect binding measurements, because we determine the [peptide] in both the supernatant and pellet. In binding assays with [^{3}H]peptides, the fraction of peptide bound to the LUVs was determined by scintillation counting of the supernatant and pellet and deconvolution of the [^{3}H]myristate and [^{14}C]PC radiolabels:

$$f_b = 1 - [^3\text{H}(\text{supernatant})/(^3\text{H}(\text{supernatant}) + ^3\text{H}(\text{pellet}))]$$

Determination of the Molar Partition Coefficient. The molar partition coefficient, K_1, for binding of the myristylated peptide to the membrane is defined as in Peitzsch and McLaughlin (1993):

$$\chi = [P]_m/[L] = K_1[P] \quad (1)$$

where χ is the mole ratio of myristylated peptide in the accessible leaflet of the membrane, $[P]_m$ is the concentration of peptide bound to the membrane, [L] is the concentration of lipid accessible to the myristylated peptide, and [P] is the concentration of peptide in the bulk aqueous phase. All concentrations are in units of M, and K_1 has units of M^{-1}. In our studies, [L] is the concentration of lipid on the outer surface of the LUVs, or one-half the total lipid concentration. Control experiments showed that the membranes are impermeable to myr-src(2–12) for the time course of the binding assay (≈1 h), and it is reasonable to extrapolate this result to all of our peptides. Equation 1 may also be written as $[P]_m = K_1[P][L]$, which has the same form as the limiting version of a mass action equation that assumes (incorrectly) that the acylated peptide forms a 1:1 complex with a lipid. Thus, K_1 may be regarded as an apparent association constant.

By defining $[P]_{tot} = ([P] + [P]_m)$ and noting that $[L] \gg [P]_m$ under our conditions, the fraction of peptide bound is

$$f_b = [P]_m/[P]_{tot} = K_1[L]/(1 + K_1[L]) \quad (2)$$

We determined K_1 from a least-squares fit of eq 2 to the data.

Electrophoretic Mobility Measurements. MLVs were prepared and the electrophoretic mobility was measured as described previously (McLaughlin et al., 1981; Cafiso et al., 1989) on a Rank Brothers Mark I instrument (Bottisham, Cambridge, UK).

The zeta potential, ζ, is calculated from the measured value of the electrophoretic mobility, the velocity of the vesicle in a unit electric field, by using the Helmholtz–Smoluchowski equation (Hunter, 1981). Under our conditions, the electrostatic potential at the membrane surface, $\Psi(0)$, can be estimated from the linearized version of the Gouy–Chapman theory (McLaughlin, 1977, 1989; Honig et al., 1986):

$$\Psi(0) \approx \zeta \approx \sigma/(\epsilon_r\epsilon_o\kappa) \quad (4)$$

where σ is the surface charge density or charge per unit area, ϵ_r is the dielectric constant of the aqueous phase, ϵ_o is the permittivity of free space, and $1/\kappa$ is the Debye length ($1/\kappa \approx 1$ nm in 0.1 M salt). Equation 4 also describes the potential difference between the two parallel plates of a capacitor separated by a distance $1/\kappa$.

The surface charge density of the membrane, σ, is

$$\sigma \approx eF(-\{PG\} + z\{P_m\}) \quad (5)$$

where $\{P_m\}$ is the surface concentration of bound peptide, {PG} (or {PS}) is the surface concentration of acidic lipid, z is the peptide valence, e is the electronic charge, and F is the Faraday constant. The partition coefficient K_2 relates the surface concentration of peptide, $\{P_m\}$ (in units of moles of peptide per area), to its concentration in bulk aqueous phase, [P] (in units of M): $\{P_m\} = K_2[P]$. K_2, expressed in units of length, corresponds to the distance one must move from a unit area of membrane to obtain a volume of solution that contains the same number of peptides that are bound to the membrane. A phospholipid occupies an area of about 0.7 nm^2 (Shipley, 1973; Rand, 1981; McIntosh et al., 1989); if the units of K_1 are M^{-1}, and the units of K_2 are nm, then

$$K_1 = 0.42K_2 \quad (6)$$

Combining eqs 4 and 5, we calculate

$$\zeta \approx (eF/\epsilon_r\epsilon_o\kappa)(-\{PG\} + zK_2[P]) = a(-\{PG\} + zK_2[P]) \quad (7)$$

Equation 7 contains several approximations [see McLaughlin (1989)]; to compensate for these approximations, we replace $eF/\epsilon_r\epsilon_o\kappa$ with the constant a, which we determine experimentally from ζ when [P] = 0. We then calculate K_2 from

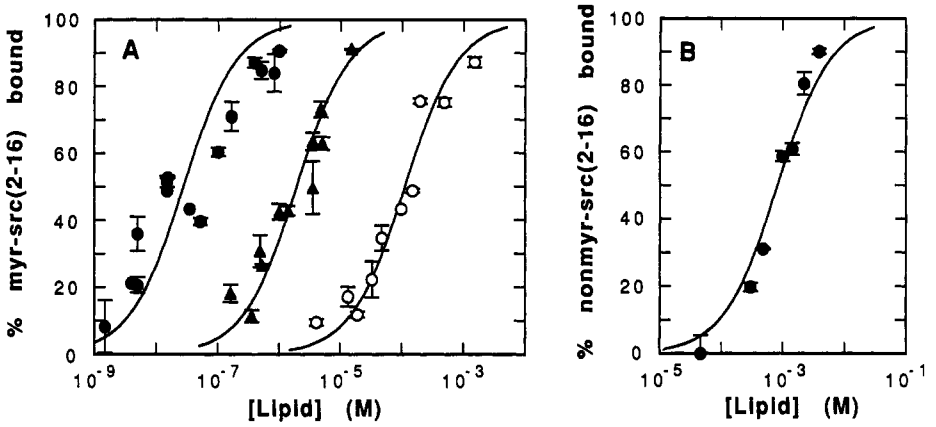

FIGURE 1: Membrane binding of myristylated (A) and nonmyristylated (B) peptides corresponding to residues 2–16 of Src (Gly-Ser-Ser-Lys-Ser-Lys-Pro-Lys-Asp-Pro-Ser-Gln-Arg-Arg-Arg). The results were obtained with a sucrose-loaded vesicle assay using LUVs formed from (○) PC, (▲) 5:1 PC/PG, and (●) 2:1 PC/PG in 0.1 M KCl buffered to pH 7.0 with 1 or 10 mM MOPS. [lipid] = accessible lipid concentration = one-half the total lipid concentration. Error bars represent the standard deviations of duplicate measurements at the indicated lipid concentration. The data were fit with eq 2 to determine the partition coefficient, K_1 (Table 1). Replacement of PG with a different monovalent acidic lipid, PS, did not affect the membrane binding of myr-src(2–16), and data from both PC/PG and PC/PS lipid mixtures are included in part A.

measured values of [P] and ζ, using eq 7 and concentrations of P that produce only small $\Delta\zeta$.

Circular Dichroism (CD). Spectra were recorded on a J-20 spectropolarimeter (JASCO, Tokyo, Japan) using a 1 mm cylindrical cell. Small unilamellar vesicles (SUVs) containing either PC or PG were prepared by ultrasonication as described by Barenholz et al. (1977), in buffer containing 0.1 M KCl, 2.5 mM K_2HPO_4, and 2.5 mM KH_2PO_4 (pH 7). Aqueous solutions containing 20 μM myr-src(2–12) or myr-src(2–16) in this buffer were prepared from stock peptide solutions in H_2O, since DMSO interfered with our CD measurements.

RESULTS

The myr-src(2–16) peptide contains three Lys, three Arg, and one Asp residues and thus has a net charge of + 5 in a pH 7 solution. Figure 1 illustrates our three most important points. First, myr-src(2–16) binds to electrically neutral PC LUVs with a molar partition coefficient $K_1 = 10^4\ M^{-1}$ (Figure 1A). This binding arises from the hydrophobic insertion of the myristate into the bilayer (Peitzsch & McLaughlin, 1993; Silvius & l'Heureux, 1994). The reciprocal of the molar partition coefficient, $1/K_1 = 10^{-4}$ M, can be regarded as an apparent dissociation constant that is equal to the accessible lipid concentration that binds 50% of the peptide.

Second, the corresponding nonmyristylated peptide, nonmyr-src(2–16), does not bind significantly to PC vesicles (data not shown), but does bind to 2:1 PC/PG vesicles, with $K_1 = 1.2 \times 10^3\ M^{-1}$ (Figure 1B). Thus, the binding mechanism of the nonmyristylated peptide differs from peptides that form an amphipathic helix and strongly adsorb to PC vesicles (e.g., melittin; Kuchinka & Seelig, 1989). The binding of nonmyr-src(2–16) to membranes containing acidic lipids can be explained by an electrostatic interaction between basic residues and acidic lipids comparable to that observed with the well-characterized basic peptide, penta(lysine) (de Kruijff et al., 1985; Roux et al., 1988; Kim et al., 1991; Mosior & McLaughlin, 1992a,b). Both Lys_5 and nonmyr-src(2–16) have a net charge of +5 and bind with a partition coefficient of $K_1 \approx 10^3\ M^{-1}$ to 2:1 PC/PG membranes in 0.1 M KCl (Mosior & McLaughlin, 1992a).[2]

Third, the combination of myristate insertion and electrostatic interaction between basic residues and acidic lipids results in a strong membrane association of myr-src(2–16). Figure 1A shows that myr-src(2–16) binds 3 orders of magnitude more strongly to membranes upon incorporation of 33% acidic lipids (Figure 1A and Table 1). Parallel studies of the intact c-Src protein show an identical 3 orders of magnitude increase in the membrane binding of the protein upon incorporation of 33% acidic lipids (Sigal et al., 1994). We obtained comparable results for myr-src peptide binding with the monovalent acidic lipids PG and PS (Figure 1), a result consistent with our hypothesis that the interaction between basic residues and acidic lipids is due to electrostatics. Moreover, the addition of PIP_2 (2% to 5:1 PC/PS vesicles), a lipid that has specific interactions with some proteins, e.g., profilin (Machesky et al., 1990; Goldschmidt-Clermont et al., 1990) and the δ isoform of phospholipase C (Rebecchi et al., 1992), did not affect the binding of myr-src(2–16) (data not shown).

The important conclusion from these data is that the binding of myr-src(2–16) arises from the synergism between the hydrophobic insertion of the myristate and the electrostatic association of basic residues and acidic lipids. This synergism is illustrated by noting that, for a lipid concentration of 10^{-6} M, 2:1 PC/PG vesicles bind >90% myr-src(2–16), while neutral PC vesicles bind <1% myr-src(2–16) and 2:1 PC/PG vesicles bind <1% nonmyr-src(2–16). Using the simple model presented in the Discussion section, the overall binding of the myr-src peptide can be estimated by multiplying the partition coefficients (or adding the binding energies) for the individual peptide–membrane interactions, each of which is well-understood from first principles.

[2] Extensive studies with basic peptides (e.g., Lys_n, n = 2–5) show that their binding to membranes can be described by a Gouy–Chapman/mass action model, assuming that the binding energy between basic residues (Lys or Arg) and an acidic lipid (PS or PG) is about 1 kcal/mol (Kim et al., 1991; Mosior & McLaughlin, 1992a,b). Both Lys_5 [Figures 1 in Mosior and McLaughlin (1992a,b)] and nonmyr-src(2–16) (not shown) exhibit a sigmoidal dependence of binding on the mole percent of acidic lipid: the data obtained with these two peptides are identical within experimental error. This sigmoidicity arises from two factors: the electrostatic potential produced by the acidic lipids and the reduction in dimensionality that occurs when the first basic residue interacts with an acidic lipid [discussed in detail by Mosior and McLaughlin (1992b)]. As predicted by the Gouy–Chapman theory, the binding of both nonmyr-src(2–16) and Lys_5 increases when the salt concentration decreases [see Table 1 for nonmyr-src(2–16) in 0.02, 0.1, and 0.5 M KCl and Mosior and McLaughlin (1992a) for Lys_5]. Thus, the electrostatic interaction of nonmyr-src(2–16) with membranes containing acidic lipids is well characterized and well understood.

Table 1: Summary of Binding Constants for Peptides Related to the NH_2-Terminal Sequence of Src

peptide	charge	lipid composition	K_1 (M^{-1}) 0.1 M KCl[a]	0.1 M KCl[b]	0.02 M KCl[a]	0.5 M KCl[a]
myr-src(2–16)	+5	PC	$9.0 \times 10^3 \pm 1.1 \times 10^3$			
		PC/PG (5:1)	$5.1 \times 10^5 \pm 6.8 \times 10^4$		$3.1 \times 10^5 \pm 8.9 \times 10^4$	$2.8 \times 10^4 \pm 4.0 \times 10^3$
		PC/PG (2:1)	$3.6 \times 10^7 \pm 8.8 \times 10^6$			
nonmyr-src(2–16)	+5	PC/PG (2:1)	$1.2 \times 10^3 \pm 1.5 \times 10^2$		$1.0 \times 10^4 \pm 2.5 \times 10^4$	$2.1 \times 10^2 \pm 1.5 \times 10^2$
myr-src(2–12)	+2	PC	$8.5 \times 10^3 \pm 8.8 \times 10^2$			
		PC/PG (10:1)	$3.4 \times 10^4 \pm 2.5 \times 10^3$	3×10^4		
		PC/PG (5:1)	$6.8 \times 10^4 \pm 3.3 \times 10^3$	8×10^4		
		PC/PG (2:1)	$2.7 \times 10^5 \pm 2.1 \times 10^4$	4×10^5		
nonmyr-src(2–12)	+2	PC/PG (2:1)	$7.1 \times 10^1 \pm 1.3 \times 10^1$			
myr-src(2–12,Y^{COO^-})	+1	PC	$6.0 \times 10^3 \pm 2.9 \times 10^2$			
		PC/PG (10:1)	$4.5 \times 10^4 \pm 7.9 \times 10^3$	2×10^4		
		PC/PG (5:1)	$7.8 \times 10^4 \pm 3.1 \times 10^3$	1×10^5		
		PC/PG (2:1)	$1.5 \times 10^5 \pm 5.8 \times 10^3$	4×10^5		
myr-src(RRR,Y^{COO^-})	+1	PC/PG (2:1)		4×10^5		
myr-src(NKN,Y^{COO^-})	−1	PC	$2.4 \times 10^3 \pm 2.5 \times 10^2$			
		PC/PG (2:1)	$2.8 \times 10^3 \pm 1.5 \times 10^2$			

[a] Binding constants were determined from the sucrose-loaded vesicle-binding assay (see Materials and Methods for details). [b] Binding constants were determined from ζ potential measurements (see Materials and Methods for details).

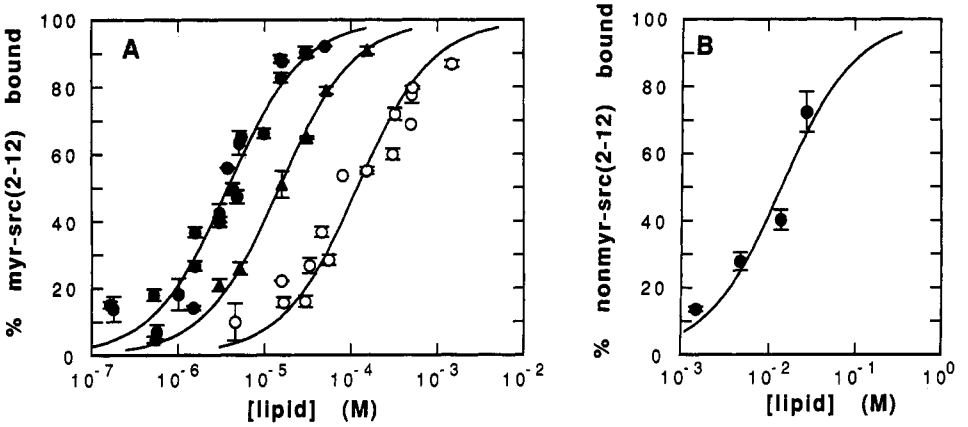

FIGURE 2: Membrane binding of myristylated (A) and nonmyristylated (B) peptides corresponding to residues 2–12 of Src. The data were obtained and analyzed as described for Figure 1; symbols are as defined for Figure 1. Again, substitution of PS for PG did not affect membrane binding of myr-src(2–12), and data from both PC/PG and PC/PS lipid mixtures are included in part A.

Earlier competitive binding measurements with myr-src peptides corresponding to residues 2–12 of Src suggested that the myristate and basic residues at positions 5, 7, and 9 are required for membrane binding of v-Src (Silverman & Resh, 1992). We studied the dependence of the electrostatic interaction on the number of basic residues by measuring the membrane binding of related peptides containing only the first 12 amino acids of Src. Figure 2A,B shows the binding of myr- and nonmyr-src(2–12)s to LUVs containing 0%, 17%, and 33% acidic phospholipids. The results are as expected. The binding of myr-src(2–12) to electrically neutral PC vesicles (Figure 2A) is the same as for myr-src(2–16) (Figure 1A). The corresponding nonmyristylated peptide, nonmyr-src(2–12), does not bind to PC vesicles (not shown), but does bind to 2:1 PC/PG vesicles (Figure 2B). The contribution of the electrostatic interaction to membrane binding depends on the number of basic residues: nonmyr-src(2–12), with a net charge of +2, binds less strongly than nonmyr-src(2–16), net charge +5, to 2:1 PC/PG vesicles (Table 1). The binding energy of myr-src(2–12) to 2:1 PC/PG membranes is approximately equal to the product of the partition coefficients for the insertion of the myristate (i.e., binding of myr-src(2–12) to PC vesicles) and for the electrostatic interaction (i.e., binding of nonmyr-src(2–12) to 2:1 PC/PG membranes).

Both the myristylated and nonmyristylated peptides in Figure 2 contain amino acids 2–12 of the Src sequence and have amide-blocked C-termini. The peptides used in the competitive binding experiments of Silverman and Resh (1992) also corresponded to amino acids 2–12 of Src, but these peptides contained an additional, unblocked C-terminal tyrosine residue and are denoted here as myr-src(2–12,Y^{COO^-}). Table 1 shows that myr-src(2–12,Y^{COO^-}) and myr-src(2–12) bind to membranes with approximately the same affinities.

We confirmed the results obtained using the sucrose-loaded vesicle technique (Figures 1 and 2) with electrophoretic mobility measurements, which also provide direct information about how the peptides affect the electrostatic potential close to the membrane surface, the zeta potential. The electrophoretic mobility is proportional to the zeta potential, ζ. Equation 4 shows that, under our conditions, ζ is proportional to the surface charge density of lipid vesicles and, thus, is linearly related to the number of bound peptides per unit area.

Figure 3A,B shows the zeta potential measurements for myr- and nonmyr-src(2–12) peptides, respectively. Figure 3A illustrates that in the presence of increasing concentrations of myristylated peptide, ζ (and the surface charge density of the vesicle) becomes less negative, indicating peptide binding. As expected, the concentration of myristylated peptide necessary to produce a given change in ζ (e.g., 10 mV) decreases with increasing mole percent acidic lipid, indicating stronger peptide binding to more acidic membranes. For example, 10^{-7} M myr-src(2–12) produces $\Delta\zeta \approx 10$ mV for the 2:1 PC/PG vesicles, but 5×10^{-7} M is required to produce the $\Delta\zeta \approx 10$ mV for 5:1 PC/PG vesicles. This agrees well with the

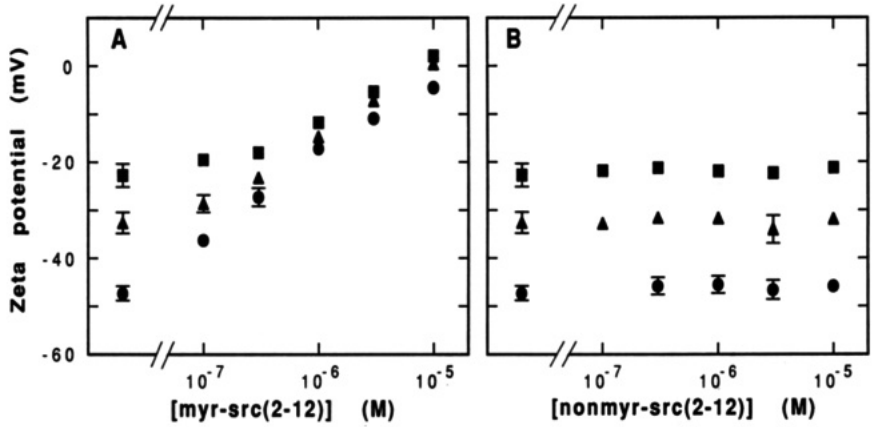

FIGURE 3: Effect of myr-src(2–12) (A) and nonmyr-src(2–12) (B) peptides on the zeta potential of multilamellar vesicles formed from mixtures of PC/PG or PC/PS at ratios of (■) 10:1, (▲) 5:1, and (●) 2:1. At least 10 measurements in two separate experiments were averaged for each data point; error bars show standard deviations. The aqueous solution contained 0.1 M KCl buffered with 1 mM MOPS to pH 7.0 at 25 °C. The symbols to the far left indicate the zeta potential of vesicles in the absence of peptide. Peptide binding to PC/PG and PC/PS lipid mixtures was equivalent, and the data were averaged.

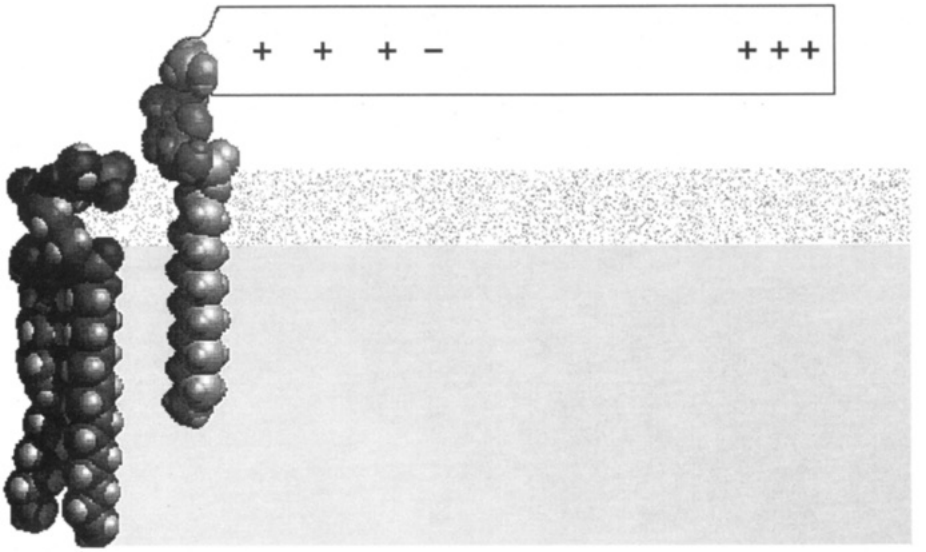

FIGURE 4: Diagram (to scale) of the interaction of the myr-src(2–16) peptide with a phospholipid bilayer containing acidic lipids. A model of a PS lipid is shown on the left. About 10 CH_2 groups of myristate penetrate into the hydrocarbon interior of the membrane (gray region), and the polar NH_2-terminal glycine remains in the aqueous phase, just outside the envelope of the polar head groups (stippled region). The rectangle illustrates the size of a random coil structure for residues 5–16 and shows the appproximate location of the charged residues.

results illustrated in Figure 2A, which show that myr-src-(2–12) binds about 5-fold more strongly to 2:1 than to 5:1 PC/PG vesicles. For peptide concentrations $\leq 10^{-5}$ M, the nonmyr-src(2–12) peptide does not bind significantly to vesicles containing 0–33% acidic lipids (Figure 3B). A comparison of columns 4 and 5 in Table 1 shows that the partition coefficients for myr-src(2–12) determined from zeta potential measurements agree well with the data from the sucrose-loaded vesicle-binding assay.

We also investigated the membrane binding of two peptides related to the myr-src(2–12,Y^{COO^-}) peptide: myr-src(R-RR,Y^{COO^-}) has the Lys residues at positions 5, 7, and 9 replaced by Arg, and myr-src(NKN,Y^{COO^-}) has the Lys residues at positions 5 and 9 substituted by Asn. Both myr-src(2–12,Y^{COO^-}) and the substituted peptide with conserved charge, myr-src(RRR,Y^{COO^-}), bind similarly to 2:1 PC/PG LUVs (Table 1). This result agrees with the binding studies of other small basic peptides, showing that Arg_5 and Lys_5, for example, bind comparably to vesicles containing acidic phospholipids (Mosior & McLaughlin, 1992a). As expected, the addition of acidic phospholipids does not increase the membrane binding of myr-src(NKN,Y^{COO^-}), net charge −1 (Table 1).

DISCUSSION

Figure 4 illustrates the simple picture that emerges from our membrane-binding studies of myristylated peptides identical or related to the NH_2 terminus of Src (Figures 1–3 and Table 1): the myristate hydrophobically inserts into the bilayer, and the basic residues electrostatically interact with acidic lipids in the membrane. As discussed in detail elsewhere (Peitzsch & McLaughlin, 1993), our results are consistent with those obtained using several different techniques, which suggest that about 10 CH_2 groups of myristate penetrate into the hydrocarbon interior of the membrane and the polar NH_2-terminal glycine residue of the myristylated peptide remains in the aqueous phase, immediately outside the envelope of the polar head group region. Circular dichroism measurements suggest that myr-src(2–12) and myr-src(2–16) are random coils in aqueous solution, in methanol, bound to PC vesicles, and bound to PG vesicles (data not shown). The increased binding observed with peptides containing a larger number of basic residues [e.g., myr-src(2–16) vs myr-src(2–12)] implies that the peptide lies parallel to the membrane surface, with its basic residues interacting with the acidic lipids. Our experiments provide strong evidence that this interaction is

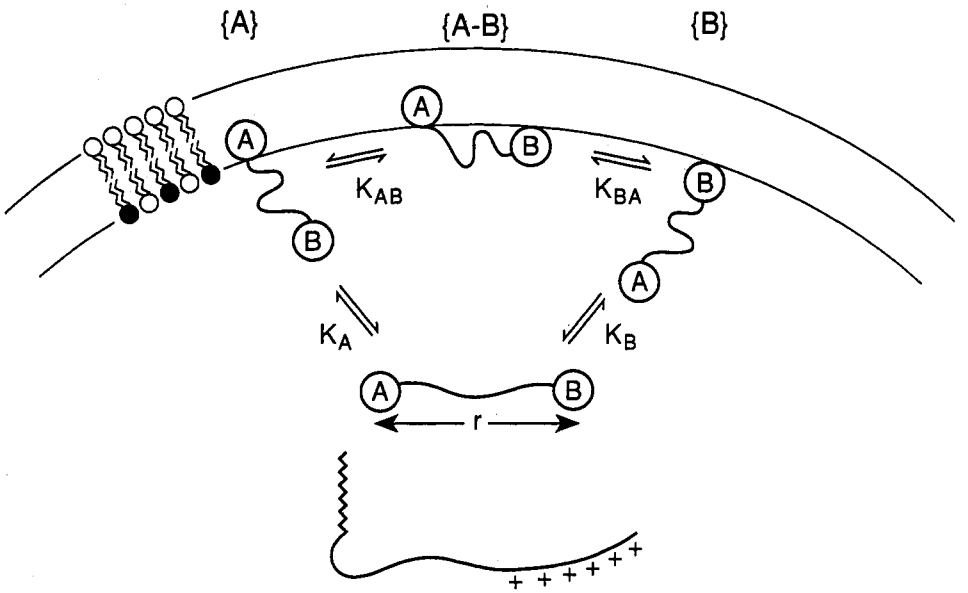

FIGURE 5: Theoretical ball and string model used to calculate the overall partition coefficient (K) for an acylated peptide or protein onto a membrane. The peptide is represented by the two binding sites A (acyl) and B (basic residues) joined by a flexible, electrically neutral string of length r. Symbols and binding constants are discussed in the text.

electrostatic. First, the membrane association of both myr- and nonmyr-src peptides increases with increasing mole percent acidic lipids (Figure 1A, Table 1, and footnote 2). Second, membrane binding depends on the number of basic residues in the peptide (Table 1). Third, binding is independent of the chemical nature of both the basic residues (Lys vs Arg; Table 1) and the acidic lipids (PG vs PS). Finally, the membrane association of nonmyr-src(2–16) increases when the ionic strength of the solution decreases (Table 1).

If we know the individual binding energies for the hydrophobic insertion of the myristate and for the electrostatic interaction between basic residues and acidic lipids, can we predict the synergism between the two binding motifs? To address this question, we consider the ball and string model illustrated in Figure 5, which assumes that the peptide, P, has two binding sites joined by a thin, electrically neutral, flexible string of length r: one binding site is the myristate (A for acyl) and the other is the cluster of basic residues (B for basic). The partition coefficient K_A, determined from binding measurements of myr-src(2–16) to neutral PC membranes, describes tethering of the peptide to the membrane by insertion of the myristate. The partition coefficient K_B, determined from binding measurements of nonmyr-src(2–16) to membranes containing 33% acidic phospholipids, describes the interaction of the basic residues on the peptide with the acidic lipids. K_{AB} describes the binding of the basic region when the myristate is already bound, and K_{BA} describes the binding of the myristate when the basic region is already bound.

Equation 8 describes the overall binding of a peptide to a membrane when $K_A > K_B$ (or $\{A–B\} + \{A\} > \{B\}$), which is true for the myr-src peptides used in our experiments:

$$K = (\{A–B\} + \{A\})/[P] \quad (8)$$

where $\{A\}$, $\{B\}$, or $\{A–B\}$ is the surface concentration of bound peptide (moles/area), [P] is the concentration of free peptide (M), and K is a partition coefficient expressed in eq 8 in units of distance. Equation 8 can also be written in terms of the other partition coefficients ($K_A = \{A\}/[P]$, $K_B = \{B\}/[P]$, and $K_{AB} = \{A–B\}/\{A\}$):

$$K = K_A(1 + K_{AB}) \quad (9)$$

To rewrite eq 9 in terms of the experimentally determined partition coefficients K_A and K_B, we consider a single molecule tethered to the membrane by the myristate. The basic region B is confined to a hemisphere of volume $V = (2/3)\pi r^3$ and exposed to an area of the membrane $a = \pi r^2$. The surface to volume ratio is given by

$$a/V = 3/(2r) \quad (10)$$

We assume that the basic residues on the tethered and free peptides have the same spatial distribution in the diffuse double layer.[3] Applying eq 9 to the ensemble of myristyl-tethered peptides, and assuming the density of peptides at the membrane surface is low (i.e., no interaction between bound peptides), we can describe the surface concentration of peptide bound by both the myristate and basic residues:

$$\{A–B\} = K_B\{A\}(a/V) \quad (11)$$

Combining eqs 9–11, we can rewrite the expression for the overall binding constant for the peptide with the membrane in terms of the observed binding constants K_A and K_B:

$$K = K_A[1 + 3K_B/2r] \quad (12)$$

where r and K_B are in units of distance (see eq 6) and K is in the same units as K_A.

Thus, in our model, the tethering of the peptide by the insertion of its myristate confines the cluster of basic residues to a small hemisphere of radius r, reducing the sampling volume of B. A direct result of this reduction in dimensionality (Adam & Delbrück, 1968; Berg & Purcell, 1977; Berg & von Hippel, 1985; Mosior & McLaughlin, 1992b; Axelrod & Wang, 1994) is that once A binds, B is confined to a region of high lipid concentration, increasing the probability that it, too, will bind to the membrane. The same conceptual point has been made previously for multivalent antigen–antibody interactions (Crothers & Metzger, 1972; Reynolds, 1979). The reduction

[3] While this assumption is not necessarily true for the src peptides and protein since r ($\approx$1 nm) is comparable to the Debye length (1 nm in 0.1 M salt), the concentrating effect of the diffuse double layer is incorporated in the experimentally determined binding constant, K_B.

in dimensionality produces an apparent cooperativity for the two independent peptide–membrane interactions. Table 1 lists the experimentally determined binding constants K_A (binding of the myr-src peptide to neutral PC membranes) and K_B (binding of the nonmyr-src peptide to 2:1 PC/PG membranes). If we assume r = 1 nm, the overall partition coefficient calculated for the binding of myr-src(2–16) to 2:1 PC/PG membranes ($K = 4 \times 10^7$ M^{-1}; eq 12) agrees surprisingly well with the measured value ($K_1 = 4 \times 10^7$ M^{-1}; Table 1). Using the same approach, we calculated the partition coefficient for myr-src(2–12) binding of PC/PG (2:1) membranes to be 2×10^6 M^{-1}, which is within an order of magnitude of the 3×10^5 M^{-1} measured value.[4]

While this model is oversimplified, particularly its assumption that the two peptide-binding sites are single points separated by a distance r (the NH_2-terminal basic residues of Src are distributed over r), it effectively describes the synergism we observed when myristylated peptides containing basic residues bound to membranes containing acidic lipids. A similar ball and chain model has proved successful in describing the inactivation of the Shaker potassium channel (Hoshi et al., 1990). It is important to note that the *product* of the partition coefficients for the two individual interactions, scaled by the distance between the two regions, predicts the strong binding of myr-src peptides to membranes with an acidic lipid concentration similar to that of the inner surface of the plasma membrane.

Our binding measurements with peptides that mimic the NH_2 terminus of Src (Figure 1) and parallel results obtained with the intact Src protein (Sigal et al., 1994) indicate that the myristate and NH_2-terminal basic residues can firmly anchor Src to membranes containing acidic lipids. Thus, the lipids in the cytoplasmic surface of a biological membrane can act as a nonspecific Src receptor; the specific localization of v-Src in the plasma and perinuclear membranes or c-Src in the endosomal membrane [see Resh (1993)], however, implies that Src also interacts with other membrane-bound proteins. For example, the Src SH2 group binds to the phosphotyrosine-containing peptide pYEEI with an association constant of 10^7 M^{-1} (Marengere et al., 1994). If there is one intrinsic v-Src receptor protein with this affinity for Src per 10^5 lipids in the plasma membrane (10^4 receptors per spherical 10 μm radius cell), the concentration of receptors in the surface phase of a few nanometers thickness is 10^{-5} M. Our model predicts that these receptors would produce a $(10^7$ M$^{-1})(10^{-5}$ M) = 100-fold increase in the binding of Src to the plasma membrane. Furthermore, it is easy to show that our model predicts significant binding of Src to the protein receptor to occur only if Src is in the surface phase, which in turn requires the interaction of both the myristate and the cluster of basic residues with lipids.

While our results specifically address the synergistic relationship between the membrane binding of myristate and basic residues in Src, this phenomenon appears to be more general. More than 100 proteins are now known to be permanently modified by either NH_2-terminal myristylation or C-terminal farnesylation. The myristate (Peitzsch & McLaughlin, 1993) and farnesyl (Silvius & l'Heureux, 1994) groups bind with identical hydrophobic energy that is not sufficient to strongly anchor a protein to a membrane. Thus, one or more additional factors are necessary to stably anchor a myristylated or farnesylated protein to the lipid bilayer. Our study of myr-src peptides and the intact Src protein (Sigal et al., 1994) identifies a second binding motif in Src: six basic residues at the NH_2 terminus that bind to the lipid bilayer via electrostatic interaction with acidic phospholipids. The combination of myristate or farnesyl and basic residues appears to be a more general motif and is important for the membrane binding of several other proteins, including MARCKS (Kim et al., 1994), HIV-gag (Zhou et al., 1994), K-ras (Hancock et al., 1990; Magee et al., 1992), and mutant Raf (Leevers et al., 1994; Stokoe et al., 1994). Other motifs that can produce synergistic binding when combined with myristylation or farnesylation include palmitylation of cysteine residues, e.g., $p56^{lck}$, $p59^{lyn}$, α-subunits of heterotrimeric G proteins, and H-ras [see Resh (1994)], and exposure of the hydrophobic residues of proteins produced by a change in conformation, e.g., adenosine ribosylation factor (Serafini et al., 1991; Walker et al., 1992; Haun et al., 1993; Randazzo et al., 1993) and recoverin (Zozulya & Stryer, 1992; Dizhoor et al., 1992, 1993).

[4] In our model for Src and the Src peptides, the overall partition coefficient can be approximated by the product of the individual partition coefficients (in units of M^{-1}). However, this is a special case in which r ($\approx$1 nm) is approximately equal to the conversion factor used in changing a partition coefficient from units of M^{-1} to units of nm (see eq 6). Note that in the limit $r \rightarrow 0$, eq 12 predicts $K \rightarrow \infty$; however, in this limit, the number of binding sites approaches 1. Thus, eq 12 is limited to $r >$ molecular dimensions.

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REFERENCES

Adam, G., & Delbrück, M. (1968) in *Structural Chemistry and Molecular Biology* (Rich, A., & Davidson, N., Eds.) pp 198–215, W. H. Freeman & Co., San Francisco.

Axelrod, D., & Wang, M. D. (1994) *Biophys. J. 66*, 588–600.

Barenholz, Y., Gibbes, D., Litman, B. J., Goll, J., Thompson, T. E., & Carlson, F. D. (1977) *Biochemistry 16*, 2806–2810.

Berg, H. C., & Purcell, E. M. (1977) *Biophys. J. 20*, 193–219.

Berg, O. G., & von Hippel, P. H. (1985) *Annu. Rev. Biophys. Biophys. Chem. 14*, 131–160.

Buss, J. E., Kamps, M. P., & Sefton, B. M. (1984) *Mol. Cell. Biol. 4*, 2697–2704.

Cafiso, D., McLaughlin, A., McLaughlin, S., & Winiski, A. (1989) *Methods Enzymol. 171*, 342–364.

Courtneidge, S. A., Levinson, A. D., & Bishop, J. M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3783–3787.

Cross, F. R., Garber, E. A., Pellman, D., & Hanafusa, H. (1984) *Mol. Cell. Biol. 4*, 1834–1842.

Cross, F. R., Garber, E. A., & Hanafusa, H. (1985) *Mol. Cell. Biol. 5*, 2789–2795.

Crothers, D. M., & Metzger, H. (1972) *Immunochemistry 9*, 341–357.

Deichaite, I., Casson, L. P., Ling, H.-P., & Resh, M. D. (1988) *Mol. Cell. Biol. 8*, 4295–4301.

de Kruijff, B., Rietveld, A., Tleders, N., & Vaandrager, B. (1985) *Biochim. Biophys. Acta 820*, 295–304.

Devaux, P. F. (1992) *Annu. Rev. Biophys. Biomol. Struct. 21*, 417–439.

Dizhoor, A. M., Ericsson, L. H., Johnson, R. S., Kumar, S., Olshevskaya, E., Zozulya, S., Neubert, T. A., Stryer, L., Hurley, J. B., & Walsh, K. A. (1992) *J. Biol. Chem. 267*, 16033–16036.

Dizhoor, A. M., Chen, C. K., Olshevskaya, E., Sinelnikova, V. V., Philipov, P., & Hurley, J. B. (1993) *Science 259*, 829–832.

Doberstein, S. K., & Pollard, T. D. (1992) *J. Cell Biol. 117*, 1241–1249.
Duronio, R. J., Towler, D. A., Heucheroth, R. O., & Gordon, J. I. (1989) *Science 243*, 796–800.
Garber, E. A., & Hanafusa, H. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 80–84.
Garber, E. A., Cross, F. R., & Hanafusa, H. (1985) *Mol. Cell. Biol. 5*, 2781–2788.
Goddard, C., Arnold, S. T., & Felsted, R. L. (1989) *J. Biol. Chem. 264*, 15173–15176.
Goldschmidt-Clermont, P. J., Machesky, L. M., Baldassare, J. J., & Pollard, T. D. (1990) *Science 247*, 1575–1578.
Hancock, J. F., Paterson, H., & Marshall, C. J. (1990) *Cell 63*, 133–139.
Haun, R. S., Tsai, S. C., Adamik, R., Moss, J., & Vaughan, M. (1993) *J. Biol. Chem. 268*, 7064–7068.
Honig, B. H., Hubbell, W. L., & Flewelling, R. F. (1986) *Annu. Rev. Biophys. Biophys. Chem. 15*, 163–193.
Hope, M. J., Bally, M. B., Webb, G., & Cullis, P. R. (1985) *Biochim. Biophys. Acta 812*, 55–65.
Hoshi, T., Zagotta, W. N., & Aldrich, R. W. (1990) *Science 250*, 533–538.
Hunter, R. J. (1981) *Zeta Potential in Colloid Science: Principles & Applications*, Academic Press, New York.
Kamps, M. P., Buss, J. E., & Sefton, B. M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4625–4628.
Kaplan, J. M., Mardon, G., Bishop, J. M., & Varmus, H. E. (1988) *Mol. Cell. Biol. 8*, 2435–2441.
Kaplan, J. M., Varmus, H. E., & Bishop, J. M. (1990) *Mol. Cell. Biol. 10*, 1000–1009.
Kim, J., Mosior, M., Chung, L. A., Wu, H., & McLaughlin, S. (1991) *Biophys. J. 60*, 135–148.
Kim, J., Blackshear, P. J., Johnson, J. D., & McLaughlin, S. (1994) *Biophys. J. 67*, 227–237.
Kuchinka, E., & Seelig, J. (1989) *Biochemistry 28*, 4216–4221.
Leevers, S. J., Paterson, H. F., & Marshall, C. J. (1994) *Nature 369*, 411–414.
Machesky, L. M., Goldschmidt-Clermont, P. J., & Pollard, T. D. (1990) *Cell Regul. 1*, 937–950.
Magee, A. I., Newman, C. M., Giannakouros, T., Hancock, J. F., Fawell, E., & Armstrong, J. (1992) *Biochem. Soc. Trans. 20*, 497–499.
Marengere, L. E. M., Songyang, Z., Gish, G. D., Schaller, M. D., Parsons, J. T., Stern, M. J., Cantley, L. C., & Pawson, T. (1994) *Nature 369*, 502–505.
McIntosh, T. J., Magid, A. D., & Simon, S. A. (1989) *Biochemistry 28*, 7904–7912.
McLaughlin, S. (1977) in *Current Topics in Membranes and Transport*, Vol. 9, pp 71–144, Academic Press, New York.
McLaughlin, S. (1989) *Annu. Rev. Biophys. Biophys. Chem. 18*, 113–136.
McLaughlin, S., Mulrine, N., Gresalfi, T., Vaio, G., & McLaughlin, A. (1981) *J. Gen. Physiol. 77*, 445–473.
Montich, G., Scarlata, S., McLaughlin, S., Lehrmann, R., & Seelig, J. (1993) *Biochim. Biophys. Acta 1146*, 17–24.
Mosior, M., & McLaughlin, S. (1991) *Biophys. J. 60*, 149–159.
Mosior, M., & McLaughlin, S. (1992a) *Biochemistry 31*, 1767–1773.
Mosior, M., & McLaughlin, S. (1992b) *Biochim. Biophys. Acta 1105*, 185–187.
Mui, B. L.-S., Cullis, P. R., Evans, E. A., & Madden, T. D. (1993) *Biophys. J. 64*, 443–453.
Newton, A. C. (1993) *Annu. Rev. Biophys. Biomol. Struct. 22*, 1–25.
Op den Kamp, J. A. F. (1979) *Annu. Rev. Biochem. 48*, 47–71.
Parsons, J. T., & Weber, M. J. (1989) *Curr. Top. Microbiol. Immunol. 147*, 80–127.
Peitzsch, R. M., & McLaughlin, S. (1993) *Biochemistry 32*, 10436–10443.
Pellman, D., Garber, E. A., Cross, F. R., & Hanafusa, H. (1985a) *Nature 314*, 374–377.
Pellman, D., Garber, E. A., Cross, F. R., & Hanafusa, H. (1985b) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1623–1627.
Pollard, T. D., Doberstein, S. K., & Zot, H. G. (1991) *Annu. Rev. Physiol. 53*, 653–681.
Rand, R. P. (1981) *Annu. Rev. Biophys. Bioeng. 10*, 277–314.
Randazzo, P. A., Yang, Y. C., Rulka, C., & Kahn, R. A. (1993) *J. Biol. Chem. 268*, 9555–9563.
Rebecchi, M. J., Peterson, A. A., & McLaughlin, S. (1992) *Biochemistry 31*, 12742–12747.
Resh, M. D. (1990a) *Methods 1*, 264–268.
Resh, M. D. (1990b) *Oncogene 5*, 1437–1444.
Resh, M. D. (1993) *Biochim. Biophys. Acta 1155*, 307–322.
Resh, M. D. (1994) *Cell 76*, 411–413.
Resh, M. D., & Ling, H.-p. (1990) *Nature 346*, 84–86.
Reynolds, J. A. (1979) *Biochemistry 18*, 264–269.
Roux, M., Neumann, J.-M., Bloom, M., & Devaux, P. F. (1988) *Eur. Biophys. J. 16*, 267–273.
Serafini, T., Orci, L., Amherdt, M., Brunner, M., Kahn, R. A., & Rothman, J. E. (1991) *Cell 67*, 239–253.
Shipley, G. G. (1973) in *Biological Membranes* (Chapman, D., & Wallach, D. F. H., Eds.) Vol. 2, pp 1–89, Academic Press, New York.
Sigal, C. T., & Resh, M. D. (1993) *Mol. Cell. Biol. 13*, 3084–3092.
Sigal, C. T., Zhou, W., Buser, C. A., McLaughlin, S., & Resh, M. D. (1994) *Proc. Natl. Acad. Sci. U.S.A.* (in press).
Silverman, L., & Resh, M. D. (1992) *J. Cell Biol. 119*, 415–425.
Silvius, J. R., & l'Heureux, F. (1994) *Biochemistry 33*, 3014–3022.
Stokoe, D., Macdonald, S. G., Cadwallader, K., Symons, M., & Hancock, J. F. (1994) *Science 264*, 1463–1467.
Taniguchi, H., & Manenti, S. (1993) *J. Biol. Chem. 268*, 9960–9963.
Walker, M. W., Bobak, D. A., Tasi, S. C., Moss, J., & Vaughan, M. (1992) *J. Biol. Chem. 267*, 3230–3235.
Weigele, M., DeBernarde, S. L., Tengi, J. P., & Leimgruber, W. (1972) *J. Am. Chem. Soc. 94*, 5927–5928.
Zheng, J., Knighton, D. R., Xuong, N.-H., Taylor, S. S., Sowadski, J. M., & Eyck, L. F. T. (1993) *Protein Sci. 2*, 1559–1573.
Zhou, W., Parent, L. J., Wills, J. W., & Resh, M. D. (1994) *J. Virol. 68*, 2556–2569.
Zozulya, S., & Stryer, L. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 11569–11573.