How signaling proteins integrate multiple inputs: a comparison of N-WASP and Cdk2

Kenneth E Prehoda and Wendell A Lim*

Signal transduction proteins that can integrate multiple upstream signals play a critical role in the complex regulatory circuits that control cellular behavior. The two signaling node proteins cyclin-dependent kinase 2 and the actin regulator neuronal Wiskott–Aldrich syndrome protein have qualitatively similar signaling properties. Recent studies, however, reveal that these proteins utilize distinct mechanisms of signal integration, leading to subtle but important quantitative differences in behavior.

Addresses
Institute of Molecular Biology and Department of Chemistry, 1229 University of Oregon, Eugene, OR 97403-1229, USA; *Department of Cellular and Molecular Pharmacology, Department of Biochemistry and Biophysics, 513 Parnassus Avenue, University of California, San Francisco, CA 94143-0450, USA;
e-mail: wlim@itsa.ucsf.edu
Correspondence: Wendell A Lim

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Abbreviations

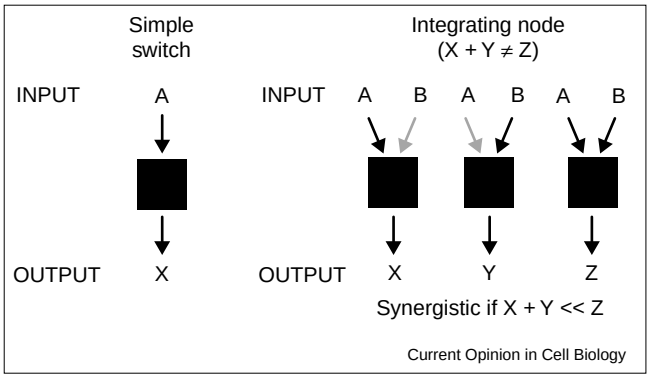
ARP 2/3	actin-related protein 2/3
CAK	Cdk-activating kinase
Cdk	cyclin-dependent kinase
GDP	G-protein-binding domain
N-WASP	neuronal Wiskott–Aldrich syndrome protein
PIP2	phosphatidylinositol (4,5)-bisphosphate
SH2/3	Src homology 2/3

Introduction: signaling nodes are points of pathway integration

Cellular responses to external stimuli are controlled by diverse signal transduction pathways. Over the past decade, it has become clear that most pathways do not transmit information in a simple linear fashion; rather, what at first appear to be individual pathways are actually highly interconnected networks. An emerging goal in modern cell biology is to understand the cellular circuitry by mapping the regulatory network connections and quantitatively understanding network properties that yield precise and sophisticated biological control.

To quantitatively understand the behavior of signaling networks, some of the most crucial elements to study are signaling nodes — the connection points between multiple pathways. Nodes can be defined as signaling elements that can detect multiple upstream inputs. Signal integrating nodes are a subset of nodes that yield differential responses depending on the combination of inputs present

Figure 1



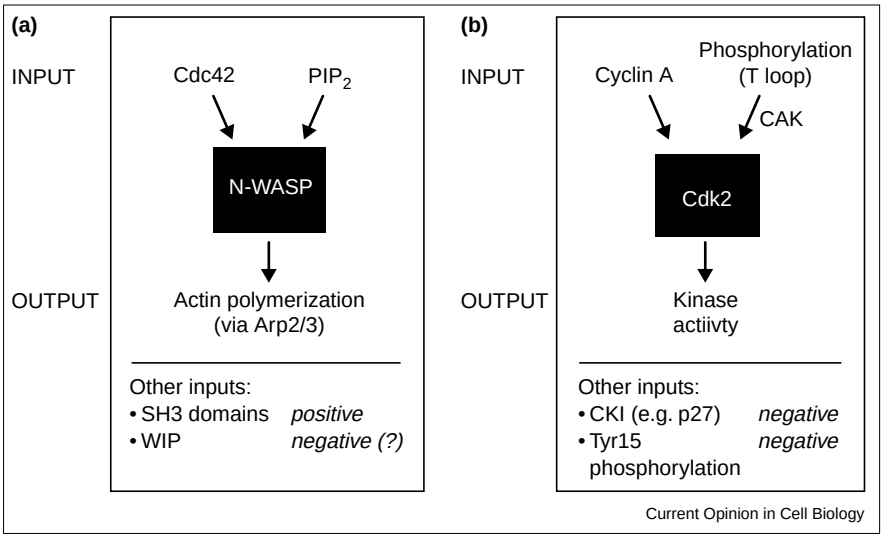
The distinction between simple switch and signal integrating node. A simple signaling switch produces only output X upon stimulation with input A. An integrating node responds to at least two different inputs, A and B, but the responses to both inputs simultaneously, Z, is distinct from the sum of the responses, X and Y, that result from each input alone. The node displays synergistic effects, or positive integration, if the response to both inputs simultaneously is greater than the sum of the individual responses.

(Figure 1). Simple signaling switches respond to an input *A* with a response *X*, but are only useful in transmitting information within a linear pathway. Signal integrating nodes, in contrast, can detect multiple inputs (*A* and *B*). A critical requirement for signal integration is that the simultaneous presentation of inputs A and B yields a response, *Z*, that is qualitatively or quantitatively distinct from the sum of the responses to input A and input B individually (responses X and Y, respectively). Integration could be either positive ($Z >> X + Y$) or negative ($Z << X + Y$).

Integrating nodes are critical for linking the effects of multiple pathways and can allow for highly specific combinatorial input control of a single response. For example, promoters often function as integrating nodes: transcription is highly dependent on the convergent presence of a particular combination of induced transcription activators, as well as the absence of inhibitory factors [1–4]. Moreover, integrating nodes allow for complex behavior such as feedback control, in which a downstream response can also act as an additional input for upstream nodes.

What are the molecular and thermodynamic mechanisms by which signal integration can be achieved? In this review, we will focus on intracellular node proteins capable of positive signal integration. This behavior is often described using the vaguely defined term ‘synergistic’, since in most cases response of these proteins to single inputs is weak, whereas the response to co-stimulation by

Figure 2



Two examples of positive integrating node proteins. (**a**) The actin regulatory protein N-WASP can activate the Arp2/3 complex, stimulating actin filament nucleation. However, under basal conditions N-WASP is repressed. The inputs Cdc42 (GTP-bound) and PIP2 are poor activators alone, but together are potent activators. (**b**) The cell-cycle regulatory protein Cdk2 requires two inputs to display maximal kinase activity: cyclin A must bind and Thr160 in the T loop must be phosphorylated by CAK. Both of these nodes integrate other positive or negative inputs, as indicated. However, we do not focus on these other inputs here.

multiple inputs is strong. Positive integrating nodes therefore qualitatively approximate logical AND gates — the digital circuit elements that require two inputs to yield a response. Here, we will examine two such protein nodes: the actin regulatory protein N-WASP (neuronal Wiskott–Aldrich syndrome protein), and the cell cycle regulatory protein Cdk2 (cyclin-dependent kinase 2). Despite their qualitative similarities, recent studies have revealed that these two nodes use different mechanisms, which leads to subtle but important quantitative differences in signal integration properties. Understanding these distinct mechanisms of signaling integration is likely to be necessary in order to understanding the behavior of larger, complex cellular networks.

N-WASP and Cdk2: two nodes that approximate AND gates

N-WASP integrates Cdc42 and phosphatidylinositol 4,5-bisphosphate stimulation of actin polymerization

Actin polymerization is regulated in a precise spatial and temporal manner, to provide the mechanical force required for cell movement, morphogenesis and endocytosis. Directed movement requires the coordinated action of many signaling pathways. WASP and its more widely expressed homologue N-WASP have emerged as central node proteins that regulate actin polymerization in response to multiple upstream signals [5–7].

Output activity of N-WASP — actin polymerization — is mediated through the actin-related protein 2/3 (Arp2/3) complex, a seven-protein complex capable of stimulating actin filament nucleation [5,7]. Basal activity of the Arp2/3 complex is low, but can be dramatically increased by interaction with N-WASP. The Arp2/3 stimulatory activity of N-WASP, however, is itself tightly regulated under basal conditions: intact N-WASP is a very poor activator of Arp2/3 but becomes fully active upon stimulation with certain upstream signaling molecules [8]. A number of these upstream activators of N-WASP (Figure 2a) have been identified, including the Rho GTPase Cdc42, the acidic phospholipid phosphatidylinositol (4,5)-bisphosphate (PIP2) [9••,10••], and several Src homology 3 (SH3)-containing proteins including Nck, Grb2 and WISH [11–13]. Cdc42 and PIP2 are the best characterized of these.

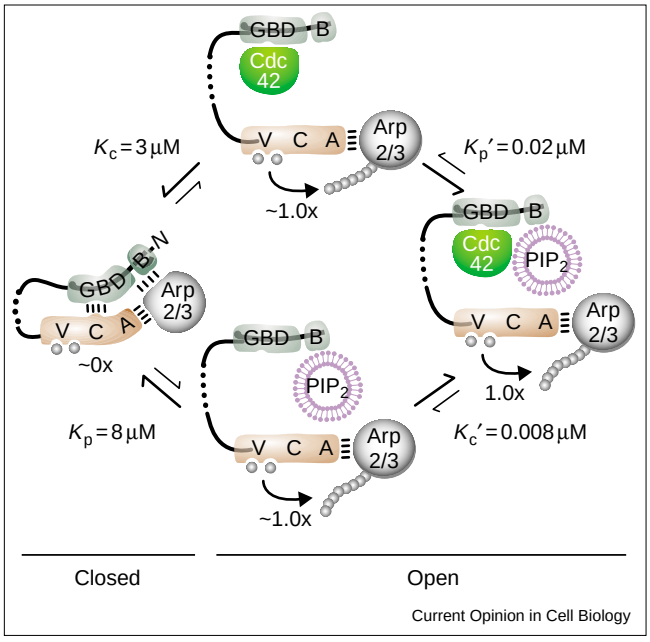
Activation of N-WASP by GTP-bound Cdc42 and PIP2 is highly synergistic: either input alone is a poor activator, but co-stimulation yields potent activation. Thus N-WASP approximates an AND gate, requiring both inputs to promote potent Arp2/3-mediated actin polymerization. Integrated activation by Cdc42 and PIP2 may help specifically target actin polymerization to membrane sites at which both inputs are present.

Cdk2 integrates the effects of cyclin binding and phosphorylation in cell cycle regulation

The eukaryotic cell cycle requires precise temporal coordination of major events, ranging from DNA synthesis to mitosis. These events are controlled largely by a class of proteins known as the cyclin-dependent kinases. As might be expected, Cdk activity is extremely tightly regulated, by multiple inputs (reviewed in greater depth elsewhere [14]). Cdks are now known to be involved in regulating several cellular processes, but here we focus only on one of the best-studied Cdks — the vertebrate Cdk2 protein.

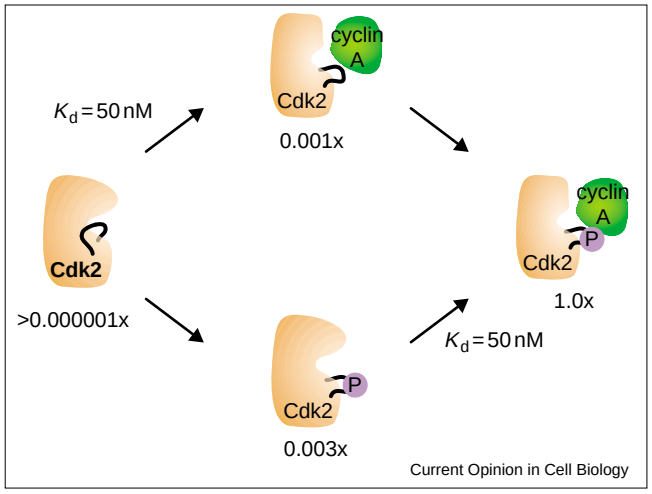
Full activation of Cdk2 requires two inputs (Figure 2b). First, Cdk2 must bind to a cyclin subunit, a polypeptide whose concentration oscillates over the course of the cell cycle. (We will focus on the interaction of Cdk2 with cyclin A, which is present during S phase and G2.) Second, Cdk2 must be phosphorylated on residue Thr160, in a segment

Figure 3



Model for N-WASP integration of Cdc42 (GTP-bound) and PIP2 inputs. N-WASP behaves as two-state ('closed' or 'open') system. The closed state is stabilized by auto-inhibitory interactions involving the B and GBD modules. These interactions inactivate the output VCA domain. The two inputs bind to these modules and thus individually stabilize the open state. However, binding of a single input is unfavorable in the absence of the other input because the initial binding ligand must disrupt the competing auto-inhibitory interactions. The number below each model indicates relative output activity (i.e. actin polymerization rate). Dissociation constants (K_c, for Cdc42; K_c', for Cdc42 in the presence of saturating PIP2; K_p, for PIP2; K_p', for PIP2 in the presence of saturating Cdc42) estimated for each reaction are also shown.

Figure 4



Model for Cdk2 integration of cyclin A and T-loop phosphorylation inputs. N-WASP behaves as multistate system. Each intermediate state is distinct and only gains partial activity far below maximal output, which is only observed when saturated with both inputs. The number below each model indicates relative output activity (k_{cat}). Dissociation constants (K_d), where known, are also shown.

referred to as the 'T loop'. Phosphorylation is catalysed by the Cdk-activating kinase (CAK). There are also several known inhibitory inputs for Cdk2, which we will not discuss here but are reviewed elsewhere [15]. Our main focus here will be on understanding how Cdk2 approximates an AND gate, displaying full activity only upon cyclin A binding and T-loop phosphorylation.

N-WASP signal integration

N-WASP is a cooperative, two-state switch

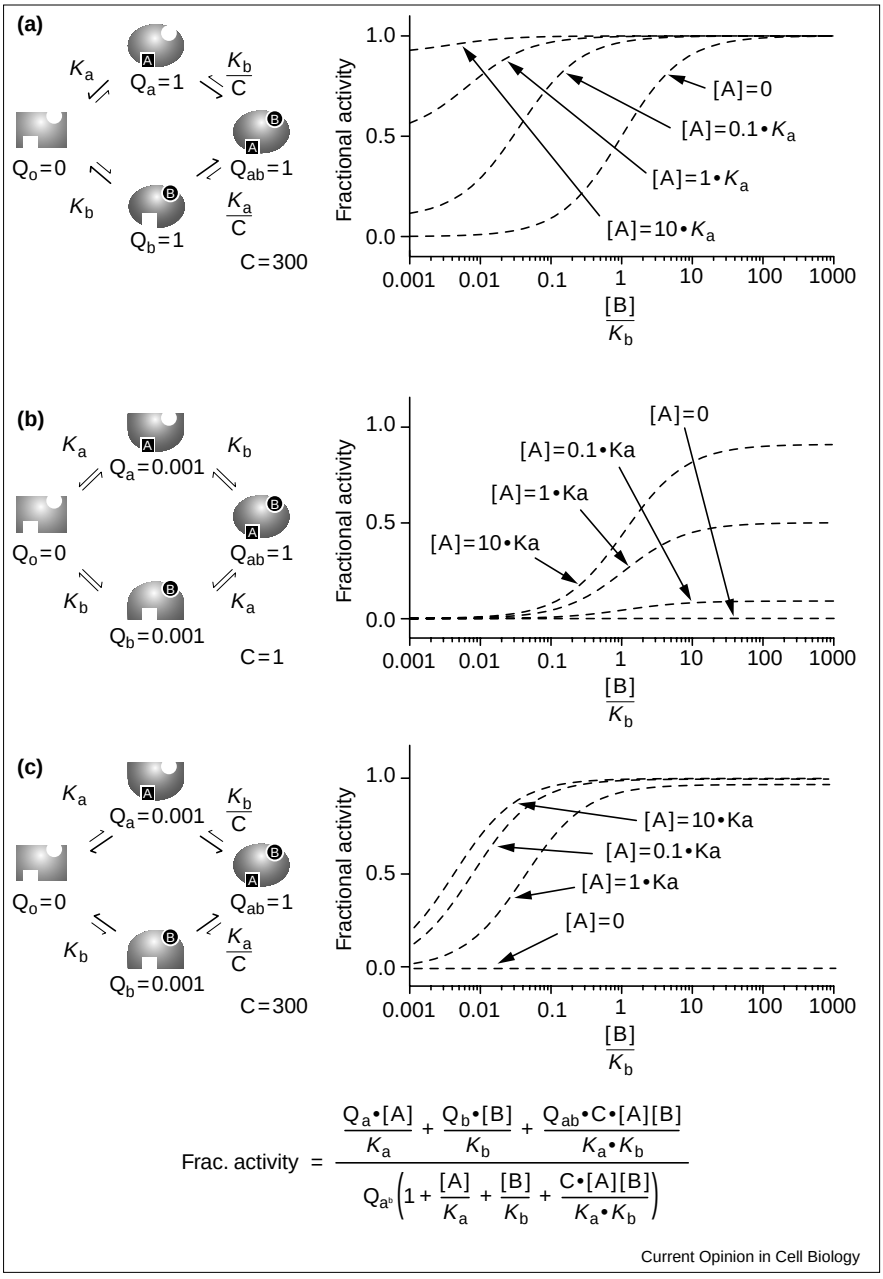
Recent mechanistic studies suggest that N-WASP, with respect to Cdc42 and PIP2 activation, acts as a two-state switch — the protein can exist in either an active 'open' state or an inactive 'closed' state (Figure 3). It is clear that N-WASP acts to integrate Cdc42- and PIP2-based signals because these two inputs act in a highly cooperative manner to stabilize the active state. More specifically, N-WASP has a constitutively active output domain (referred to as the VCA domain) at its carboxyl terminus. This domain is sufficient to bind and fully activate the Arp2/3 complex [8]. However, in the intact protein amino-terminal regulatory regions act in concert to lock the protein in an inactive, closed state, through a set of auto-inhibitory interactions. Specifically, two domains are critical for auto-inhibition: a highly basic motif (B) and the adjacent G-protein-binding domain (GBD) [9••,10••]. PIP2 and Cdc42 activate the protein by binding the B and GBD modules, respectively, in a manner that disrupts the auto-inhibitory interactions. Thus, binding of the inputs acts to stabilize the open state in which the VCA domain is released and fully activated.

Cooperativity of input binding is observed — initial binding of one input pays the energetic cost of disrupting the closed state, thereby increasing the apparent affinity of the second input by a cooperativity factor c. *In vitro* experiments indicate that the c factor for N-WASP activation is approximately 300 [10••]. Such data were generated using soluble Cdc42. *In vivo*, where Cdc42 is prenylated and membrane-localized, the high effective concentration of Cdc42 and PIP2 at membrane sites may yield an even higher apparent cooperativity factor.

N-WASP approximates an AND gate only at low input concentrations

N-WASP is not an absolute AND gate. In fact, both Cdc42 and PIP2 can fully activate model N-WASP constructs alone, albeit only when present at very high concentrations. However, at low concentrations (below the apparent dissociation constant [K_d] for each isolated activator) N-WASP does *approximate* an AND gate. Under these conditions, each input alone is insufficient to induce significant activation. However, both inputs together, because they cooperatively bind and stabilize the open state, can yield potent activation. Thus, the behavior observed for this switch depends highly on the concentrations of the

Figure 5



Input/output behavior of the general classes of two-input switches. Activity as a function of concentration of the two inputs (A and B) is calculated using the general equation given at the bottom of the figure. Each class differs in the relative activities (Q_n [activity coefficient for state n]) of the different input bound states, and the degree of cooperativity (C) between inputs A and B. **(a)** A cooperative, two-state switch (e.g. N-WASP) integrates input effects based on cooperativity. One input increases sensitivity to the other but does not alter maximal output activity. **(b)** The multistate switch (e.g. Cdk2) attains only minimal fractional activity when bound to either individual input (Q_a and Q_b <> Q_{ab}), and lacks input binding cooperativity. One input does not affect the other's sensitivity but alters maximal output activity. This type of switch is ideal for generating stable intermediate activity levels. **(c)** A cooperative, multistate switch best approximates the all-or-none behavior of a digital AND gate. No significant activity is observed with a single input, but nearly maximal activity is attained with minimal concentrations of both inputs. Frac, fractional.

inputs. At high concentrations (greater than the K_d for each isolated activator), little or no integration is observed (effects are additive or less than additive). At low concentrations (below K_d), however, high levels of integration are observed (i.e. the effects are more than additive). The main driving force for integration in this case is the cooperativity of input binding.

Cdk2 signal integration

Cdk2 is a multistate switch

Mechanistic and structural studies indicate that Cdk2 functions in a very different manner from N-WASP (Figure 4). Isolated Cdk2 has very low activity, for two apparent reasons: first, ATP is bound in a catalytically non-productive orientation; and second, the T-loop segment occludes the Cdk2 substrate-binding site [16••]. However, addition of each of the multiple positive inputs results in distinct kinase states, with incrementally increased kinase activity.

Binding of one input, cyclin A, to Cdk increases kinase activity (k_{cat}) by several orders of magnitude. (Exact estimates are difficult, given the problems in detecting any activity for unbound Cdk.) [17•]. Cyclin A binding leads to conformational changes that place ATP into a catalytically productive orientation [18••]. In addition, the T loop moves and no longer occludes the substrate-binding site.

For full activation of the Cdk2–cyclin A complex, however, a second input is required: phosphorylation of Thr160 in the T loop by CAK. Phosphorylation of the T loop in the presence of cyclin A leads to subtle conformational shifts thought to increase peptide substrate affinity and orient the peptide to optimize phosphoryl transfer [19••,20•]. This second input further increases maximal k_{cat} around 1000-fold [21•]. The increase in catalytic efficiency (k_{cat}/K_M), however, is around 100,000-fold [21•].

T-loop phosphorylation alone (in the absence of cyclin A) also appears to result in an incremental increase in kinase activity, comparable to that observed for cyclin binding alone [22•]. The activity of the phosphorylated-only intermediate state is ~300-fold lower than the fully activated Cdk2 complex. T-loop phosphorylation alone results in increased affinity for both ATP and protein substrate [22•]. In summary, both individual inputs increase kinase activity to intermediate levels. The activity of these intermediate states is ~300–1000-fold lower than that of the fully activated kinase.

T-loop phosphorylation and cyclin A binding appear to be non-cooperative

Recent biophysical studies estimate that cyclin A binds Cdk2 with a dissociation constant of ~50 nM [22•], and this affinity appears to be unaffected by the phosphorylation state of Cdk2. Thus, the two inputs — cyclin binding and T-loop phosphorylation — do not appear to be strongly linked thermodynamically (in contrast to the strong coupling of the two inputs for N-WASP). It should be noted that this may not be true for all classes of Cdk–cyclin pairs [23]; however, for Cdk2 and cyclin A at least, cooperativity between the two inputs does not appear to play a role in signal integration.

Cdk2 approximates an AND gate under all conditions

Cdk2 appears to more rigorously fit the definition of an AND gate than N-WASP. AND-gate-like behavior is independent of input levels (i.e. cyclin A concentration or the degree of phosphorylation). Each input generates a separate intermediate state that, even under saturation conditions (or full phosphorylation), has only minimal kinase activity (1000-fold lower than fully activated). Thus under all conditions, both inputs must be present to achieve full activity. Cooperativity does not drive signal integration; signal integration is driven by the requirement that both inputs be present to assemble a unique, fully active state, distinct from that stabilized by either input alone.

Comparison of integration mechanisms

These two integrating switches, N-WASP and Cdk2, appear to represent two very different mechanism for achieving qualitatively similar signal-processing behavior. We have schematically modeled the behavior of these switches in Figure 5. Here, for simplicity, we have treated phosphorylation as a binding event. On the basis of these models, we have also plotted activity as a function of the concentration of one input (B), under conditions of different constant concentrations of the other input (A). These results reveal subtle but important functional differences between these switches.

Two-state cooperative switches allow tuning of input sensitivity

This type of switch (Figure 5a) is not an absolute AND gate, since addition of high concentrations of the input B yields maximal activation, even in the absence of input A. However, it is clear that with increasing constant concentrations of input A, the concentration of B required to trigger activation decreases. Thus, input A increases the sensitivity of the switch to input B, although it does not change the maximal output achievable by input B. At low concentrations of both inputs (i.e. those lower than K_d), however, the system will closely approximate an AND gate. The stronger the cooperativity, the more this AND-gate-like behavior will be accentuated. However, in all cases this behavior will be restricted to the low input concentration range.

Multistate switches allow stable tuning of output levels

A multistate switch such as Cdk2 more closely approximates an absolute AND gate (i.e. full activity requires both inputs). Figure 5b reveals that this type of switch can be used to tune effectively output levels — changing the concentration of input A does not change the sensitivity of the switch to input B. Rather, it changes the maximal output level achievable by input B. Thus, this type of switch may control activity levels in a more robust and stable manner (independent of small input-B concentration fluctuations). The tight, noise-free control of activity levels afforded by this type of switch may be required for a process such as cell-cycle regulation, for which consequences of incorrect activity levels could be dramatic.

One cost for this kind of control, however, is that overall sensitivity is low compared with the cooperative two-state switch — either high amounts of input or high input affinities are required to reach maximal activity (the curves in Figure 5b are shifted to the right compared with those in Figure 5a). Thus, unlike the above case, for a given concentration range of inputs, the required high-affinity interactions may yield a less dynamic and reversible switch. This type of switch therefore may be better suited for relatively slow time-scale processes, such as regulation of the cell cycle, but poorly suited for highly dynamic processes, such as actin-mediated cell motility. Covalent modification, such as phosphorylation, may be a particularly good input to control this type of switch. Phosphorylation is essentially an irreversible reaction (infinite affinity) that can be kinetically controlled by upstream kinases and phosphatases.

Conclusions

We have focused on two types of node proteins capable of positive signal integration. The two mechanisms for

integration probably represent extreme cases, and other node proteins may use mechanisms that have aspects of both (i.e. both cooperative and multistate properties; Figure 5c). It is striking that two such different mechanisms can yield qualitatively similar behavior; however, it is also clear that the different mechanism have critical quantitative differences in behavior. These different properties lend themselves to particular biological situations, both with respect to the relevant input concentrations and fluctuations and to the required degree of output control.

The differences between integrating nodes also demonstrates how critical it is to understand the quantitative behavior of these nodes. If we are to understand and model the behavior of complex signaling networks, we must understand the precise mechanism of signaling nodes and the relevant parameters that describe this integration. As such, researchers will need to study such proteins in a complete manner. It is insufficient to qualitatively state that two inputs show synergistic effects. It is critical to know how each input effects activity over a wide concentration range, up to and including saturation. It is critical to observe the quantitative degree of integration over a wide range of both input concentrations. It is also critical to know the biologically relevant concentrations of inputs. With this knowledge, we can then proceed to understand how ensembles of many such nodes can form networks capable of generating the sophisticated behavior of a cell.

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