Electrostatic interaction of myristoylated proteins with membranes: simple physics, complicated biology

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Cell membrane association by several important peripheral proteins, such as Src, MARCKS, HIV-1 Gag, and K-Ras, requires nonspecific electrostatic interactions between a cluster of basic residues on the protein and acidic phospholipids in the plasma membrane. A simple theoretical model based on the nonlinear Poisson–Boltzmann equation describes well the experimentally measured electrostatic association between such proteins and the cell membrane.

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Hydrophobic and electrostatic interactions act in concert to anchor to cell membranes several important proteins that are either myristoylated (e.g. Src, HIV-1 Gag and myristoylated alanine-rich C kinase substrate, MARCKS) or farnesylated (e.g. K-Ras). Two recent reviews focused on how the attachment of acyl and isoprenyl groups to proteins influences their association with membranes [1,2]. Here, we consider the role electrostatic interactions play in enhancing the partitioning of myristoylated proteins onto membranes, using the proto-oncogene product c-Src as an example [1,3].

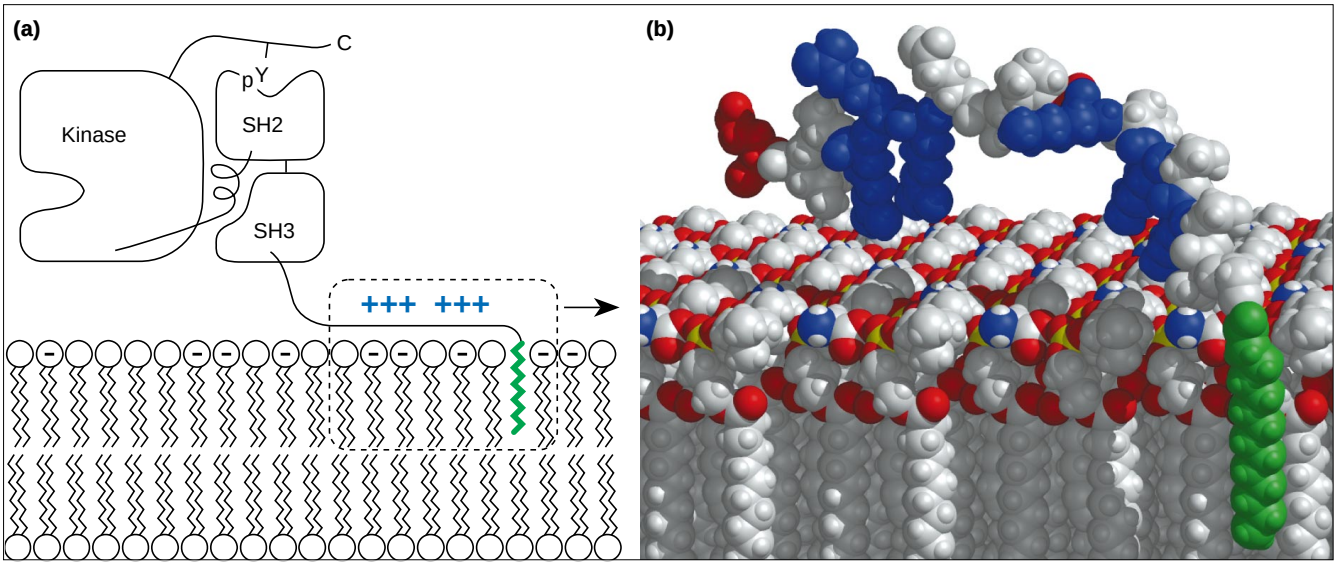
Figure 1 shows the domain organization of the tyrosine kinase c-Src. Recent structural studies have revealed how the intramolecular binding of a C-terminal phosphorylated tyrosine (Tyr527) to the SH2 domain juxtaposes the SH3 domain with the polyproline type-II helix that links the SH2 and kinase domains [3]. The intramolecular interaction between the SH3 and kinase domains maintains the protein in an inactive conformation. Because the viral, oncogenic version, v-Src, lacks the C-terminal tyrosine, it is constitutively active. The N-terminal portion was not included in the recently determined crystal structure of Src [3]. This region contains the two motifs required for membrane association (shown in Figure 1a) — the myristate (colored green) and a cluster of basic residues (blue plus signs), which are shown in more detail in Figure 1b. Membrane binding increases the effective concentration of Src in a thin (d ~1 nm) surface layer adjacent to the membrane. For a spherical cell with a radius r equal to a few micrometers, the volume of the surface phase ($V = 4\pi r^2 d$) is about 1/1000 the volume of the cell ($V = 4\pi r^3/3$); thus, anchoring Src to the membrane increases its effective concentration 1000-fold, thereby greatly enhancing its ability to phosphorylate its membrane-bound substrates.

Two factors contribute to Src membrane binding

Myristate (14-carbon fatty acid) is attached cotranslationally through an amide bond to the N-terminal glycine of Src by the enzyme N-myristoyl transferase [1,2]. Myristate is required for Src membrane binding which, in turn, is required for Src to function — nonmyristoylated v-Src mutants are found in the cytoplasm and do not transform cells, even though the kinase activity of the protein is unaffected [1,4,5]. Small myristoylated peptides bind to electrically neutral phospholipid vesicles with a unitary binding energy of 8 kcal/mol (or a molar partition coefficient of 104 M–1) [6,7]. Measurements of the membrane partitioning of acylated peptides show that the binding energy increases 0.8 kcal/mol for each CH2 group added to the acyl chain [6], which is in agreement with measurements of the hydrophobic partitioning of fatty acids into oil from water [8]. The model in Figure 1 is consistent with these results: 8 kcal/mol ÷ 0.8 kcal/mol per CH2 = 10 CH2 groups, which penetrate the hydrocarbon core of the membrane; the remaining four traverse the polar head group region and the N-terminal glycine is located just outside the envelope of the polar head group region (Figure 1b). Monolayer and circular dichroism measurements [7] and, more importantly, direct structural electron paramagnetic resonance (EPR) measurements of spin-labeled peptides (D Cafiso, personal communication) confirm that the N-terminal residues of Src do not penetrate the membrane and indicate that the peptide has an extended conformation, as illustrated in Figure 1.

Although myristate is required for Src membrane binding, when alone it is not sufficient to anchor Src to its target membranes [1,2,9]. The Src protein partitions onto electrically neutral membranes with a molar partition coefficient of 10^3 M^{-1} [10]; the concentration of lipid in the plasma membrane of a cell of 10 μm radius is about 10^{-3} M, so myristate alone would anchor only half of the number of protein molecules to the membrane. As discussed elsewhere [1,2], other Src family kinases (e.g. Lck and Fyn) augment the binding due to myristate with an N-terminal palmitate (16-carbon fatty acid). Src, in contrast, has an N-terminal cluster of basic residues. Studies with peptides corresponding to the N terminus of Src show that adding

Figure 1



Domain structure of c-Src. **(a)** Cartoon (approximately to scale) illustrating the domain structure of c-Src as determined by recent X-ray crystallographic analysis [3]. See text for details. **(b)** Exploded view of Src's N terminus interacting with a 2:1 phosphatidylcholine: phosphatidylserine membrane. The conformation of Src(2–19), myristate–GSSKSKPKDPSQRRRSLE, is consistent with experimental measurements (see text). The myristate is colored green, basic residues blue and acidic residues red. In the membrane, the acidic lipid, phosphatidylserine, is identified by its exposed nitrogen, colored blue.

33% acidic lipid to electrically neutral membranes increases the binding 1000-fold [7]. The same 1000-fold enhancement is seen with the intact Src protein [10]. Mutations that remove the N-terminal basic residues weaken the partitioning of Src onto phospholipid vesicles containing acidic lipids and produce non-transforming phenotypes in living cells [1,10,11]. These observations provide strong evidence that the N-terminal basic residues contribute to the membrane binding of Src by interacting electrostatically with acidic lipids.

Energetics of membrane binding

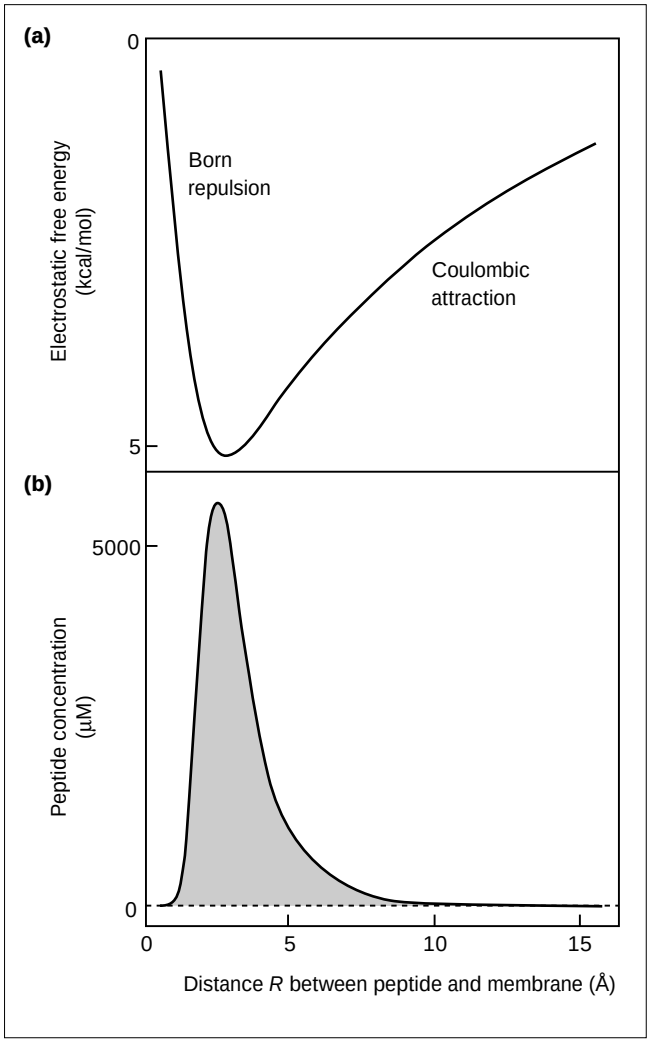
To a first approximation, the hydrophobic and electrostatic binding energies can be added together to give the total binding energy of Src (or the molar partition coefficients can be multiplied). This observation follows from models that consider the acyl chain and basic cluster as points connected by a flexible string of length L [7,12]. Binding of myristate to the membrane confines the basic cluster to a hemisphere of radius L above the membrane surface and facilitates its adsorption to the membrane. Although these simple models account for the synergism between electrostatic and hydrophobic interactions, they are descriptive rather than predictive. Using atomic models of Src's N terminus and phospholipid bilayers (Figure 1) and a continuum representation of the solvent [13,14], we can predict the electrostatic partitioning by solving the nonlinear Poisson–Boltzmann equation [15,16]. This mean field theory ignores ion–ion correlation effects and the finite size of ions in the aqueous phase; theoretical work has justified these assumptions for physiological conditions [17]. The assumption that water molecules adjacent to a membrane can be treated theoretically as a dielectric continuum is supported by surface force, X-ray diffraction and other experiments [18]. The theoretical methods accurately describe the electrostatic potentials adjacent both to proteins [13] and to phospholipid bilayers [19].

We illustrate the theoretical model by considering the interaction of a nonmyristoylated (nonmyr) peptide corresponding to the N terminus of Src, nonmyr-Src(2–19), with a phospholipid membrane containing 33% acidic lipid in 100 mM salt solution. The peptide is docked in the aqueous solution above the membrane (e.g. in an orientation similar to that depicted in Figure 1). Each atom is assigned a radius and a partial charge, and the peptide–membrane model is mapped onto a three-dimensional lattice of points [20]. The nonlinear Poisson–Boltzmann equation is solved numerically [20] for the electrostatic potential due to the peptide and the membrane when they are far apart and when they are close together, as described by Ben-Tal *et al.* [15]. These potentials are used to calculate the changes in the electrostatic free energy as the peptide approaches the membrane [21]. Figure 2a shows the electrostatic free energy of interaction as a function of the distance R between the van der Waals surfaces of the peptide and

membrane. The free energy curve illustrates the long-range Coulombic attraction and short-range Born repulsion (i.e. the free energy cost of desolvating charged and polar groups) that result in the free energy minimum at R ~3 Å. The peptide concentration at each distance R (Figure 2b) is a product of the peptide concentration at infinity (in the bulk solution) and the exponent of the interaction energy. (In practice, the peptide concentration at R is calculated by averaging over many orientations of the peptide with respect to the membrane in order to approximate a complete ensemble of different configurations) Integrating the excess peptide concentration over R (grey area in Figure 2b) gives the Gibbs surface excess, which represents the number of moles of peptide bound per unit area of membrane surface. The Gibbs surface excess is simply related to the molar partition coefficient that is measured experimentally [15]. As illustrated in Figure 2b, the bound peptides, which associate with the membrane through long-range electrostatic interactions, can be located at an appreciable distance from the membrane surface (e.g. a significant amount of peptide is bound at R = 5 Å). This is consistent with experimental results that show the binding of model peptides to membranes depends only weakly on the chemical nature of either the basic residues or the monovalent acidic lipid [15]. As discussed in detail elsewhere, this nonspecific association is more accurately described by a partition coefficient [6,16,22] rather than by a binding constant which assumes the formation of a 1:1 complex between protein and membrane lipid (as is seen, for example, with the binding of phosphatidylinositol 4,5-bisphosphate (PIP_2) to the pleckstrin homology domain of phospholipase C-δ_1 [23]).

This theoretical methodology has been used to describe the membrane partitioning of basic peptides [15], charybdotoxin and its analogs [16], and Src(2–19) (DM, NB, BH and SM, unpublished results) — the model correctly predicts how the binding is affected by changes in the ionic strength of the solution, the net positive charge of the peptide or the mole % acidic lipid in the membrane. For example, the model predicts that the binding of charybdotoxin (net charge +4) decreases by five orders of magnitude when the salt concentration is increased from 10 mM to 150 mM, in agreement with the measured partitioning [16]. The results of these studies indicate that the model describes well the long-range ($R \geq 3$ Å) electrostatic attraction that gives rise to the membrane binding. Nevertheless, the calculated binding energies based on electrostatics alone consistently underestimate the observed values by 1–2 kcal/mol. This implies that the model ignores some attractive interactions [16], overestimates the repulsive interactions, or both. Further theoretical and experimental work, such as that previously described [16,24,25], is required to obtain a more accurate description of the short-range ($R < 3$ Å) interactions, particularly when hydrophobic residues penetrate the polar head group region.

Figure 2



Membrane electrostatics. **(a)** Electrostatic free energy curve. The electrostatic free energy of the interaction between nonmyristoylated (nonmyr)-Src(2–19) and a 2:1 phosphatidylcholine: phosphatidylserine lipid bilayer in 100 mM monovalent salt as a function of the distance R between the van der Waals surfaces of the peptide and the membrane (DM, NB, BH and SM, unpublished calculations). See Ben-Tal *et al.* [15] for theoretical methods. **(b)** The concentration of nonmyr-Src(2–19), [P(R)], as a function of the distance R. The bulk peptide concentration, [P(∞)], is chosen as 1 μM to match the experimental conditions [7]. The Gibbs surface excess is defined as the integral of [P(R)]–[P(∞)] over distance R . This quantity, the grey area in the figure, may be considered as the number of moles of nonmyr-Src(2–19) adsorbed per unit area of membrane surface.

Acyl groups and basic clusters: other proteins, other functions

Other proteins that use either myristate or farnesyl groups and a cluster of basic residues to bind to membranes include HIV-1 Gag [26], K-Ras 4B [27] and MARCKS [28, 29] (Figure 3). The N-terminal cleavage product of HIV-1 Gag, the viral matrix protein, contains the protein's two

Figure 3

Clusters of basic residues in four proteins that interact electrostatically with acidic phospholipids in membranes. Basic residues are shown in blue, acidic residues are in red.

membrane-binding motifs [26]. Structural studies of the HIV-1 matrix protein show the basic residues, in contrast to the extended conformation of Src's N-terminus, are clustered into a β-sheet region that forms a membrane-binding surface [30]. K-Ras, a small GTPase, uses farnesyl (a 15-carbon isoprenoid) rather than myristate to bind to membranes. The farnesyl chain, like myristate, does not provide sufficient hydrophobic energy to anchor the protein to membranes [31]. Adding 20% acidic lipid to electrically neutral membranes enhances the binding of farnesylated peptides corresponding to the C terminus of K-Ras 300-fold [12]. Although separated by 150 residues, both the N-terminal myristate and the cluster of basic residues are required for the membrane anchoring of MARCKS [2,9, 28,29]. EPR measurements of 15 spin-labeled peptides corresponding to the basic effector region of MARCKS, MARCKS (151–175), showed that the peptide lies at the membrane interface in an extended conformation with its five phenylalanines penetrating the polar head group region [32]. MARCKS is interesting because protein kinase C (PKC) catalyzed phosphorylation of three serines within the basic effector region weakens the electrostatic interaction and causes the MARCKS protein to translocate from the plasma membrane to the cytoplasm in many cell types [33]; this mechanism has been termed the 'myristoyl–electrostatic' switch [1,2,9]. Other myristoylated proteins use a different switch mechanism, the 'myristoyl–ligand' switch [1,2], for reversible membrane binding; for example, recoverin binds to membranes when its sequestered myristate moiety is exposed in response to Ca^{2+} [34]. Other lipid-modified proteins have clusters of basic residues that may interact with acidic lipids: for example, endothelial nitric-oxide synthase (eNOS) [35,36], diacylglycerol kinase ζ [37], GAP–43/neuromodulin [38], Src-suppressed C kinase substrate (SSeCKS) [39], some G-protein coupled receptor kinase (GRK) family members [40], the heterotrimeric G-protein subunit α12 (TLZ Jones, personal communication) and Rho family GTPases [41]. As an aside, we note that myristate and clusters of basic residues are not always involved in membrane binding — cAMP-dependent protein kinase, for example, keeps its myristate permanently tucked away in a hydrophobic cleft, and many proteins use clusters of basic residues as nuclear localization signals.

In addition to attaching proteins to membranes, acyl chains and clusters of basic residues may direct peripheral proteins to lateral domains in the plasma membrane. Many important signaling molecules (e.g. Src family members Lck and Fyn, some G-protein alpha subunits, H-Ras, eNOS and PIP_2) are concentrated in plasma membrane organelles called caveolae [42]. Src, PKCα, SSeCKS and focal adhesion kinase are co-localized with cytoskeletal elements in focal adhesion plaques [39,43]. MARCKS has a punctate distribution in the plasma membrane of macrophages and is concentrated, with PKCα, in nascent phagosomes [44]. In phospholipid vesicles, the basic effector region of MARCKS forms discrete lateral domains enriched in monovalent acidic phospholipids and PIP_2, as visualized by digital imaging fluorescence microscopy [45]. Similar results are obtained with the simpler basic peptide pentalysine [45], the domains of which have also been observed using magic angle spinning NMR (G Gröbner and A Watts, personal communication). The results indicate that electrostatic interactions can play a major role in lateral domain formation in membranes, and that PIP_2 can be reversibly sequestered in domains by nonspecific electrostatic interactions [45], a phenomenon with interesting biological implications. In summary, hydrophobic and electrostatic interactions act synergistically to anchor several important myristoylated and farnesylated proteins to membranes; the electrostatic interactions can be described well using simple physical models.

Acknowledgements

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