Leading Edge
Minireview
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Tuning Bulk Electrostatics to Regulate Protein Function

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Cyclin-dependent kinase activation can prevent yeast cells from responding to mating pheromone. Strickfaden et al. (2007) now show that this block arises from the multisite phosphorylation of Ste5. This provides a beautiful example of how phosphorylation can produce decisive changes in protein function through bulk electrostatics, without the necessity of intricate conformational changes.

Current thinking about the regulation of proteins by phosphorylation is dominated by allosteric regulation and conformational changes. The classic example is glycogen phosphorylase; when phosphorylated, it undergoes a dramatic reorganization that allows access of substrate to the otherwise buried catalytic site (Barford et al., 1991). Phosphorylation-driven conformational changes often involve the coordinated repositioning of multiple residues. These changes depend on subtle details of the folding energy landscape of the protein, and thus mutating or moving the phosphosite is rarely tolerated.

However, the consequences of protein phosphorylation can be much simpler. For example, the phosphorylation of a residue near the active site of isocitrate dehydrogenase inactivates the enzyme without inducing any conformational change; the phosphate simply blocks substrate binding (Hurley et al., 1990). Phosphorylation can also add function to a protein without a conformation change, as in the creation of SH2-binding epitopes by the phosphorylation of tyrosine.

A splendid example of a simple mechanism for transducing changes in phosphorylation state into changes in protein function is provided by recent work on the regulation of Ste5, a MAPK cascade scaffold protein critical for mating pheromone responses in *S. cerevisiae.* In this issue, Strickfaden and colleagues (2007) show that phosphorylation of a cluster of eight poorly conserved SP/TP residues near the polybasic membrane-binding domain of Ste5 prevents binding to the inner leaflet of the plasma membrane and thence prevents picking up an activation signal. This inhibition appears to arise from the electrostatic repulsion of the negatively charged plasma membrane lipids by these phosphorylated residues. The idea of regulating protein function through bulk electrostatics is highly appealing; in contrast to allostery, it seems simple, robust, and easy to evolve. In addition, the involvement of a large number of phosphorylation sites should allow for the generation of a decisive, ultrasensitive switch without a requirement for classical cooperativity.

Regulation of Ste5 by Cln/CDK

Mating is initiated when a mating pheromone binds a G protein-coupled receptor on a haploid yeast cell. This causes the release of a Gβγ dimer from a trimeric G protein, which then recruits the Ste5 scaffold to the plasma membrane. This recruitment then promotes the activation of the MAPKKK Ste11 by the membrane-localized Ste20. Mutations that abolish membrane localization of Ste5 abolish signaling, and mutations that allow membrane localization in the absence of Gβγ cause constitutive signaling. However, the mating pheromone pathway is not always responsive to pheromone. The pathway is maximally responsive in G1-phase cells and becomes unresponsive once cells have activated Cln/CDKs and traversed START when cells commit to cell division (Oehlen and Cross, 1994; Wassmann and Ammerer, 1997). The converse is also true; activation of the mating pathway prevents the cell from activating Cln/CDK complexes and entering S phase (Chang and Herskowitz, 1992).

The identification of Ste5 as an important Cln/CDK target comes from studies that examine how and when Ste5 is localized to the membrane (Strickfaden et al., 2007; Winters et al., 2005). Winters and coworkers observed that an N-terminal peptide from Ste5 localizes constitutively to the membrane with or without pheromone present and in yeast lacking the Gβ subunit normally required for Ste5 recruitment. Molecular dissection of Ste5 revealed a crucial lysine- and arginine-rich stretch near the N terminus that partitions into the plasma membrane. There is a striking dependence on the charge and density of the acidic phospholipids, suggesting that electrostatic interactions are important (Winters et al., 2005). The partitioning is strong enough to localize an N-terminal peptide fragment of Ste5 but insufficient to localize the full-length protein (Winters et al., 2005). Likewise, in the absence of the basic region, the affinity of Ste5 for the receptor-activated Gβγ dimer is not enough to bring the scaffold to the membrane (Winters et al., 2005).

Strickfaden et al. (2007) now show that Ste5 is multiply phosphorylated by Cln/CDK at sites close to the basic region. These phosphorylations inhibit membrane binding and pheromone signaling by repelling the negatively charged lipids of the inner leaflet of the plasma membrane. Thus, Ste5 is acting as an AND gate, or a coincidence counter, requiring both an active Gβγ dimer to provide one increment of binding energy and a dephosphorylated basic region to provide another. Substituting the eight phosphorylation sites with glutamates yields a protein that has an impaired, but not completely blocked, response to pheromone (Strickfaden et al., 2007). One might suspect that the carboxylic acid only partially mimicked the allosteric role of the phosphoryl group, and under such circumstances, replacing the adjacent proline with an additional glutamate should only make matters worse. Strikingly, replacing the SPs and TPs with EE completely blocked the pheromone response, and further mutational analysis revealed that membrane binding depends on the number, but not the exact position, of the negative charges (Strickfaden et al., 2007). Therefore, Ste5 differs from glycogen phosphorylase in two fundamental and ultimately interconnected ways. First, glycogen phosphorylase requires only a single phosphorylation to dramatically change the behavior of the protein, whereas each of the many phosphosites within Ste5 contributes fractionally to the inhibition. Second, glycogen phosphorylase is allosterically regulated, whereas Ste5 is not.

The Electostatic Switch Model

In the mid-1990s, Stuart McLaughlin suggested an intriguing mode of phospho-dependent regulation that he called the myristoyl-electrostatic switch (McLaughlin and Aderem, 1995). He and others had shown that polybasic peptides would bind through coulombic attraction, albeit weakly, to acidic phospholipids (Ben-Tal et al., 1996). Furthermore, he observed that the MARCKS protein contains a cluster of basic residues that, in conjunction with a myristate chain, provide enough binding energy to anchor the protein to the plasma membrane. Embedded within this basic region are three phosphorylation sites and, upon phosphorylation, MARCKS falls off the membrane (Kim et al., 1994). A similar biophysical study with the myristoylated and basic N terminus of Src and a pair of phosphosites had the same outcome (Murray et al., 1998). McLaughlin found that "the membrane association increases with a larger percentage of acidic lipid in the membrane, a greater number of basic residues in the peptide, and a reduction in the ionic strength, whereas it is independent of the chemical nature of both the basic residues and the acidic lipid." These observations strongly suggest that the binding is due to nonspecific electrostatic attraction and that phosphorylation reduces the affinity by reducing the net positive charge of the peptide.

The net free energy of association of a modest macromolecular interaction (K_d = 1 μM) is 8.3 kcal/mol. McLaughlin's biophysical measurements indicate that each phosphorylation lowers the equilibrium constant (K_{eq}) by about a factor of 10, corresponding to $\Delta\Delta G$ of 1.4 kcal (Murray et al., 1998). Thus, given that the electrostatic repulsive force provided by the addition of two negative charges is small compared to the scale of most biologically meaningful binding energies, only the cumulative effective of several phosphorylation sites will completely disrupt an interaction. In addition the location and primary structural context of the phosphosite, provided that it is close to the polybasic region, is relatively unimportant, and thus might be relatively easy to evolve.

The essential features of McLaughlin's model are found prominently in the regulation of Ste5's association with the plasma membrane. For Ste5, the role of myristoylation is played by the weak protein-protein interaction with the receptor-activated Gβγ dimer. The multisite phosphorylation of Ste5 first weakens the association of Ste5 with the membrane and then ultimately reverses it. If the McLaughlin data apply, each Ste5 phosphorylation decreases the binding energy by (probably) about 1.4 kcal/mol.

Switch or Rheostat?

There are clear parallels between the regulation of Ste5 as elucidated here and that of Sic1, a well-studied stoichiometric inhibitor of CDKs (Nash et al., 2001). Both proteins are inhibited by CDK phosphorylation at multiple poorly conserved sites. In addition, both are embedded in double-negative feedback loops: Ste5 inhibits the activation of CDKs through Far1 and CDKs block Ste5 localization through phosphorylation; Sic1 inhibits CDKs stoichiometrically and CDKs inhibit Sic1 by triggering its degradation. These systems could allow the cell to toggle between two mutually exclusive states. Bistable systems such as these are easiest to engineer if at least one leg of the double-negative feedback loop exhibits a switch-like, sigmoidal, ultrasensitive response to its upstream inhibitor. Sigmoidal responses can be generated through allostery in multisubunit cooperative enzymes. As Strickfaden et al. (2007) point out, multisite phosphorylation and bulk electrostatics might be a simpler way of producing a qualitatively similar response.

There are also parallels between Ste5 and Ets1, a transcription factor whose DNA binding is regulated by three clustered CaMKII phosphorylation sites. Each phosphorylation in Ets1 successively decreases the affinity of Ets1 for DNA, by about 0.4 kcal/mol (Pufall et al., 2005), less than that measured by McLaughlin and assumed here for Ste5. This occurs by shifting Ets1 from an autoinhibited state to a DNA-binding state through bulk electrostatic interactions between the phosphorylated cluster and the DNA-binding ETS domain (Pufall et al., 2005). However, in this case, the regulation of Ets1 is said to resemble a rheostat, not a switch. There are other examples, such as the multisite phosphorylation of the Kv2.1 potassium channel, which produces graded, additive changes in channel properties (Park et al., 2006). This is a conundrum. Does multisite phosphorylation and bulk electrostatics produce a graded response in some con-

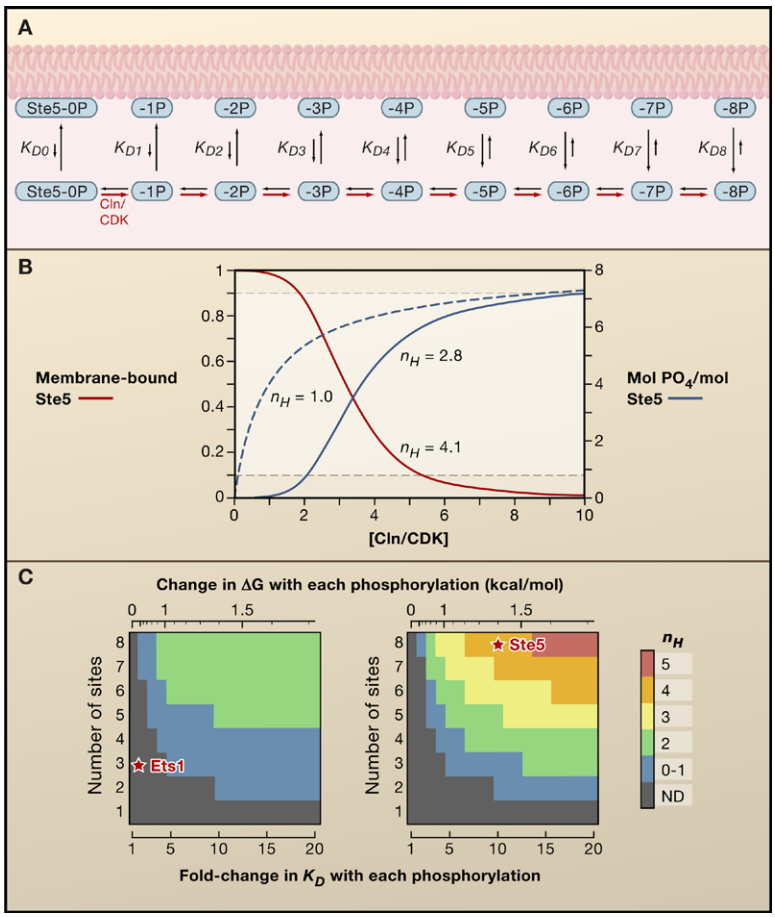

Figure 1. Electrostatic Switches and Rheostats

(A) Schematic model of Ste5 phosphorylation and dephosphorylation. For simplicity we omit the interaction of Ste5 with Gβγ, as well as the interaction of Ste5's PH domain with PIP_2.

(B) Calculated steady-state levels of phosphorylation (blue lines) and membrane binding (red line) for Ste5. The dashed blue line assumes no membrane binding. The solid lines assume that there is membrane binding and that the membrane shields Ste5 from kinases and phosphatases. Details of the modeling can be found as Supplemental Data.

(C) Heat map representation of the effective Hill coefficients for membrane binding as a function of the number of phosphorylation sites and the free energy increment produced by each phosphorylation. Hill coefficients are calculated based on the fold-change in kinase activity needed to drive the system from 90% membrane binding to 10% membrane binding by the formula

$$n_H = \frac{\log[81]}{\log[EC10/EC90]}$$

For parameter choices that yielded maximum levels of binding of less than 90%, minimum levels of binding of more than 10%, or both, the Hill coefficients are depicted as "ND." The map on the right assumes that the kinases and phosphatases act only on the free species, whereas the map on the left assumes that the enzymes act equally well on the free and bound forms. The expected Hill coefficients for Ste5 membrane binding and Ets1 DNA binding are indicated by stars.

texts and a switch-like response in others? If so, what determines the qualitative character of the response?

To address this question we begin with a simple kinetic model of the phosphorylation and dephosphorylation of Ste5 at eight sites (Figure 1A). A system like this produces a switch-like, ultrasensitive response to changes in the concentration of active CDK only if the phosphorylation of the last few sites is more favorable than the phosphorylation of the first few (Gunawardena, 2005). This requirement is analogous to the concept of cooperativity in multisubunit, allosteric enzymes. So does the phosphorylation of Ste5 at the first few sites promote its phosphorylation at the last few?

In the absence of membrane binding, our guess is that it would not, based on simple combinatorics. There are eight ways to convert Ste5-0P (with no phosphorylations) to Ste5-1P and only one way to convert Ste5-7P to Ste5-8P, so that other things being equal, we would expect the effective rate constant for the first phosphorylation to be eight times that of the eighth. When the phosphorylation rate constants become successively smaller and the dephosphorylation rate constants successively larger as phosphorylation increases, the result is that phosphorylation becomes a very graded function of kinase concentration (Figure 1B, dashed blue curve). Phosphorylation is a rheostat here.

However, introducing membrane binding can dramatically change things. If one assumes that only free Ste5 can be phosphorylated and dephosphorylated, with membrane binding shielding Ste5's phosphorylation sites from Cln/CDK and the relevant phosphatase, then as each phosphate is added to Ste5, there is successively more and more free Ste5 available for phosphorylation. In this way, phosphorylation promotes more phosphorylation, and the result can be a highly switch-like response (Figure 1B, solid blue line). The system now shifts from having most of the Ste5 hypophosphorylated to most of it hyperphosphorylated over a relatively narrow range of kinase concentrations (Figure 1B).

Some additional sharpening of the response arises because of the exponential dependence of binding constants on binding energy. Changing the phosphorylation state of Ste5 from, say, four phosphates to five increases the phosphorylation by only 1.25-fold but would be expected to change the membrane binding constant by 10-fold. This sharpens the response further still (Figure 1B, red line). A high Hill coefficient would be expected for Ste5 membrane binding as long as the number of phosphorylation sites involved is not much less than 8 and the change in binding constant per phosphorylation is not much less than 10-fold (Figure 1C, right).

So is Ets1 actually a rheostat in terms of binding energy but a switch in terms of binding constants? Probably not. The degree of ultrasensitivity produced by bulk electrostatics depends both on the amount of binding energy produced (or taken away) by each phosphorylation and also on the number of sites. For the number of sites (3) and the amount of binding energy (0.4 kcal/mol) measured for Ets1, the protein acts like a rheostat, not a switch (Figure 1C, left).

Evolutionary Plasticity

Phosphorylation sites are frequently found in regions of proteins predicted to be unstructured (Dunker et al., 2002), which are often poorly conserved (Brown et al., 2002). Both observations are at odds with an allosteric model of phosphorylation but are consistent with mechanisms employing bulk electrostatic properties. Even though Ste5 homologs in *Ashbya gossypii* and *Kluyveromyces lactis* have less than 30% identity with *S. cerevisiae* Ste5, helical wheels anchored by a conserved basic-basic-W-T-E motif reveal basic and hydrophobic faces. In all three proteins, the regions on either side of the basic stretch do contain an abundance of SP and TP motifs, though their positions vary widely, supporting the notion that position is relatively unimportant. In principle both the binding affinity and the cooperativity of a response to kinase activity in these multiply phosphorylated proteins can be fine-tuned through the straightforward evolutionary emergence or disappearance of phosphosites.

The loose spatial requirements of the phosphosites may also allow for overlapping regulation by different upstream kinases. David Morgan's group has recently shown that the kinases Ime2 and Cdk1 have different optimal peptide substrate specificities, but nonetheless both phosphorylate an overlapping set of substrates. Cdh1, for example, has 11 consensus CDK sites and 5 distinct consensus Ime2 sites, and phosphorylation by either kinase inhibits Cdh1's ability to activate the APC (D. Morgan, personal communication). The reliance on multiple spatially distributed phosphosites suggests a mechanism akin to the electrostatic switch.

Recently our group has been studying the control of another important cell-cycle regulator, Wee1, by multisite phosphorylation. Like Ste5, Wee1 is phosphorylated at several poorly conserved CDK sites. In this case, these sites appear not to function as an electrostatic switch but rather to buffer the activity of Cdk1, generating ultrasensitivity through a competition mechanism (Kim and J.E.F., unpublished data). As with the electrostatic model, the structure and local sequence surrounding the phosphosites in this competition model need not be rigidly defined.

Final Word

Ste5 has provided the field of protein regulation with a strikingly large number of important insights down through the years, contributing to our ideas of signaling scaffolds (Choi et al., 1994), docking domains (Kusari et al., 2004), and enforced proximity (Park et al., 2003), among others. All of these concepts are widely appreciated to apply to virtually all signaling pathways. Now Ste5 has provided a beautiful example of how multisite phosphorylation can be read out via bulk electrostatic effects to yield a powerful biochemical switch (Strickfaden et al., 2007). Post-translationally tuned bulk electrostatics provides a simple and effective alternative to allostery for regulating protein function. We suspect that this too will prove to be a recurring theme in protein regulation.

Supplemental Data

Supplemental Data include details of the modeling and can be found with this article online at http://www.cell.com/cgi/content/full/128/3/441/DC1/.

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