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Binding of Basic Peptides to Membranes Produces Lateral Domains Enriched in the Acidic Lipids Phosphatidylserine and Phosphatidylinositol 4,5-Bisphosphate: An Electrostatic Model and Experimental Results

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ABSTRACT   Direct fluorescence digital imaging microscopy observations demonstrate that a basic peptide corresponding to the effector region of the myristoylated alanine-rich C kinase substrate (MARCKS) self-assembles into membrane domains enriched in the acidic phospholipids phosphatidylserine (PS) and phosphatidylinositol 4,5-bisphosphate (PIP$_2$). We show here that pentalysine, which corresponds to the first five residues of the MARCKS effector region peptide and binds to membranes through electrostatic interactions, also forms domains enriched in PS and PIP$_2$. We present a simple model of domain formation that represents the decrease in the free energy of the system as the sum of two contributions: the free energy of mixing of neutral and acidic lipids and the electrostatic free energy. The first contribution is always positive and opposes domain formation, whereas the second contribution may become negative and, at low ionic strength, overcome the first contribution. Our model, based on Gouy-Chapman-Stern theory, makes four predictions: 1) multivalent basic ligands, for which the membrane binding is a steep function of the mole fraction of acidic lipid, form domains enriched in acidic lipids; domains break up at high concentrations of either 2) basic ligand or 3) monovalent salt; and 4) if multivalent anionic lipids (e.g., PIP$_2$) are present in trace concentrations in the membrane, they partition strongly into the domains. These predictions agree qualitatively with experimental data obtained with pentalysine and spermine, another basic ligand.

GLOSSARY

$A_{(i)}$ area of the ith phase ($i = h, d,$ and n) (m^2)
A_L area per one lipid (m^2)
B $= (8\epsilon_0 \epsilon_r RT C_e)^{-1/2}$ (see Eq. 1)
C capacitance/area (CV^{-1}m^{-2})
C_e bulk univalent electrolyte concentration (kmol $\cdot$ m^{-3})
C_p bulk peptide concentration (kmol $\cdot$ m^{-3})
F Faraday number (C $\cdot$ kmol^{-1})
$f_{(i)}$ fraction of acidic lipid in the ith phase (i = h, d, n)
G free energy of phase (kJ)
g free energy per unit area of a given phase (kJ $\cdot$ m^{-2})
K_p intrinsic binding constant of the peptide (m$^3 \cdot$ kmol^{-1})
N_A Avogadro's number (kmol^{-1})
R gas constant (kJ $\cdot$ kmol$^{-1} \cdot$ K^{-1})
T temperature (K)
z peptide valence
Z average or effective surface charge per acidic lipid (in units of an elementary charge)

Greek letters

ϵ_r dielectric constant of the aqueous solution
ϵ_0 dielectric permittivity of free space (F $\cdot$ m^{-1})
κ reciprocal of Debye screening length (m^{-1})
μ electrochemical potential (J $\cdot$ mol^{-1})
π surface pressure (J $\cdot$ m^{-2})
σ surface charge density (C $\cdot$ m^{-2})
θ degree of occupancy of binding sites
ψ surface potential (V)

Subscripts

(h)   homogeneous (nonseparated) membrane consisting of 1 mole of lipids
(d)   domain phase
(n)   nondomain phase of the separated membrane
e   electrostatic term
m   mixing term

INTRODUCTION

Stimulation of the calcium/phospholipid second messenger system activates protein kinase C (PKC), as reviewed by Berridge (1993) and Clapham (1995). The major PKC substrate in many cell types is the myristoylated alanine-rich C kinase substrate (MARCKS) protein (reviewed by Aderem, 1992; Blackshear, 1993). The cellular function of MARCKS is not known, but there is good evidence that it binds membranes, calmodulin, and actin in a phosphorylation-dependent manner. It appears to be involved in phagocytosis, exocytosis, and membrane trafficking. Several recent studies have focused on the mechanism by which MARCKS binds to membranes, which requires both hydrophobic insertion of the myristate into the bilayer and elec-

trostatic interaction of a cluster of basic residues in its "effector" region with acidic lipids (George and Blackshear, 1992; Taniguchi and Manenti, 1993; Kim et al., 1994a,b; Swierczynski and Blackshear, 1995, 1996; Seykora et al., 1996). Phosphorylation of MARCKS by PKC introduces negative charges into the cluster of basic residues, weakening the electrostatic interaction and producing translocation from membrane to cytoplasm in many cell types. This has been termed the "myristoyl electrostatic switch" mechanism (McLaughlin and Aderem, 1995; Bhatnagar and Gordon, 1997). Although there is general agreement that both hydrophobic and electrostatic interactions are required to anchor MARCKS to membranes, we know little about the factors that produce a nonuniform lateral distribution of this protein in biological membranes. For example, MARCKS has a punctate distribution in the membranes of macrophages (Rosen et al., 1990), and recent work indicates that these domains formed by MARCKS are localized to nascent phagosomes (Allen and Aderem, 1995). MARCKS also has a nonuniform distribution in the plasma membrane of fibroblasts (Myat et al., 1997). Many factors can contribute to the formation of domains in biological membranes, e.g., interactions with cytoskeletal proteins. To understand the physical factors that contribute to the spontaneous self-assembly of proteins into lateral domains, studies on model systems are useful.

The MARCKS(151–175) peptide (KKKKKRFSFKKS-FKLSGFSFKKNKK), which corresponds to the effector region of bovine MARCKS, forms lateral domains when it binds to phospholipid vesicles formed from a mixture of the acidic lipid phosphatidylserine (PS) and the zwitterionic, electrically neutral lipid phosphatidylcholine (PC); these domains are enriched in PS (Yang and Glaser, 1995). The multivalent acidic lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) is also concentrated in the domains if it is present in the vesicles, but phospholipase C, the enzyme that hydrolyzes PIP_2, is not (Glaser et al., 1996). This lateral separation of enzyme and substrate decreases the rate of PLC-catalyzed hydrolysis of PIP_2. Upon phosphorylation of MARCKS(151–175) by PKC, the peptide (and the intact MARCKS protein) desorbs from the membrane (Kim et al., 1994a,b), and PIP_2 is hydrolyzed rapidly by PLC (Glaser et al., 1996).

We would like to understand the molecular mechanism by which MARCKS(151–175) forms domains enriched in acidic lipids when it adsorbs to phospholipid vesicles. Unfortunately, three factors make analysis of domains difficult, even in a model system that comprises only phospholipid vesicles and MARCKS(151–175) peptide. First, this peptide is amphipathic (it contains 5 Phe hydrophobic groups and 13 basic residues) and could self-aggregate either in solution or when adsorbed to a vesicle; thus peptide-peptide interactions could contribute to domain formation. Second, the peptide penetrates the polar headgroup region of the membrane when it binds (Qin and Cafiso, 1996; Glaser et al., 1996), because of the five Phe residues; Wimley and White (1996) have shown that a Phe residue can con-

tribute 1 kcal/mol to the binding energy of a peptide when it partitions into the interface. It has been argued that membrane penetration contributes to the domain formation observed with another amphipathic molecule, polymyxin (Hartmann et al., 1978; Sackmann, 1978). Third, MARCKS(151–175) binds to membranes in an extended form and may be represented as a rod ∼100 Å long (Qin and Cafiso, 1996). Onsager's (1949) elegant analysis shows that entropic effects can cause long rods to separate into ordered (concentrated) and disordered (dilute) phases in three dimensions; this effect may contribute to domain formation when long peptides adsorb to the two-dimensional surface of a membrane. These three factors may contribute to domain formation with the MARCKS effector region peptide, and we postulate that a fourth factor, electrostatics, also contributes significantly to the formation of lateral domains when basic peptides bind to membranes.

To investigate this possibility, we studied pentalysine (Lys_5, or KKKKK), a basic peptide that corresponds to the first five residues of bovine MARCKS(151–175). There are three advantages to using this peptide: first, it does not self-aggregate in solution, so attractive interactions between the peptides adsorbed to membranes are almost certainly negligible; second, NMR, surface potential, and surface pressure measurements show that Lys_5 does not penetrate the membrane when it binds (Roux et al., 1988; Kim et al., 1991; Ben Tal et al., 1996); third, because it is relatively small (dimensions ∼4 Å × 14 Å × 21 Å), the long rod effects described by Onsager (1949) do not contribute to domain formation. We show here, using fluorescence digital imaging microscopy, that when Lys_5 binds to PC/PS phospholipid vesicles, it forms domains enriched in the acidic lipid PS (and PIP_2 if the vesicles also contain PIP_2). We obtained similar results with another basic ligand, spermine.

We present a simple theoretical model that can account qualitatively for several aspects of this domain formation. Although there have been many theoretical studies of domain formation in membranes (for reviews see, e.g., Vaz, 1994; Mouritsen and Jorgensen, 1994; Thompson et al., 1995; Raudino, 1995; Sackmann, 1995), to the best of our knowledge this is the first proposal that a change in the electrostatic free energy can produce lateral domains.

Träuble (1977) discussed how domains will form if they decrease the free energy of the system. As illustrated in Fig. 1 *A*, the acidic lipids (*filled circles*) in a fluid membrane are not in domains but appear to be distributed randomly in the plane of the membrane in the absence of basic peptides. Two factors oppose the formation of domains enriched in acidic lipids (Fig. 1 *B*): first, electrostatic work must be done to bring negatively charged lipids together; second, the decrease in the entropy of mixing is unfavorable. When a basic peptide (e.g., pentalysine) binds to a PC/PS vesicle (Fig. 1 *C*), it stabilizes domains enriched in PS (Fig. 1 *D*). Our explanation for this observation hinges on the assumption that pentalysine is attracted electrostatically to the domain phase and thus is preferentially bound to this phase (*lower right-hand portion* of Fig. 1 *D*). Less pentalysine is
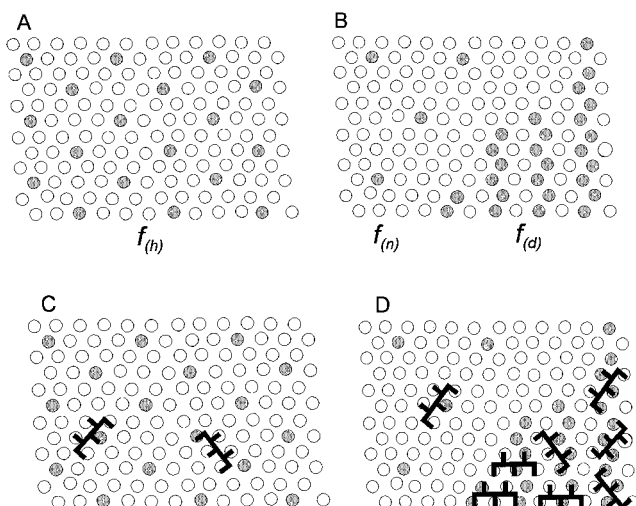
FIGURE 1 Schema for domain formation. The open circles represent zwitterionic lipids (e.g., PC), and the stippled circles represent acidic phospholipids (e.g., PS). The fractions of acidic lipids in the homogeneous membrane, domain, and nondomain phase are designated $f_{(h)}$, $f_{(d)}$, and $f_{(n)}$, respectively. In the absence of basic peptides, acidic lipids do not form domains (*A*); we assume that they are distributed randomly in the plane of the membrane. Acidic lipids will not spontaneously form domains (*B*) because the change in the Gibbs free energy due to both entropy of mixing and electrostatics is unfavorable. When pentalysine is added to the membrane (*C*), it stabilizes domains (*D*) because it binds much more strongly to regions of the membrane that contain high fractions of acidic lipids.

bound to a unit area of either the nondomain phase (*upper left-hand portion* of Fig. 1 *D*) or a nonseparated membrane (Fig. 1 *C*).

When pentalysine binds to a membrane containing acidic lipids, it reduces the net charge density and the electrostatic free energy stored in the diffuse double layer. Specifically, the negatively charged lipids in the membrane (e.g., PS) and the counterions (e.g., K^+) in the aqueous diffuse double layer adjacent to the surface may be considered as a parallel plate capacitor, at least when the surface potential, ψ, is small (McLaughlin, 1989). The Debye length, $1/\kappa$, the average distance between the surface and the counterions (~10 Å in a 0.1 M monovalent salt solution) corresponds to the distance between the plates of this capacitor. Electrical energy equal to $\frac{1}{2}C\psi^2$ is stored in a unit area of the diffuse double layer, just as it is stored in a conventional parallel plate capacitor; here $C = \epsilon_0\epsilon_r\kappa$ is the capacitance/area of the diffuse double layer, ϵ_0 is the permittivity of free space, and ϵ_r is the dielectric constant of the aqueous phase. Thus reducing the charge density and surface potential of the membrane reduces the free energy of the system. The increased binding of pentalysine to the membranes due to the formation of domains must reduce the electrostatic free energy sufficiently to overcome the increase in free energy due to an entropy of mixing term, which represents the tendency of PS to diffuse out of the domain, where its mole fraction is high (e.g., Träuble, 1977). In this report we present a quantitative, albeit highly oversimplified model of how electrostatics could drive domain formation, and then

compare the predictions of the model with the experimental results.

THEORETICAL MODEL

Outline of the model

We consider a membrane formed from a mixture of two lipids: a zwitterionic (electrically neutral) lipid such as PC and a monovalent acidic (negatively charged) lipid such as PS. The lipids have the same cross-sectional area and are distributed randomly in the initial homogeneous membrane. Upon the addition of peptide, the membrane separates into two macroscopic phases, characterized by their mole fractions of acidic lipid and surface potentials. The phase with the higher mole fraction of acidic lipid is defined as the domain phase; the phase with the lower mole fraction of acidic lipid is defined as the nondomain phase. We apply the Gouy-Chapman-Stern theory of the diffuse double layer and ligand binding to each phase, as described briefly below. Domain formation results from a decrease in the Gibbs free energy of the system, which our model treats as the sum of two contributions: the electrostatic free energy and the free energy of mixing of the acidic and zwitterionic lipids.

Although the domain and nondomain phases have different surface potentials and surface charge densities, PS and PC both must be at electrochemical equilibrium. In our model (Appendix B), the sum of three generalized forces produces the equilibrium between the acidic lipids in the two phases (Fig. 2): the generalized force resulting from a decrease in the electrostatic free energy of the system that occurs when more peptide binds to the membrane upon domain formation drives PS from the nondomain to the domain phase ($\Delta\mu_\psi$); the generalized diffusional force moves PS down its concentration (or, more correctly, mole
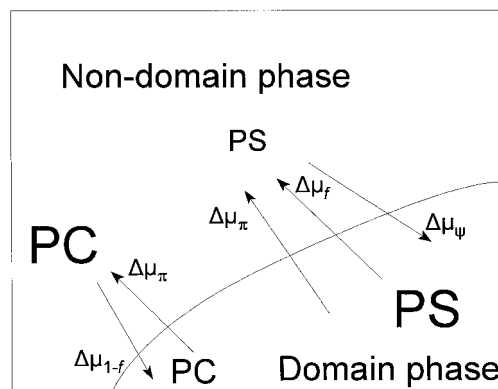

FIGURE 2 Sketch of the generalized forces that maintain equilibrium between lipids in the domain and nondomain phase. $\Delta\mu_\psi$ is the generalized electrostatic force that arises because the electrostatic free energy of the system decreases when PS moves from the nondomain to the domain phase, and pentalysine binds more strongly to the membrane; $\Delta\mu_f$ and $\Delta\mu_{1-f}$ are the generalized diffusional forces on PS and PC, respectively; and $\Delta\mu_\pi$ is the generalized force due to the surface pressure difference between the phases. See Appendix B for details.

fraction) gradient from the domain to the nondomain phase ($\Delta\mu_f$); and the force due to a difference between the surface pressure of the phases moves lipids out of the domain ($\Delta\mu_\pi$). The sum of two generalized forces produces the equilibrium between the PC in the two phases: the generalized diffusional force ($\Delta\mu_{1-f}$) directs PC from the nondomain to the domain phase, and the force due to a difference in surface pressure ($\Delta\mu_\pi$) acts in the opposite direction.

Gouy-Chapman-Stern model for ligand binding

We use the standard assumptions inherent in the Gouy-Chapman-Stern theory: 1) The homogeneous (h), domain (d), and nondomain (n) phases of the membrane are slabs of uniformly smeared surface charge, which arises from both the acidic lipids and bound peptides. For simplicity, we ignore the binding of monovalent cations to acidic lipids (McLaughlin, 1989), which does not affect the qualitative features of how pentalysine is predicted to form domains (calculations not shown). 2) The solution contains a univalent electrolyte (concentration C_e) and multivalent ions (the peptide, present at trace concentration $C_p \ll C_e$; valence $z > 1$) that are treated by using a mean field theory. 3) The electrostatic potential at the membrane surface, $\psi_{(i)}$, is described by the Gouy equation (McLaughlin, 1989):

$$\sinh\frac{F\psi_{(i)}}{2RT} = B\sigma_{(i)}; \qquad B = (8\epsilon_0\epsilon_r RTC_e)^{-1/2} \qquad (1)$$

where the subscript i designates the phase (i = h, d, or n), F is the Faraday constant, R is the gas constant, T is the temperature, $\sigma_{(i)}$ is the surface charge density, ϵ_0 is the permittivity of free space, and ϵ_r is the relative permittivity or dielectric constant of the solution. 4) The peptide concentration in the aqueous phase immediately adjacent to the membrane surface, C_p^0, is linked to its bulk concentration C_p through a Boltzmann-like relationship:

$$C_p^0 = C_p \exp(-z_{eff}F\psi_{(i)}/RT) \qquad (2)$$

where the effective valence $z_{eff} \leq z$. The use of an effective valence, which partially compensates for the discreteness of charge effects (Mosior and McLaughlin, 1992, and references therein; Heimburg and Marsh, 1995), is not required to account qualitatively for domain formation.

In addition, we assume that ligand binding to the membrane obeys either the Langmuir isotherm,

$$\theta_{(i)} = \frac{K_p C_p^0}{1 + K_p C_p^0} \qquad (3)$$

which describes 1:1 binding to localized binding sites (acidic lipids), or the Volmer isotherm, which describes nonlocalized binding of the ligand to the surface (see Appendix A); in Eq. 3 K_p is the intrinsic binding constant and $\theta_{(i)}$ is the fraction of occupied binding sites on the surface, e.g., the fraction of PS that has formed 1:1 complexes with penta-

lysine. The predictions of our model are qualitatively similar with either isotherm; for simplicity, we consider only the more familiar Langmuir isotherm in the body of this paper. The Volmer isotherm probably is more appropriate for pentalysine, however, because the available evidence suggests that this peptide does not bind specifically to acidic lipids (Ben Tal et al., 1996).

Free energy of domain formation

Domain formation at constant pressure and temperature is driven by the decrease in the Gibbs free energy of the system, ΔG:

$$\Delta G = \Delta G_m + \Delta G_e + \ldots \qquad (4)$$

We consider only two contributions to ΔG: ΔG_m, the increase in free energy of mixing resulting from the decrease in entropy of mixing when acidic and neutral lipids separate, and ΔG_e, the change in electrostatic free energy of the membrane when the two phases form.

As shown schematically in Fig. 2, one of the generalized forces that maintains the equilibrium between the domain and nondomain phases is the difference in the surface pressure between the phases. This means that Eq. 4 should include a pressure term resulting from nonelectrostatic interactions between the lipids. However, we consider the membrane as an incompressible two-dimensional fluid (i.e., the area per lipid remains constant), so no free energy will be stored because of these nonelectrostatic forces. Equation 4 should also contain other nonelectrostatic contributions to the free energy that have been discussed in the literature; see, e.g., Träuble (1977) and Raudino (1995). At low ionic strength, however, the electrostatic contribution should dominate the other terms.

We consider a membrane consisting of 1 mole of lipids having an area $A_{(h)} = N_A A_L$, where N_A is Avogadro's number and A_L is the cross-sectional area of one lipid. The fraction of acidic lipids in the membrane is $f_{(h)}$ ($0 < f_{(h)} < 1$). Upon binding of peptide, the membrane separates into two phases: the domain phase (d) has an area $A_{(d)}$ and a mole fraction of acidic lipids $f_{(d)} > f_{(h)}$; the nondomain phase (n) has an area $A_{(n)}$ and a fraction of acidic lipids $f_{(n)} < f_{(h)}$ (Fig. 1). We ignore edge effects, so the shapes of the phases are not important. Our analysis also assumes that the domains are much larger than $1/\kappa$, the Debye length.

It follows from the definitions (and the assumptions that the area per lipid, A_L, is both the same for PC and PS and constant in the h, d, and n phases) that

$$A_{(h)} = A_{(d)} + A_{(n)}; \qquad f_{(h)}A_{(h)} = f_{(d)}A_{(d)} + f_{(n)}A_{(n)} \qquad (5)$$

or, combining Eq. 5,

$$A_{(d)} = A_{(h)}\frac{f_{(h)} - f_{(n)}}{f_{(d)} - f_{(n)}}; \qquad A_{(n)} = A_{(h)}\frac{f_{(d)} - f_{(h)}}{f_{(d)} - f_{(n)}} \qquad (6)$$

Note that $f_{(n)} = f_{(h)}$ corresponds to $A_{(d)} = 0$ and $A_{(n)} = A_{(h)}$, that is, to the absence of domains.

The first term in the right-hand side of Eq. 4 is given by

$$\Delta G_m = G_{m(d)} + G_{m(n)} - G_{m(h)} \qquad (7)$$

where

$$G_{m(i)} = \frac{A_{(i)}}{A_{(h)}} RT[f_{(i)} \ln f_{(i)} + (1 - f_{(i)})\ln(1 - f_{(i)})]$$
$$(i = h, d, n) \qquad (8)$$

are the free energies of lipid mixing in each of the phases (Träuble, 1977; Hiemenz, 1986). For simplicity, we assume in Eq. 8 that only two species are present in the membrane: the zwitterionic lipid PC and the acidic lipid PS; specifically, we do not distinguish between free PS and PS bound in a 1:1 complex with a ligand. Equation 8 also assumes that PC and PS are randomly distributed in the homogeneous membrane, the domain, and the nondomain phase.

The second term in Eq. 4 can be represented in a similar form:

$$\Delta G_e = G_{e(d)} + G_{e(n)} - G_{e(h)} \qquad (9)$$

where the electrostatic free energy of each phase is calculated within the framework of Gouy-Chapman theory for a univalent electrolyte (Jähnig, 1976; Träuble et al., 1976; Cevc and Marsh, 1987):

$$G_{e(i)} = A_{(i)}\left[\sigma_{(i)}\psi_{(i)} - \frac{2RT}{FB}\left(\cosh\frac{F\psi_{(i)}}{2RT} - 1\right)\right] \qquad (10)$$

where $\sigma_{(i)}$ and $\psi_{(i)}$ are the surface charge density and surface potential of the ith phase, and the parameter B is defined in Eq. 1; $\sigma_{(i)}$ and $\psi_{(i)}$ can be calculated for each phase from Eqs. 1, 2, and either A2 or A3 in Appendix A.

Note that in the limit of low surface potential $\psi_{(i)}$, $F\psi_{(i)}/2RT \ll 1$, Eq. 10 reduces to the well-known formula for the electrical energy of a capacitor with surface charge $A_{(i)}\sigma_{(i)}$, capacitance/area C, voltage $\psi_{(i)}$, and distance between the plates equal to the thickness of the diffuse double layer, $1/\kappa$:

$$G_{e(i)} = A_{(i)}\frac{1}{2}C\psi_{(i)}^2; \qquad C = \frac{F}{2BRT} = \epsilon_0\epsilon_r\kappa \qquad (11)$$

Because the lipid vesicles used in our experiments can be regarded as macroscopic, closed, and nonisolated systems, their Gibbs free energy assumes its minimum value at equilibrium (Hill, 1960). Thus we minimized the energy ΔG calculated from Eq. 4 as a function of lipid compositions, $f_{(d)}$ and $f_{(n)}$, to obtain the theoretical results illustrated in Figs. 6 and 8.

MATERIALS AND METHODS

Vesicle preparation and fluorescence digital imaging microscopy

Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), dioleoyl-*sn*-glycero-3-phosphoserine (DOPS), and NBD-PS were obtained from Avanti Polar Lipids (Alabaster, AL). The triammonium salt of bovine brain phosphatidylinositol 4,5-bisphosphate (PIP$_2$) was from Calbiochem (San Diego, CA). NBD-PIP$_2$, synthesized as described by Chen et al. (1996), was a generous gift of Glenn Prestwich. Large unilamellar vesicles for microscopy were prepared according to the procedure of Haverstick and Glaser (1987). Each sample for microscopy contained 0.1 mM lipid vesicles, either pentalysine or spermine, and 0.5% agarose to immobilize the vesicles. Instrumentation and image processing are described by Yang and Glaser (1995).

Binding measurements

The centrifugation technique for measuring the binding of peptides, such as pentalysine to sucrose-loaded large unilamellar vesicles, was described by Ben Tal et al. (1996). Buser and McLaughlin (1997) provide additional experimental details. Pentalysine was synthesized by Multiple Peptides Systems (San Diego, CA) and was >95% pure, as determined by mass spectrographic analysis and analytical high-performance liquid chromatography. Spermine, 98% pure, was from Aldrich Chemical Co. (Milwaukee, WI).

EXPERIMENTAL RESULTS AND THEORETICAL CALCULATIONS

Fig. 3 shows how the binding of pentalysine to large unilamellar vesicles depends on the mole fraction of acidic lipid in the membrane. (In this experiment, we used phosphatidylglycerol (PG) instead of PS for technical reasons discussed by Ben Tal et al. (1996). As shown in figure 1 of Kim et al. (1991), zeta potential measurements on multilamellar vesicles show that Lys$_5$ has no specificity for PS versus PG.) Fig. 3 *A* plots the percentage peptide bound (at a constant total peptide concentration) as a function of the concentration of accessible lipid for vesicles of four different mole fractions of acidic lipid. Note that the affinity of the peptide for the vesicles increases markedly as the mole fraction of acidic lipid in the vesicles increases. Fig. 3 *B* plots the percentage of the bound pentalysine as a function of the mole fraction of acidic lipids in the membrane at one accessible lipid concentration (10^{-3} M). The filled circles illustrate that there is a steep (sigmoidal) dependence of the binding on the mole fraction of acidic lipid. We showed elsewhere that the fraction of spermine bound to a membrane is also a steep sigmoidal function of the mole fraction of acidic lipid in the membrane (Chung et al., 1985). The pentalysine data can be described satisfactorily by the Gouy-Chapman-Stern theory, as illustrated by the two curves in Fig. 3 *B*. These curves show two sets of the parameters z_{eff} and K_p that describe the data adequately. Note that when the peptide concentration is low, which it is in these experiments, the fit does not depend on the use of the Langmuir or Volmer isotherm, because at low $\theta_{(h)}$, both isotherms reduce to Henry's law. We found that using either isotherm (with any parameter set that describes the data in Fig. 3 *B*) leads to qualitatively similar predictions regarding domain formation. We use a Langmuir isotherm and assume that $z_{eff} = 2.5$ and $K_p = 20$ M^{-1} in most calculations shown here.
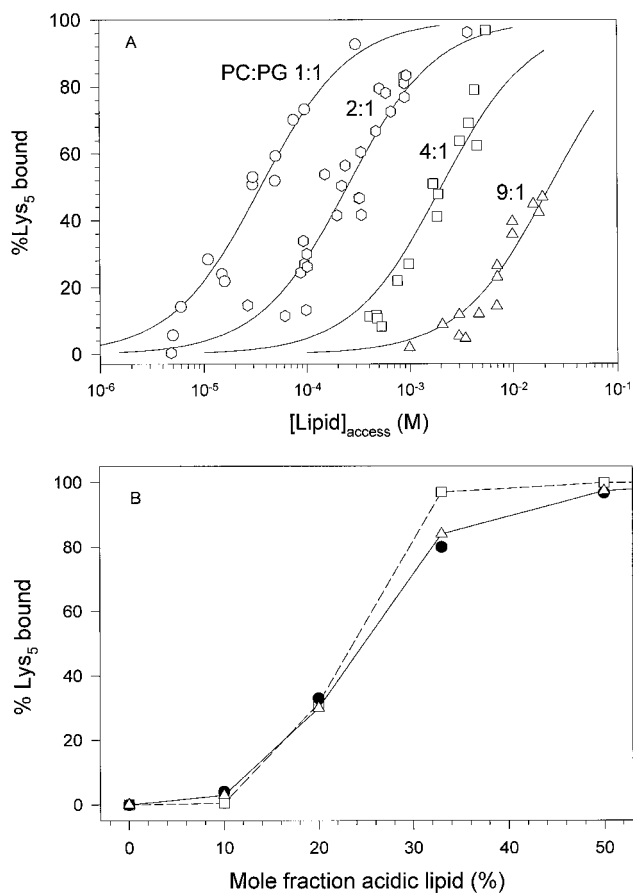
FIGURE 3 (*A*) Binding of pentalysine to large (100 nm) unilamellar sucrose-loaded PC:PG vesicles of different compositions under conditions where domains do not form. The external solution contains 0.1 M KCl buffered to pH 7.0 with 1 mM 3-(*N*-morpholino)propanesulfonic acid. Peptide (typical concentration 10^{-6} M, much lower than the lipid concentration) was equilibrated with the vesicles for 15 min, then the vesicle-bound peptide was separated by centrifugation ($T = 25°C$). The concentration of peptide in the supernatant was measured by fluorescamine assay, as described by Buser et al. (1994), Ben Tal et al. (1996), and Buser and McLaughlin (1997). Part *A* plots the percentage pentalysine bound versus the concentration of accessible lipid, the concentration of lipid in the outer leaflet of the membrane ($[L]_{access} = \frac{1}{2}[L]_{tot}$). The mole fraction of acidic lipid in the membranes is 10% (*triangles*), 20% (*squares*), 33% (*hexagons*), and 50% (*circles*). The curves represent the fit of the data to the equation: percentage of peptide bound $= 100K[L]_{access}/(1 + K[L]_{access})$, where K is the molar partition coefficient (e.g., Peitzsch and McLaughlin, 1993). (*B*) The percentage of pentalysine bound is plotted as a function of the mole fraction of acidic lipid in the vesicle at an accessible lipid concentration of 10^{-3} M. The filled circles are experimental values from the curves in *A* for $[Lipid]_{access} = 10^{-3}$ M; they illustrate the steep (sigmoidal) dependence of binding on mole fraction of acidic lipid in the membrane. The data are described by our model (Gouy-Chapman-Stern theory), assuming a 1:1 binding of pentalysine to acidic lipids (Langmuir isotherm) with either the parameter set $K_p = 0.1$ M^{-1} and $z_{eff} = 5$ (*squares, dashed curve*), or the set $K_p = 20$ M^{-1} and $z_{eff} = 2.5$ (*triangles, solid curve*).

We now consider how pentalysine decreases the free energy of the system upon domain formation, ΔG. To illustrate the essential features of the model, we first assume a membrane containing 10% acidic lipid (i.e., $f_{(h)} = 0.1$) separates into domain and nondomain phases with constant

fractions of PS: $f_{(d)} = 0.5$ and $f_{(n)} = 0.09$. (Equation 6 shows that the domain occupies ~2% of the membrane area.) We explore how ΔG depends on the concentrations of peptide, C_p, and univalent electrolyte, C_e. If the calculated ΔG is negative, domains will form. If the calculated ΔG is positive, however, domains may still form, because the chosen $f_{(d)}$ and $f_{(n)}$ may not correspond to the minimum of ΔG; we then minimize ΔG more correctly as a function of $f_{(d)}$ and $f_{(n)}$.

Fig. 4 illustrates the key feature of our model of domain formation. Fig. 4 *A* plots the calculated surface potentials of a membrane as a function of the pentalysine concentration: the filled circles illustrate the predicted potentials if domains do not form; the triangles and squares illustrate the predicted potentials of the nondomain and domain phases, respectively, if domains do form. At a very low C_p ($< 10^{-10}$ M), peptide does not bind significantly to the membrane, so the surface potential of the domain phase is much more negative (~ -150 mV) than the surface potential of the
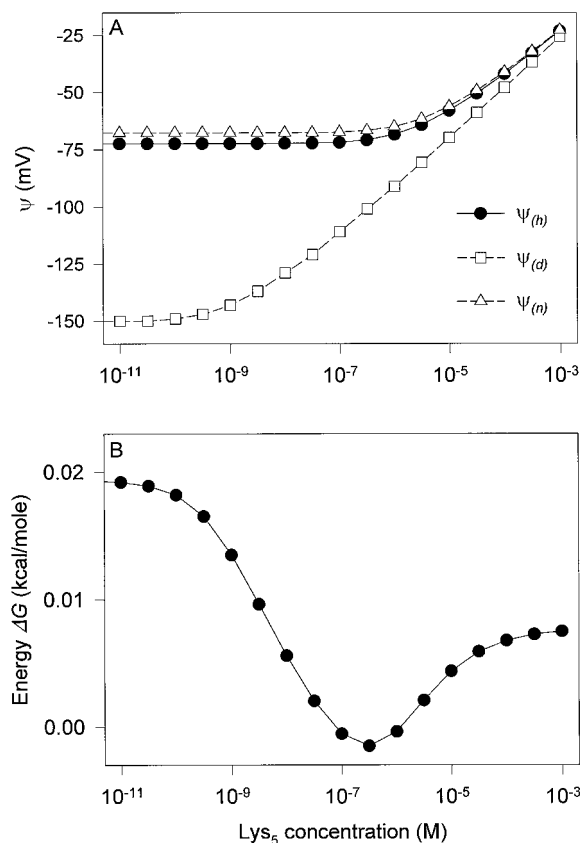

FIGURE 4 The theoretically predicted effect of pentalysine on (*A*) the surface potentials of the homogeneous membrane (*h*), and the domain (*d*) or the nondomain (*n*) part of the membrane; and (*B*) the change in free energy of the system upon domain formation. Calculations in *A* were carried out by inserting Eqs. 2, 3, and B2 into Eq. 1, then solving with respect to the surface potential as a function of peptide concentration, assuming localized 1:1 binding of a peptide to an acidic lipid. Calculations in *B* were carried out using Eqs. 1 and 4–10, with the surface potentials shown in *A*. Parameter values: $f_{(m)} = 0.1$, $f_{(d)} = 0.5$, $f_{(n)} = 0.09$, $C_e = 0.01$ M, $K_p = 20$ M^{-1}, and $z_{eff} = 2.5$.

nondomain phase (~ -70 mV). As the peptide concentration increases from 10^{-9} to 10^{-6} M, the surface potential of the domain, $\psi_{(d)}$, decreases rapidly in magnitude and approaches the values for the (h) and (n) phases, whereas $\psi_{(h)}$ and $\psi_{(n)}$ remain essentially constant; the peptide binds preferentially to the domain phase because of the Boltzmann factor in Eq. 2. At high peptide concentrations ($>10^{-5}$ M), the surface potentials of all of the phases tend to the same value.

Fig. 4 B shows the corresponding change in the free energy of the system, ΔG, upon domain formation. The contribution due to the entropy of mixing, $\Delta G_m \approx 0.008$ kcal $\cdot$ mol^{-1}, does not vary with C_p because it depends only on the fractions $f_{(d)}$ and $f_{(n)}$, which we assume are constant in these calculations; thus the change in ΔG as C_p increases is due to the change in the electrostatic contribution, ΔG_e. At low peptide concentrations, $C_p < 10^{-10}$ M, when the electrostatic potential of the domain is high (Fig. 4 A), the electrostatic free energy of the partitioned membrane exceeds the electrostatic energy of the homogeneous membrane, $\Delta G_e > 0$. In other words, bringing the negatively charged lipids together requires additional work. Under these conditions, the electrostatic free energy term, 0.01 kcal $\cdot$ mol^{-1}, and the mixing term, 0.008 kcal $\cdot$ mol^{-1}, contribute about equally to preventing domains like those illustrated in Fig. 1 B from forming in the absence of peptide. In the intermediate range of peptide concentrations, 10^{-9} to

10^{-6} M, there is significant peptide binding to the domain phase, but not to the nondomain phase. This neutralizes the charge of the domain substantially, reducing the electrostatic free energy of the partitioned membrane relative to that of the homogeneous membrane. Fig. 4 B predicts that domain formation becomes energetically favorable for 10^{-7} M $< C_p < 10^{-6}$ M. At higher peptide concentrations ($>10^{-5}$ M), the homogeneous membrane, as well as the domain and nondomain phases, have significantly reduced surface potentials (Fig. 4 A). In this case, lateral reorganization of the membrane into domains does not produce a significant change in the electrostatic free energy, $\Delta G_e \approx 0$, and domains do not form, because the change in mixing free energy is unfavorable ($\Delta G \approx \Delta G_m > 0$ in Fig. 4 B for $C_p > 10^{-5}$ M).

Thus a simple electrostatic model predicts that domains first form, then break up as the peptide concentration increases. As discussed in Appendix A, this is true not only for the model assuming localized 1:1 binding of the peptide to acidic lipids (Langmuir isotherm, Eq. 3), but also for the model assuming nonlocalized binding (Volmer isotherm).

Experimental data obtained with pentalysine (Fig. 5, *upper row*) agree qualitatively with this prediction. Before the addition of pentalysine, phosphatidylserine is not found in domains, but appears to be distributed randomly in the vesicles (left vesicle denoted *control* in *upper row* of Fig. 5). As the concentration of the basic peptide increases,
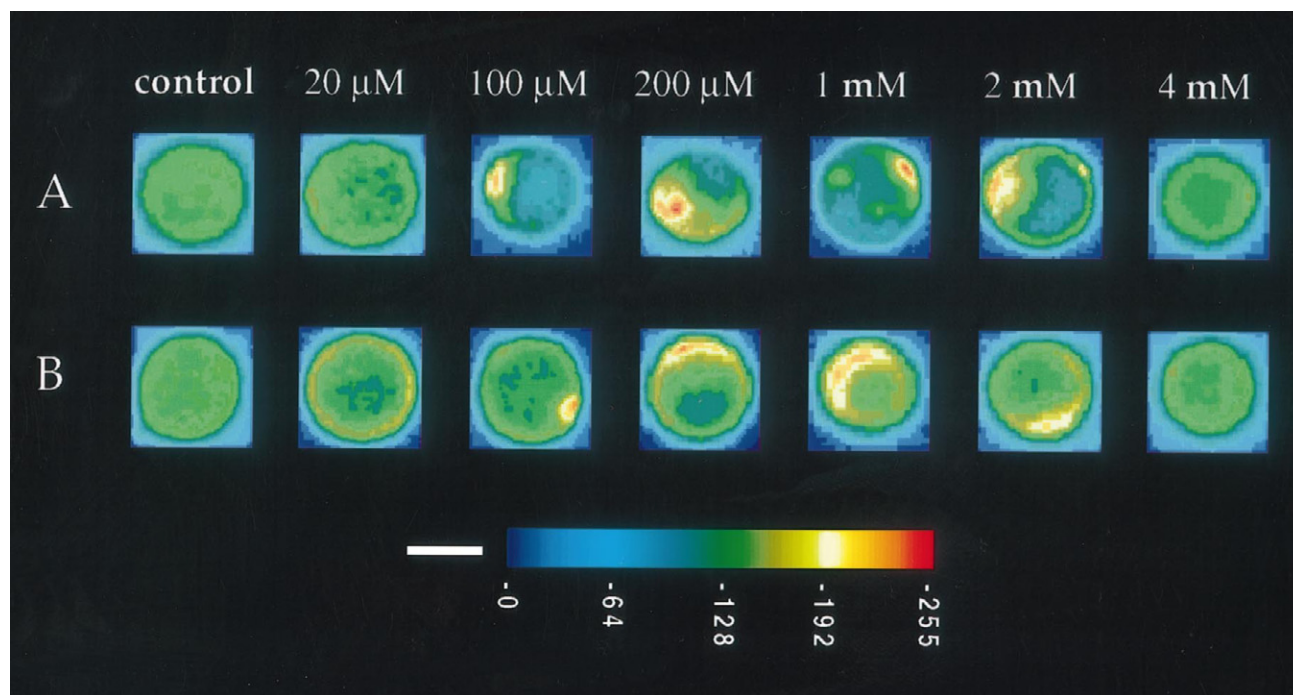

FIGURE 5   Formation of membrane domains by pentalysine and spermine in large unilamellar vesicles visualized using fluorescence microscopy. (*A*) Seven different vesicles containing 90 mol% DOPC, 9.5% mol% DOPS, and 0.5 mol% NBD-PS in 10 mM Tris buffer (pH 7.4), after the addition of the indicated concentrations of pentalysine. (*B*) Seven different vesicles of the same composition after the addition of the indicated concentrations of spermine. Vesicles were visualized for NBD fluorescence. Domains containing PS form as the concentrations of pentalysine (*A*) or spermine (*B*) increase. At higher concentrations (>2 mM), the domains break up and PS is distributed randomly. The images were normalized to a mean radiance value of 100 ± 5, and the corresponding intensity values are displayed according to the pseudocolor scheme at the bottom of the figure. The white bar equals 4 μm.

domains form that are enriched in acidic lipid. At higher peptide concentrations (>2 mM), however, the acidic lipids are again distributed randomly in the membrane. Although the predictions illustrated in Fig. 4 agree qualitatively with the experimental data, the concentration of peptide required to form domains is higher than predicted. We obtained similar data with spermine, a basic ligand with valence $+4$ (Fig. 5, *lower row*): domains form, then break up as the concentration of spermine increases.

Fig. 6 shows how the model predicts the change in free energy of the system on domain formation will vary as the ionic strength of the solution increases; the curves were calculated from the Langmuir model, assuming that the peptide concentration in solution remains fixed. Note that ΔG becomes positive as the ionic strength increases. The physical interpretation of this prediction is straightforward: when the salt concentration increases, the thickness of the diffuse double layer, $1/\kappa$, decreases, and less electrical energy is stored in the diffuse double layer (see Eq. 11). Less electrical energy, ΔG_e, is available to overcome the (positive) mixing term ΔG_m, and domains cannot form.

This prediction agrees well with the experimental results we have obtained with spermine (Fig. 7) and pentalysine (data not shown). Pentalysine and spermine form stable domains enriched in PS in a solution containing 10 mM salt, but not in a solution containing 100 mM NaCl. Yang and Glaser (1995) observed that domain formation induced by MARCKS(151–175) is favored when the ionic strength of

the solution is low, although domains also form in solutions containing 100 mM salt. The different results obtained with pentalysine and MARCKS(151–175) suggest that additional factors stabilize the domains formed by MARCKS(151–175) at physiological salt concentrations.

For the theoretical calculations presented in Fig. 4, we assumed that the domain and nondomain phases contained fixed fractions of acidic lipid. Fig. 8 illustrates predictions obtained for more realistic conditions, where we calculate the fractions $f_{(d)}$ and $f_{(n)}$ that yield the most negative value of ΔG. The curves in Fig. 8 have deeper minima than the curve in Fig. 4 B and tend to zero at very low and very high peptide concentrations, where domains do not form. Our model predicts that the fraction of acidic lipid in the domain, $f_{(d)}$, increases steeply to ~0.5 when the domain begins to form at $C_p = 10^{-7}$ M, then decreases gradually to 0.1 as C_p increases to 10^{-5} M and the domain breaks up (not shown). The size (Eq. 6) of the domain phase also is predicted to change as the peptide concentration increases (not shown).

We then used the model to estimate the fraction of PIP$_2$ that partitions into domains formed by pentalysine. In these calculations, PIP$_2$ was regarded as a trivalent acidic lipid (Toner et al., 1988) that binds pentalysine with the same binding constant K_p as PS. Two direct binding measurements (not shown) support the assumption that PIP$_2$ does not form high-affinity complexes with pentalysine. First, PC/PG/PIP$_2$ vesicles containing 18% PG and 0.5% PIP$_2$ bind pentalysine with the same affinity as PC:PG vesicles containing 20% PG (*squares* in Fig. 3 A). This result can be contrasted with the results obtained with the PH domain of PLC-δ (Garcia et al., 1995; Lemmon et al., 1996), which forms high (μM) affinity 1:1 complexes of known structure with PIP$_2$ and inositol trisphosphate (Ferguson et al., 1995). Second, PC/PIP$_2$ vesicles containing 5% PIP$_2$, which have about the same electrostatic surface potential as PC/PG vesicles containing 20%PG, bind pentalysine with only ~10-fold higher affinity than the PC/PG vesicles: the data are shifted ~10-fold to the left of the squares in Fig. 3 A. Thus pentalysine interacts somewhat more strongly with PIP$_2$ than with monovalent acidic lipids such as PS and PG, but less strongly with PIP$_2$ than does the PH domain of PLC-δ. We assumed that the mole fraction of PIP$_2$ in the vesicles was small compared to the fractions of PC and PS and that it did not form a third membrane phase. The areas of the two phases were calculated from Eq. 6. However, the energy ΔG was minimized as a function of three parameters: $f_{(d)}, f_{(n)}$, and the fraction of PIP$_2$ in one of the phases. Our calculations predict that when domains form, PIP$_2$ should partition strongly into the domain phase.

This prediction agrees qualitatively with two different types of experimental data. First, direct fluoresence measurements show that PIP$_2$ is sequestered in domains formed by pentalysine. Fig. 9 B shows that NBD-PIP$_2$ is distributed randomly in a PC/PS/PIP$_2$ vesicle when the concentration of pentalysine is low (<100 μM), and that the addition of 100 μM, 500 μM, 1 mM, or 2 mM Lys$_5$ produces domains enriched in NBD-PIP$_2$ (Fig. 9 B) as well as NBD-PS (Fig.
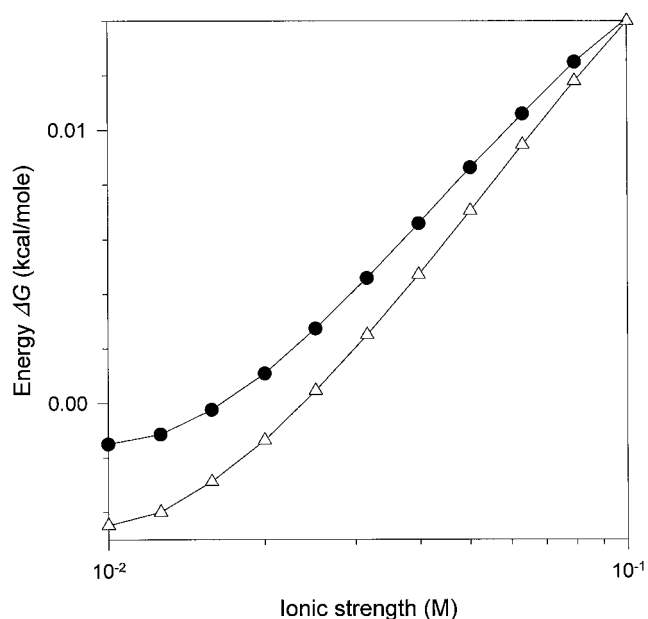
FIGURE 6   Change in free energy of the system on domain formation versus electrolyte concentration, as calculated from the model with localized 1:1 binding of pentalysine to acidic lipids. Parameter values: $K_p = 0.1$ M^{-1}, $z_{eff} = 5$, $C_p = 10^{-8}$ M ($\triangle$), and $K_p = 20$ M^{-1}, $z_{eff} = 2.5$, and $C_p = 10^{-6}$ M ($\bullet$). The values of $f_{(d)}$ and $f_{(n)}$ are calculated as those giving the most negative value to the free energy. These values change with salt concentration. The model predicts that domains will form at low (0.01 M) but not high (0.1 M) concentrations of salt.
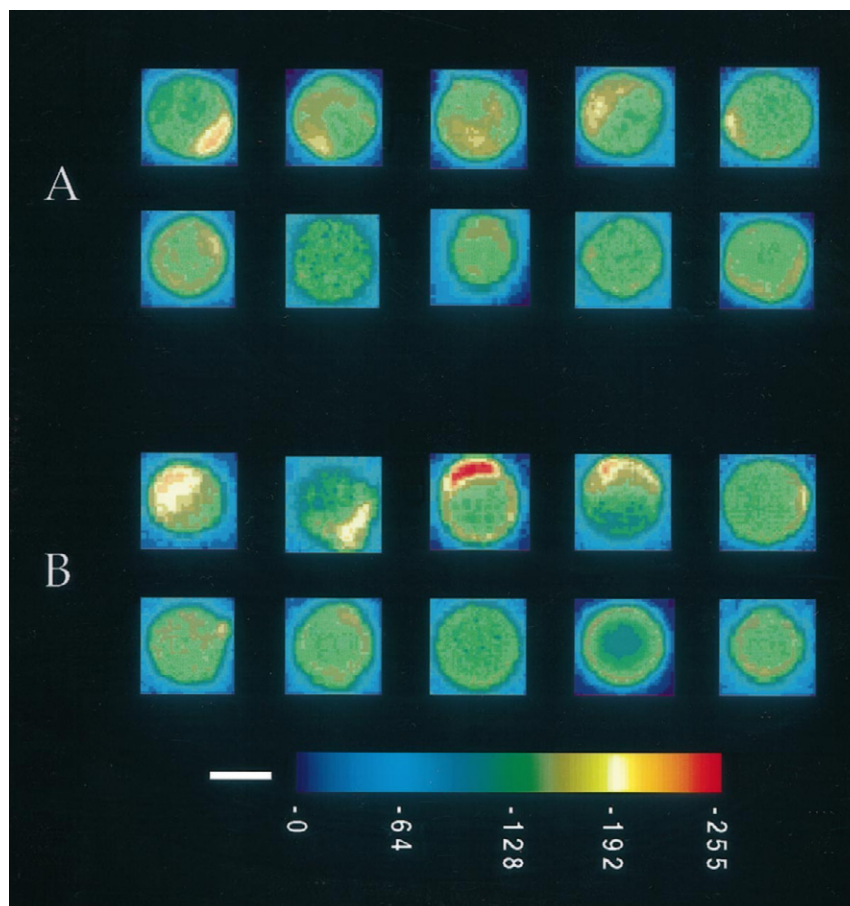
FIGURE 7   Effect of ionic strength on domain formation in large unilamellar vesicles by spermine. The upper row in *A* shows five different vesicles (90 mol% DOPC, 9.5 mol% DOPS, and 0.5 mol % NBD-PS) in 10 mM Tris buffer (pH 7.4), in the presence of 100 μM spermine. The lower row in *A* shows five different vesicles of the same composition in 10 mM Tris buffer (pH 7.4), in the presence of 100 μM spermine plus 100 mM NaCl. (*B*) Five different vesicles in either low ionic strength (*upper row*) or high ionic strength (*lower row*), as in *A*, except that the spermine concentration was increased to 200 μM. The figures show that domains formed in the presence of the spermine at low ionic strength break up when the ionic strength increases. The images were normalized to a mean radiance value of 100 $\pm$ 5, and the intensity values are displayed according to the pseudo-color scheme at the bottom of the figure. The white bar equals 4 μm.

9 *A*). Pentalysine concentrations greater than 4 mM cause the domains enriched in PIP$_2$ and PS to break up. The MARCKS(151–175) peptide also produces domains enriched in PIP$_2$ as well as PS (Glaser et al., 1996). Second, millimolar concentrations of pentalysine and spermine inhibit the phosphoinositide specific-PLC-catalyzed hydrolysis of PIP$_2$ ~10-fold (data not shown). The results we observed with millimolar concentrations of spermine and pentalysine are very similar to those reported by Glaser et al. (1996) for micromolar concentrations of MARCKS(151–175). These observations with pentalysine and spermine support our previous suggestion that the ability of MARCKS(151–175) to sequester the biologically important multivalent lipid PIP$_2$ in lateral domains is a nonspecific electrostatic phenomenon.

DISCUSSION

Our simple theoretical model predicts that small basic peptides use electrostatic interactions to form lateral membrane domains enriched in acidic lipids. The model assumes that more peptides bind to the membrane when domains form; this decreases the electrostatic free energy stored in the diffuse double layer adjacent to the membrane. To a first approximation, the double layer may be represented as a parallel plate capacitor (see Eq. 10). The charged lipids and adsorbed peptides represent one plate; the counterions located a distance $1/\kappa$ from the surface represent the oppositely charged capacitor plate. At low ionic strength, when the Debye length ($1/\kappa$) is large, this decrease in electrostatic energy due to peptide binding can overcome the change in free energy due to the entropy of mixing of the lipids, which always increases upon domain formation. Our experiments provide qualitative support for the four salient predictions of the model. First, we showed that the small multivalent basic ligands Lys$_5$ and spermine do form lateral domains enriched in acidic lipids (Fig. 5). When these ligands are present at very low concentrations, their binding to vesicles exhibits a steep sigmoidal dependence on the mole fraction of acidic lipid in the membrane (Fig. 3 *B* and Chung et al., 1985),
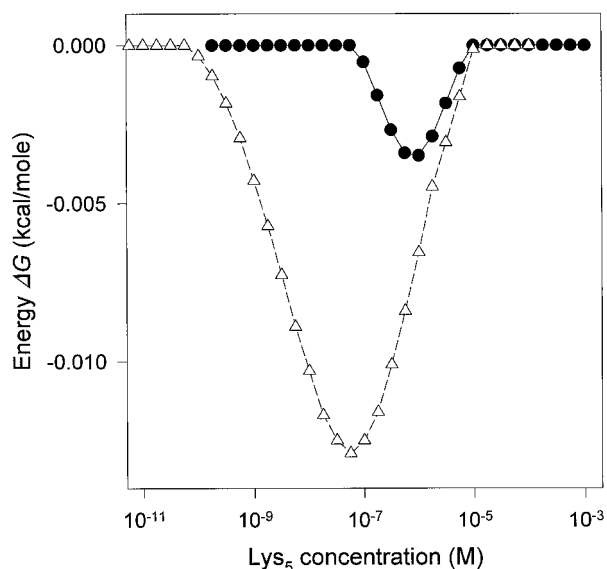
FIGURE 8   Change in the free energy of the system on domain formation versus peptide concentration calculated from the model with localized 1:1 binding of pentalysine to acidic lipids. Parameter values: $K_p = 0.1$ M^{-1} and $z_{eff} = 5$ ($\triangle$), and $K_p = 20$ M^{-1} and $z_{eff} = 2.5$ ($\bullet$). The fraction of acidic lipids in the nonseparated, or homogeneous, membrane is $f_{(h)} = 0.1$, and the values of $f_{(d)}$ and $f_{(n)}$ are chosen as those giving the most negative value to the free energy. $C_e = 0.01$ M.

which is consistent with the fundamental assumption of the model. Second, the domains break up as the concentration of ligand increases to high values (Fig. 5). This observation

strongly supports our assumption that domain formation is not due to attractive interactions between the peptides; in the latter case, domains should be more stable at high peptide concentrations. Third, decreasing the ionic strength of the solution favors domain formation (Fig. 7), which supports our assumption that electrostatic interactions drive domain formation. Fourth, when the membrane contains trace concentrations of the multivalent acidic lipid PIP$_2$, this lipid partitions strongly into the domains formed by basic peptides (Fig. 9); this inhibits hydrolysis of PIP$_2$ catalyzed by phospholipase C (see above). We stress that this partitioning, which is seen with the MARCKS(151–175) peptide as well as with Lys$_5$, does not require the formation of a high-affinity specific 1:1 complex between PIP$_2$ and either peptide. Direct measurements show these peptides, in contrast to the PH domain of PLC-δ, do not bind PIP$_2$ with high affinity (see above).

The results from fluorescence digital imaging microscopy reported here agree very well with results obtained recently with a different technique (G. Gröbner and A. Watts, personal communication), monitored lateral domain formation induced by pentalysine in PC/PG membranes by means of solid-state static and magic angle spinning (MAS) ^{31}P NMR.

The qualitative agreement we observed between the experimental results and the predictions of the model suggests that electrostatics plays an essential role in domain formation under our conditions. Not surprisingly, however, this oversimplified model fails to describe the data quantita-
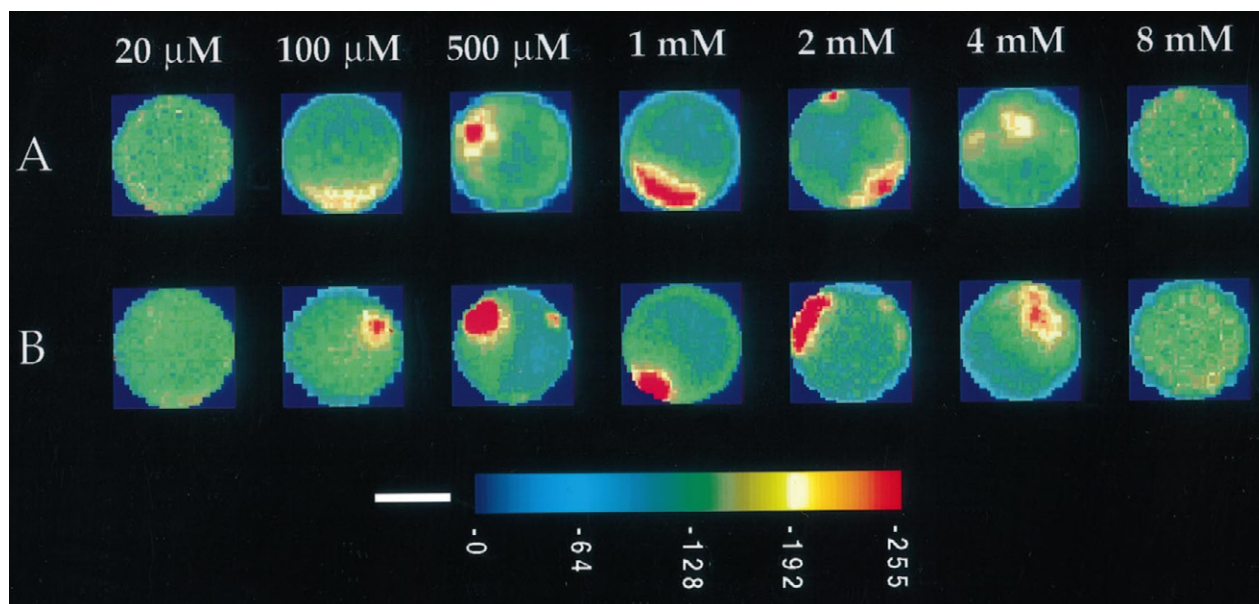

FIGURE 9   The domains produced by pentalysine contain high concentrations of PIP$_2$ as well as PS. (A) Images of seven different vesicles with the same composition (89 mol% DOPC, 9.5 mol% DOPS, 1 mol% PIP$_2$, and 0.5 mol% NBD-PS) formed in a solution containing 10 mM Tris (pH 7.4). Addition of the indicated concentration of pentalysine produces domains enriched in NBD-PS, which break up at high concentrations of pentalysine (>4 mM). (B) Images of seven different vesicles with the same composition (89 mol% DOPC, 10 mol% DOPS, 0.5 mol% PIP$_2$, and 0.5 mol% NBD-PIP$_2$) formed in a solution containing 10 mM Tris (pH 7.4). Addition of the indicated concentration of pentalysine produces domains enriched in NBD-PIP$_2$, which break up at high concentrations of pentalysine (>4 mM). The images were normalized to a mean radiance value of 100 ± 5, and the intensity values are displayed according to the pseudo-color scheme at the bottom of the figure. The white bar equals 4 μm.

tively. Specifically, the calculated pentalysine concentration required to form domains (10^{-7} to 10^{-6} M in a 0.01 M salt solution; *circles* in Fig. 8) is three orders of magnitude lower than the experimentally observed value (10^{-4} to 10^{-3} M; see Fig. 6). Calculations of the electrostatic contribution to domain formation based on realistic molecular models of phospholipid membranes and pentalysine (Ben Tal et al., 1996) are in progress; these calculations consider the highly nonuniform nature of the potential adjacent to a membrane with adsorbed basic peptides, and should provide a more realistic description of how electrostatic interactions contribute to domain formation. Specifically, the calculations show that PIP_2 can be sequestered in domains formed from basic peptides like pentalysine by a discreteness-of-charge mechanism (D. Murray and S. McLaughlin, unpublished observations).

In contrast to the domains formed by pentalysine and spermine, the domains formed by MARCKS(151–175) do not break up at high peptide concentration and are stable in physiological (0.1 M) salt solutions (Yang and Glaser, 1995; Glaser et al., 1996). This MARCKS peptide contains five Phe that penetrate the polar headgroup region of the bilayer (Qin and Cafiso, 1996; Glaser et al., 1996), is much longer than pentalysine, and has more basic residues (13 versus 5). Experiments are in progress to assess the importance of these three factors in domain formation.

An interesting biological implication of this work relates to the ability of basic peptides to sequester the biologically important lipid PIP_2 in lateral domains. The evidence reported here and in Glaser et al. (1996) suggests that the partitioning of PIP_2 into the lateral domains formed by pentalysine or MARCKS(151–175) is due to a nonspecific electrostatic effect. Clusters of basic residues on proteins such as MARCKS and caveolin, which are found in lateral domains in biological membranes, also should be able to sequester PIP_2. Thus we expect that PIP_2 will be found at high concentrations in the nascent phagosomes of macrophages, which contain high concentrations of MARCKS (Allen and Aderem, 1995), and within caveolae, which contain high concentrations of caveolin (Simons and Ikonen, 1997). Two recent reports show that PIP_2 is localized at high concentrations within caveolae (Hope and Pike, 1996; Liu et al., 1997).

APPENDIX A: LANGMUIR VERSUS VOLMER BINDING ISOTHERMS

A general form of the binding isotherm is

$$K_p C_p^0 = \Phi(\theta_{(i)}) \tag{A1}$$

for i = h, d, n. The Langmuir isotherm describes 1:1 binding to localized binding sites (e.g., acidic lipids), and the Volmer isotherm describes nonlocalized binding to the surface (Aveyard and Haydon, 1973; Heimburg and Marsh, 1995). For the Langmuir isotherm, where $\theta_{(i)} = K_p C_p^0/(1 + K_p C_p^0)$, the function $\Phi(\theta_{(i)})$ and surface charge density are

$$\Phi(\theta_{(i)}) = \frac{\theta_{(i)}}{1 - \theta_{(i)}}, \qquad \sigma_{(i)} = \frac{e}{A_L} f_{(i)}[\theta_{(i)}(z - 1) - (1 - \theta_{(i)})] \tag{A2}$$

For the Volmer isotherm,

$$\Phi(\theta_{(i)}) = \frac{\theta_{(i)}}{1 - \theta_{(i)}} \exp\left(\frac{\theta_{(i)}}{1 - \theta_{(i)}}\right), \qquad \sigma_{(i)} = \frac{e}{A_L}[-f_{(i)} + \theta_{(i)}z] \tag{A3}$$

Here, e is an elementary (positive) charge, A_L is the area of one lipid, and $f_{(i)}$ is the mole fraction of acidic lipids in the ith phase.

In the main body of this paper we consider only the Langmuir isotherm. Use of the Volmer isotherm, however, leads to qualitatively similar predictions about domain formation. For example, a plot of the change in the free energy of the system upon domain formation versus peptide concentration, as calculated from the model with the Volmer isotherm, shows that domains form, then break up with increasing peptide concentration, as shown in Fig. 4 B for the Langmuir isotherm. The change in the electrostatic free energy calculated from the model with the Volmer isotherm, however, is less favorable for domain formation than with the Langmuir isotherm. Domains are predicted to form only at lower ionic strengths (e.g., 10^{-3} M) than those used in our experiments (10^{-2} M). Both of these isotherms are less realistic than the treatment discussed by Ben Tal et al. (1996).

APPENDIX B: ELECTROCHEMICAL EQUILIBRIUM BETWEEN LIPIDS IN THE DOMAIN AND NONDOMAIN PHASE

At equilibrium, the electrochemical potential of PC in the domain phase must be equal to the electrochemical potential of this lipid in the nondomain phase, $\mu_{PC(d)} = \mu_{PC(n)}$. The same is true for the electrochemical potentials of PS in the domain and nondomain phases: $\mu_{PS(d)} = \mu_{PS(n)}$. We consider here expressions for these potentials, then give a physical interpretation of the generalized forces that maintain the equilibrium between the lipids in the two phases (see Fig. 2).

Consider a phase i (i = d or n) that consists of n_1 acidic lipids (e.g., PS) and n_0 zwitterionic lipids (e.g., PC). The total area of the phase is $A_{(i)} = A_L N$, where $N = n_1 + n_0$, the total number of lipids in the phase (for simplicity, we will omit the subscript (i) in the remainder of this appendix unless otherwise specified). Let $G = Ag = A(g_m + g_e)$ be the total surface free energy of the phase, where g_m, g_e, and g are the free energy of mixing, the electrostatic free energy, and the total free energy (each per unit area), respectively:

$$g_m = \frac{RT}{A_{(h)}}[f \ln f + (1 - f)\ln(1 - f)];$$

$$g_e = \sigma\psi(\sigma) - \frac{2RT}{FB}\left(\cosh\frac{F\psi(\sigma)}{2RT} - 1\right); \qquad g = g_m + g_e$$

$A_{(h)} = N_A A_L$ is the area per one mole of lipids; N_A is Avogadro's number; $f = n_1/N$ is the fraction of acidic lipid in the phase; and ψ is the surface potential of the phase, which can be expressed as a function of the surface charge density, σ, from Eq. 1. Note that in the absence of peptide, $\sigma = -ef/A_L$. In the presence of the peptide that binds to the acidic lipid with 1:1 stoichiometry, we have

$$\sigma = \frac{Zef}{A_L},$$

$$Z = Z(C_p) = \frac{(z - 1)K_p C_p \exp(z_{eff}F\psi/RT) - 1}{1 + K_p C_p \exp(z_{eff}F\psi/RT)}$$

where Z could be interpreted as the surface charge per acidic lipid (expressed in units of an elementary charge). Note that for $C_p = 0$, $Z = -1$.

By definition, the electrochemical potential of PC, μ_{PC}, is the free energy per mole of this lipid, or the partial derivative of the total surface

free energy of the membrane, G, with respect to the number of moles of PC in it, n_0/N_A:

$$\mu_{PC} = \frac{\partial G}{\partial(n_0/N_A)} = N_A \frac{\partial A}{\partial n_0} g + N_A A \frac{\partial g}{\partial f}\frac{\partial f}{\partial n_0}$$

$$= A_{(h)}g - A_{(h)}f\frac{\partial g}{\partial f} \tag{B1}$$

Similarly, the electrochemical potential of PS is given by

$$\mu_{PS} = \frac{\partial G}{\partial(n_1/N_A)} = N_A \frac{\partial A}{\partial n_1} g + N_A A \frac{\partial g}{\partial f}\frac{\partial f}{\partial n_1}$$

$$= A_{(h)}g + A_{(h)}(1-f)\frac{\partial g}{\partial f} \tag{B2}$$

We wish to represent these electrochemical potential expressions as a sum of the surface pressure, concentration, and electrostatic terms. The surface pressure, π, is calculated as a partial derivative of the surface free energy with respect to the area under the conditions where the total number of lipids remains constant (see, e.g., Aveyard and Haydon, 1973; Marsh, 1996):

$$\pi = \frac{\partial G}{\partial A} = \frac{1}{N}\frac{\partial G}{\partial A_L} = \frac{1}{N}\frac{\partial A}{\partial A_L}g + \frac{1}{N}A\frac{\partial g}{\partial A_L}$$

$$= g + A_L\frac{\partial(g_m + g_e)}{\partial A_L} \tag{B3}$$

Note that g_m does not depend on A_L and that A_L enters into the expression for g_e only in combination with f, so that $g_e = g_e(\sigma)$, where $\sigma = Zef/A_L$. This allows the following relationships:

$$\frac{\partial g_e}{\partial A_L} = \frac{\partial g_e}{\partial \sigma}\frac{\partial \sigma}{\partial A_L} = \frac{\partial g_e}{\partial \sigma}\left(-\frac{\sigma}{A_L}\right);$$

$$\frac{\partial g_e}{\partial f} = \frac{\partial g_e}{\partial \sigma}\frac{\partial \sigma}{\partial f} = \frac{\partial g_e}{\partial \sigma}\frac{\sigma}{f}; \qquad \frac{\partial g_e}{\partial A_L} = -\frac{\partial g_e}{\partial f}\frac{f}{A_L}$$

Now Eq. B3 can be rewritten in the form $\pi = g - f\,\partial g_e/\partial f$. By combining this equation with Eqs. B1, B2, and B3, we arrive at the desired representations for the electrochemical potentials of PC and PS in our simple model:

$$\mu_{PC} = A_{(h)}\pi - A_{(h)}f\frac{\partial g_m}{\partial f} = A_{(h)}\pi + RTf\,\ln\frac{1-f}{f} \tag{B4}$$

$$\mu_{PS} = A_{(h)}\pi + A_{(h)}\frac{\partial g}{\partial f} - A_{(h)}f\frac{\partial g_m}{\partial f}$$

$$= A_{(h)}\pi + RT(1-f)\ln\frac{f}{1-f} + ZF\psi + F\psi f\frac{\partial Z}{\partial f} \tag{B5}$$

The difference between the electrochemical potentials of the PC in the domain and nondomain phases can be written as

$$\Delta\mu_{PC} = \mu_{PC(d)} - \mu_{PC(n)}$$

$$= A_{(h)}(\pi_{(d)} - \pi_{(n)}) + RT\left[f_{(d)}\ln\frac{1-f_{(d)}}{f_{(d)}} - f_{(n)}\ln\frac{1-f_{(n)}}{f_{(n)}}\right]$$

$$\equiv \Delta\mu_\pi + \Delta\mu_{1-f} \tag{B6}$$

At equilibrium ($\Delta\mu_{PC} = 0$), the two terms of the far right hand-side of Eq. B6 must have opposite signs, which is indeed the case, because $\pi_{(d)} > \pi_{(n)}$ and $f_{(d)} > f_{(n)}$:

$$\Delta\mu_\pi > 0; \qquad \Delta\mu_{1-f} < 0 \tag{B7}$$

The first term in Eq. B6, the generalized force that results from the surface pressure difference between the two phases, pushes PC out of the domain phase. The second term, the generalized diffusional force, drives PC down its concentration gradient, from the nondomain to the domain phase. At equilibrium, the two forces balance each other (Fig. 2). To estimate a typical value for $\Delta\pi$, we consider the values $f_{(d)} = 0.5$ and $f_{(n)} = 0.09$ used in our other analyses and obtain $\Delta\pi \approx 1$ mN/m from Eq. B6. This is a low pressure relative to the surface pressure in the membrane, which is 30–35 mN/m (Marsh, 1996).

Now consider the equilibrium that exists for PS. For simplicity, we assume that the fraction of charged lipid in both phases is low, $f \to 0$; it follows from Eq. 1 and the expression for σ presented above that $\psi \propto \sigma \propto f$. Thus the last term in Eq. B5 is proportional to f^2 and is negligible compared to the first three terms. The difference between the electrochemical potentials of the PS in the domain and nondomain phases then becomes

$$\Delta\mu_{PS} = \mu_{PS(d)} - \mu_{PS(n)}$$

$$= A_{(h)}(\pi_{(d)} - \pi_{(n)}) + RT\ln\frac{f_{(d)}}{f_{(n)}} + F(Z_{(d)}\psi_{(d)} - Z_{(n)}\psi_{(n)})$$

$$\equiv \Delta\mu_\pi + \Delta\mu_f + \Delta\mu_\psi \tag{B8}$$

Two of the terms in the final expression are positive, $\Delta\mu_\pi > 0$ and $\Delta\mu_f > 0$, so the third term, $\Delta\mu_\psi$, must be negative at equilibrium ($\Delta\mu_{PS} = 0$).

We consider two specific cases: 1) The solution does not contain basic peptide. In this case $Z_{(d)} = Z_{(n)} = -1$ and $\Delta\mu_\psi = F(-\psi_{(d)} + \psi_{(n)}) > 0$ (see Fig. 4 A); thus electrochemical equilibrium does not exist and stable domains do not form. 2) The solution contains basic peptides. In this case, the values of $Z_{(i)}$ are different in the domain and nondomain phases. Specifically, the basic peptide binds preferentially to the domain phase, which has a higher fraction of acidic lipids, compared to the nondomain phase, so the average charge per acidic lipid will be less negative in the domain than in the nondomain phase: $|Z_{(d)}| < |Z_{(n)}|$. Thus the last term in Eq. B8 may become negative, $\Delta\mu_\psi = F(Z_{(d)}\psi_{(d)} - Z_{(n)}\psi_{(n)}) < 0$, and the first two terms become balanced, leading to electrochemical equilibrium, $\Delta\mu_{PS} = 0$.

The first two terms of the final expression in Eq. B8 are similar to those in Eq. B6: they represent generalized forces that drive PS from the domain to the nondomain phase because of differences in the surface pressure ($\Delta\mu_\pi$) and concentration ($\Delta\mu_f$) in the two phases. The last term in Eq. B8 is the generalized electrostatic force acting on PS. Although the domain phase has a more negative surface potential than the nondomain phase in our simple model (see Fig. 4 A), electrostatic effects drive PS from the nondomain to the domain phase, because when peptide is present, the effective charge per PS in the domain is lower. Thus the generalized electrostatic force on PS will be directed from the nondomain to the domain phase and balance the other two generalized forces at equilibrium (Fig. 2).

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