The modular logic of signaling proteins: building allosteric switches from simple binding domains

Wendell A Lim

Many eukaryotic signal transduction proteins have component-based architectures: they are built from combinations of protein interaction domains and catalytic domains. Intact, these proteins display the sophisticated allosteric behavior required for cellular regulation; the protein's output activity is tightly repressed under basal conditions, but can be robustly activated by a specific set of input effector ligands.
A combination of structural, biophysical and computational studies is beginning to shed light on the fundamental principles governing this type of modular allostery.

Addresses
Department of Cellular and Molecular Pharmacology, Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94143-0450, USA; e-mail: wlim@itsa.ucsf.edu

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Abbreviations

Arp2/3	actin-related protein 2/3
B	basic
Csk	C-terminal Src kinase
GBD	G protein binding/switching domain
N-WASP	neuronal WASP
PIP_2	phosphatidylinositol-4,5-bisphosphate
SH	Src homology
SHP-2	SH2-containing phosphatase-2
VCA	verprolin cofilin acidic
WASP	Wiskott–Aldrich syndrome protein

Introduction

Many intracellular signaling proteins function as molecular switches: stimulation by upstream input events such as ligand binding, covalent modification or a change in subcellular localization results in the induction of an output activity that can, in turn, modulate downstream signaling elements. These individual proteins, when assembled into interacting networks, yield the sophisticated circuits that control cellular behavior.

What are the general principles governing the structure and function of signaling proteins? In eukaryotes, many of these proteins appear to have a component-based architecture: they are built from multiple modular domains, some of which are catalytic (e.g. kinases and phosphatases), but the majority of which mediate protein–protein or protein–lipid interactions [1,2]. A comparison of metazoan genomes suggests that these domains have been extensively recombined and shuffled during evolution. The human genome has essentially the same number of types of individual modular protein domains as the worm and fly; however, almost twice the number of multidomain combinations (occurrences in the same polypeptide) are found in human proteins [3]. Thus, the fundamental modular domain toolkit, despite changing little since the evolution of the roundworm, appears to have been used in a combinatorial fashion to increase phenotypic complexity.

Although much is known about the structure and mechanism of individual signaling domains, our understanding of how these simple modules are used in combination to build complex switches lags far behind. This review will therefore focus on the mechanism of 'modular allosteric switches': multidomain proteins with a core output activity that, although efficiently repressed under basal conditions, is robustly activated by a specific set of input ligands. We would like to understand how these domains are assembled to yield sophisticated switch behavior, analogous to the way in which an electrical engineer can describe how simple transistors are used to fabricate the electronic AND, OR and NOT logic gates that form the backbone of digital control circuits.

Modular allosteric switches

We will focus on three example switch proteins in order to understand how their modular architecture mediates repression, activation and, in some cases, sophisticated integration of multiple inputs. There are many other related switch proteins that space limitations preclude discussion of here. The functional input/output properties of the example proteins are summarized in Table 1 and their modular architectures are shown in Figure 1.

Src family kinases

Src family proteins are tightly regulated tyrosine kinases that contain a catalytic kinase domain and two N-terminal interaction domains: a Src homology 2 (SH2) and a Src homology 3 (SH3) domain (Figure 1a; [4–6]). SH2 domains bind specific phosphotyrosine motifs, whereas SH3 domains bind specific proline-rich motifs [7–9]. The C terminus of the protein contains a specific tyrosine motif that, under basal conditions, when Src kinases are inactive, is phosphorylated by the C-terminal Src kinase (Csk). Src kinases, however, can be activated by several inputs: SH2 or SH3 ligands, or dephosphorylation of the C-terminal tyrosine [10]. The most potent activating inputs are multivalent ligands containing both SH2- and SH3-binding motifs [11].

SH2-containing phosphatase

The SH2-containing phosphatase-2 (SHP-2; also known as SH-PTP2) contains a catalytic tyrosine phosphatase domain fused to two SH2 domains (Figure 1b; [12–14]). Under basal conditions, the phosphatase is inactive; however, SH2-binding motifs can stimulate activity. A composite bisphosphotyrosine motif, with proper spacing, is the most potent activator of this switch [15].

Table 1

Input/output properties observed for protein switches.

Protein	Output	Inputs	Complex behavior	References
Src family kinases	Targeted kinase activity	*Positive* SH2-binding motifs (p-Tyr) SH3-binding motifs (proline-rich) Activation loop phosphorylation *Negative* Csk C-terminal tyrosine phosphorylation	Composite SH2/SH3-binding motifs activate most potently	[11]
SHP-2 phosphatase	Targeted phosphatase activity	*Positive* SH2-binding motifs (p-Tyr)	Properly spaced bisphosphotyrosine motifs activate most potently	[15]
N-WASP	Targeted actin polymerization (via stimulation of Arp2/3 complex)	*Positive* Cdc42 (GTP-bound) PIP_2 SH3 domains/proteins	Synergistic (nonadditive) activation upon co-stimulation by Cdc42 and PIP_2 (AND-gate-like behavior)	[21]

N-WASP

The Wiskott–Aldrich syndrome protein (WASP; the gene product mutated in the immunodeficiency disease Wiskott–Aldrich syndrome) [16] and its more widely expressed neuronal homolog, N-WASP [17], regulate actin polymerization and cell motility in response to multiple inputs. These proteins contain multiple N-terminal modules (Figure 1c) linked to a C-terminal output domain (termed the verprolin cofilin acidic [VCA] domain) that, when isolated, binds and constitutively activates the actin-related protein 2/3 (Arp2/3) complex, a seven-protein complex that promotes actin filament nucleation [18–20]. This Arp2/3 stimulatory activity, however, is repressed in intact WASP proteins [21,22]. Multiple inputs have been reported to activate N-WASP [23]. Here, however, we focus only on the two best-understood inputs: the Rho family GTPase Cdc42 (GTP-bound state) and phosphatidylinositol-4,5-bisphosphate (PIP_2) [24,25]. One of the most striking properties of N-WASP is that, with respect to these two inputs, it behaves like a logical AND gate. Individually, Cdc42 and PIP_2 are weak activators, but together they act synergistically: co-stimulation with low concentrations of both yields potent activation [21]. Thus, N-WASP acts as a signal integration device that can precisely target actin polymerization to sites at which both PIP_2 and activated Cdc42 are present.

Repression through autoinhibition

The output activities of these protein switches are controlled by autoinhibitory intramolecular interactions (or, in some cases, intracomplex interactions) involving their modular domains. Autoinhibition can be either 'direct' or 'indirect'. SHP-2 provides an example of direct auto-inhibition: in the repressed state structure, the N-terminal SH2 domain binds and directly occludes the phosphatase active site (Figure 1b; [26]). This repression can occur even when the SH2 domains are added to the phosphatase domain in *trans* [14].

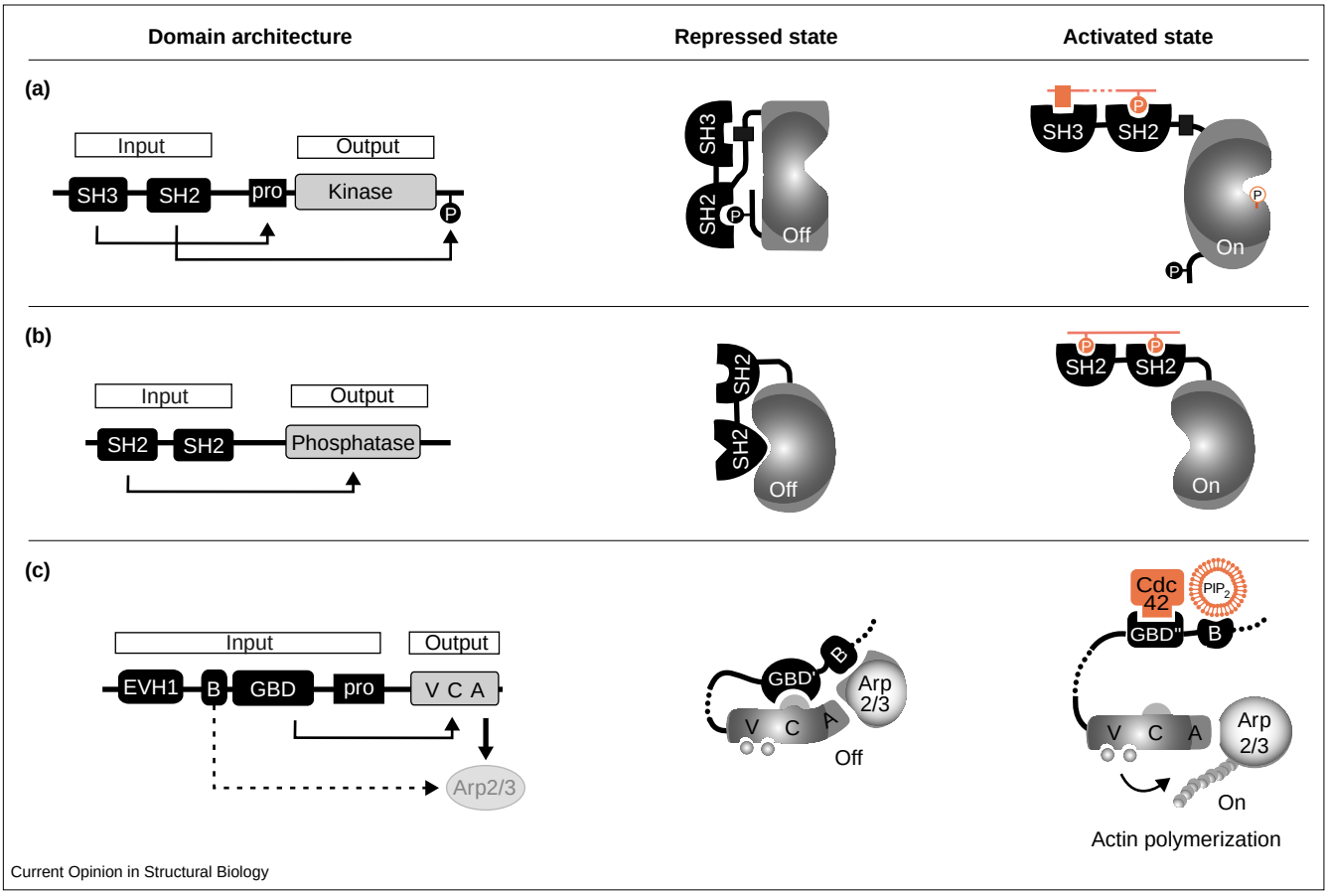
Src and N-WASP, however, provide examples of indirect, or conformational, autoinhibition. In these cases, two simultaneous intramolecular interactions are required for autoinhibition, neither of which can independently repress the output domain. In the repressed state of Src kinases, two intramolecular interactions occur: the SH2 domain binds the C-terminal phosphotyrosine motif, whereas the SH3 domain binds a proline-containing motif in the linker between the SH2 and kinase domains (Figure 1a). Neither interaction directly blocks the active site. Repression instead is allosteric; the pair of interactions leads to structural perturbations in the kinase active site [27–29].

In N-WASP, two interaction domains are also required for repression: a short basic (B) motif and the G protein binding/switching domain (GBD) (Figure 1c; [30,31••]). In the absence of activating inputs, the B motif binds the Arp2/3 complex (an intracomplex interaction), whereas the GBD binds to a small α helix in the VCA output domain (intramolecular interaction). Although neither domain represses VCA output domain activity individually, the composite B–GBD segment is strongly repressive, even when added in *trans* [31••]. Covalent linkage between the two domains is required for repression. Although the structure of the repressed N-WASP state is not known, these interactions may allosterically force the VCA domain–Arp2/3 complex to adopt an inactive conformation, as observed in the Src kinases. Alternatively, the pair of interactions might exert their effects by preventing motions required for activity.

Activation by external inputs

These switch proteins can be activated, or 'derepressed', by the binding of external ligands to the regulatory domains involved in autoinhibition. There appear to be at least two general classes of activating ligands: simple competitive activators and conformational activators. Simple competitive activators are external ligands that are closely related to the intramolecular ligands; for example, canonical SH2-binding and SH3-binding peptides or proteins can displace the

Figure 1



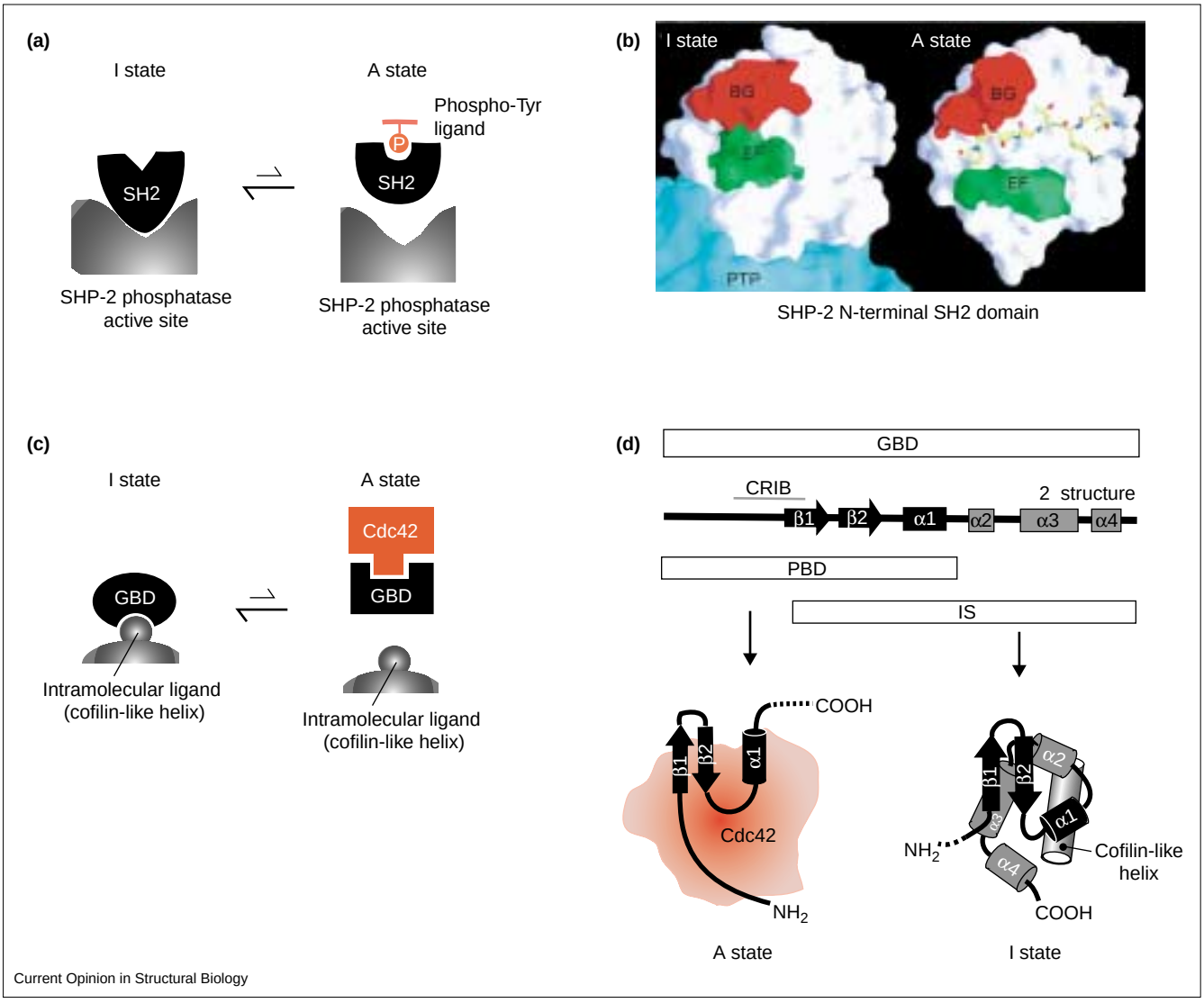
Domain architecture and autoinhibitory interactions in modular switch proteins. **(a)** Src family kinases contain N-terminal SH3 and SH2 domains, and a kinase domain flanked by intramolecular SH3-binding and SH2-binding sites (when the C-terminal motif tyrosine is phosphorylated by Csk). The crystal structures of several family members show that both intramolecular domain interactions function in concert to lock the kinase in an inactive conformation. Activating stimuli (red) include external SH2 or SH3 ligands. After initial activation, the kinase is maintained in an active state by autophosphorylation of its activation loop. **(b)** SHP-2 phosphatase contains two SH2 domains and a phosphatase domain. The crystal structure of the phosphatase shows that the N-terminal SH2 domain participates in an autoinhibitory interaction that directly blocks the phosphatase active site. Binding of external SH2 ligands activates by disrupting the autoinhibitory interaction. **(c)** N-WASP contains an Enabled VASP homology 1 (EVH1) domain, a B motif, a GBD, a proline-rich segment (pro) and an output region (VCA) that alone binds the Arp2/3 complex and stimulates its actin nucleation activity. The B and GBD motifs are required to repress activity and, by current models, are thought to participate in intracomplex interactions (only the structure of the GBD intramolecular complex for WASP is known). GTP-bound Cdc42 and PIP_2 synergistically activate N-WASP.

related intramolecular motifs in Src kinases, resulting in activation [10,32].

Conformational activators function by a somewhat more complex mechanism: although unrelated to the intramolecular ligands, they disrupt binding by causing a conformational change in the regulatory module. In SHP-2, the N-terminal SH2 domain has two binding surfaces: one for external phosphotyrosine motifs and another that docks against the catalytic domain, mediating autoinhibition (Figure 2a) [26]. This SH2 domain appears to adopt two slightly different conformational states: an inhibitory (I) state, in which the autoinhibitory interaction with the phosphatase active site is favored, and an active (A) state, in which interaction with external phosphotyrosine motifs is favored (Figure 2b). Thus, the two binding sites display negative cooperativity.

The GBD module in the WASP family proteins is also regulated by conformational activation. Like the SHP-2 N-terminal SH2 domain, the GBD can exist in two alternative states (Figure 2c): an I state, in which it binds the cofilin-like α helix in the VCA domain as a part of autoinhibition [33••], and an A state, in which it binds to the GTP-bound form of Cdc42 [34]. Structural studies indicate that the two states are mutually exclusive. Thus, Cdc42 binding disrupts the GBD autoinhibitory interaction. A related GBD module found in the P_{21} (Cdc42 or Rac) activated kinases appears to play a similar switching role to that in the WASP family proteins [35–37,38••,39]. The

Figure 2

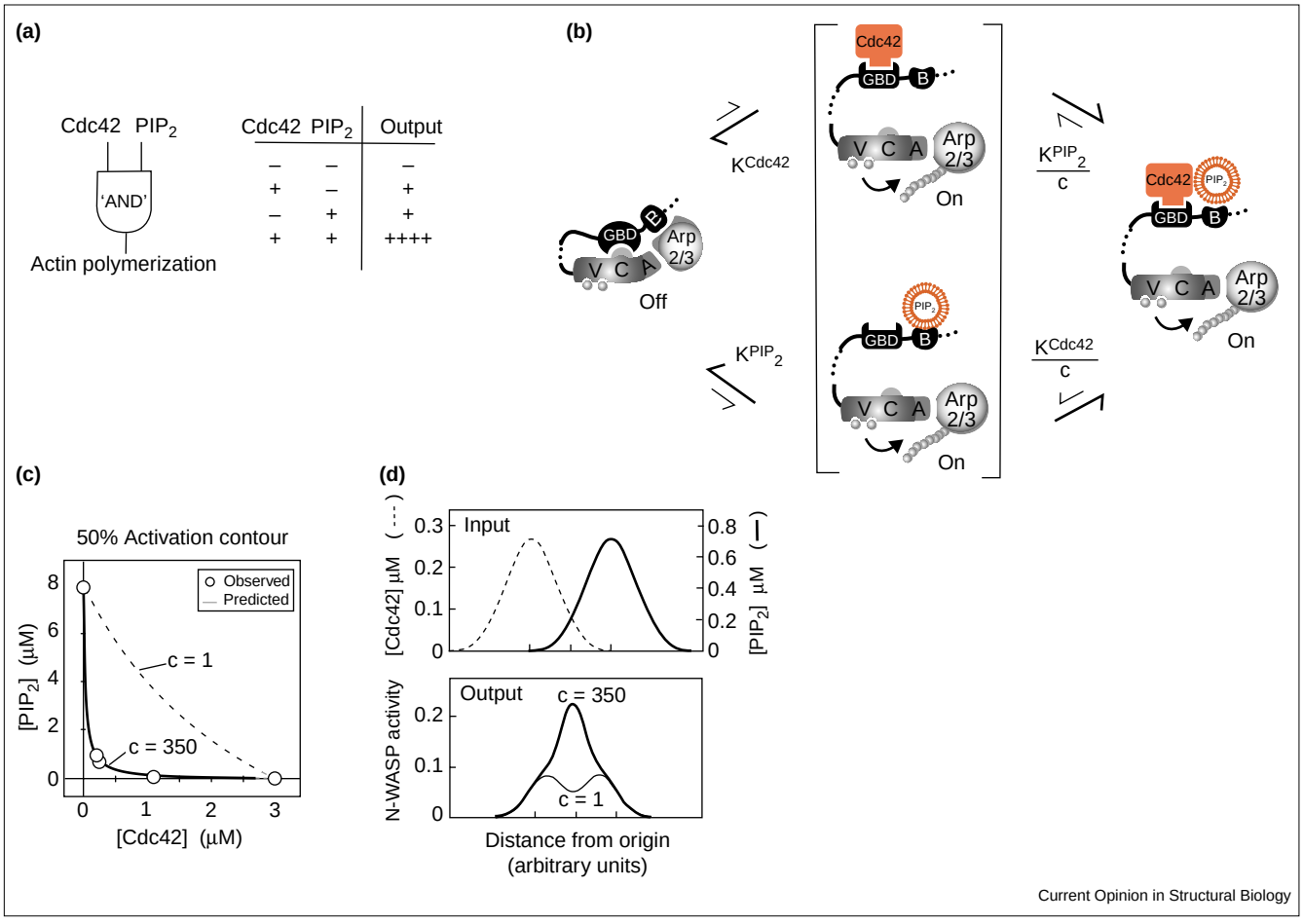


'Switching' domains allow the disruption of autoinhibitory interactions by conformational activators. **(a)** The N-terminal SH2 domain from SHP-2 can adopt two states: the inactive (I) state, which binds the phosphatase domain active site, and the active (A) state, which preferentially binds an external phosphotyrosine ligand. The two bindings events show negative cooperativity. **(b)** Comparison of the I and A states of the SHP-2 N-terminal SH2 domain. Specific loops in the domain (BG, red; EF, green) are colored to highlight the conformational displacement that occurs in the two states **(c)** WASP, N-WASP and P_{21}-activated kinases (PAKs) share the GBD, which can adopt two distinct conformational states. In the I state (black oval), the GBD binds an intramolecular α-helical ligand (in WASP family members, the cofilin-like segment within the VCA output domain). In the A state (black rectangular), the GBD binds GTP-bound Cdc42. **(d)** Structures of both states of the GBD reveal the basis for switching. The GBD is composed of two overlapping binding functions: an N-terminal P_{21}-binding domain (PBD) that includes the conserved CRIB motif and a C-terminal inhibitory segment (IS) that binds the cofilin-like helix. The overlapping regions of these two fragments (β1, β2, α1) are packed in a mutually incompatible manner in the two states. In the absence of either intramolecular or external ligands, the GBD is poorly structured.

B motif of N-WASP also behaves as a switching domain, as its autoinhibitory interactions with the Arp2/3 complex appear to be displaced by binding to the external input PIP_2 [31••]. However, little is known about the basis of the B motif interactions.

Interestingly, the GBD itself has a modular architecture: it is composed of two functionally distinct but overlapping subdomains (Figure 2d) [31••,33••,35,36,38••,40]. The first subdomain is the P_{21}-binding domain (PBD), which is homologous to other simple (nonswitching) Cdc42-interacting regions (including the canonical Cdc42/Rac interactive binding [CRIB] motif). The second is the inhibitory segment (IS), which binds to the intramolecular α-helical ligand. These two fragments share an overlapping region of sequence that adopts noncompatible structures in the A and I states. Thus, the GBD may have evolved from the overlap of two simple binding modules.

Figure 3



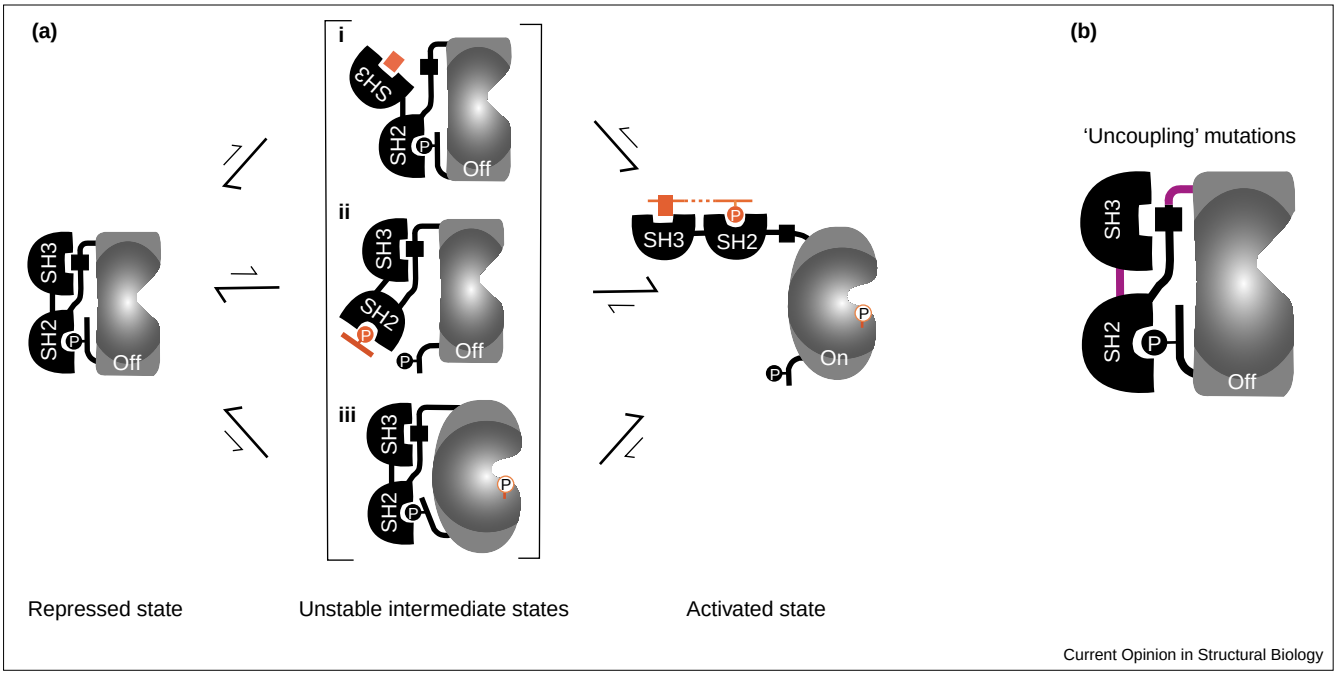
Signal integration in N-WASP is a result of interdomain cooperativity. (a) N-WASP, which regulates Arp2/3-mediated actin polymerization, approximates a logical AND gate – individually, the two input ligands, Cdc42 and PIP_2, are poor activators, but together they are potent activators. (b) Two-state model for the mechanism of signal integration. The size of reaction arrows reflects the favorability of the reaction. N-WASP can exist in two major states: first, a closed inactive state in which the two regulatory domains, B and GBD, are involved in coordinated autoinhibitory interactions; and second, an open state in which these autoinhibitory interactions are released and the output (VCA) domain is active. In the closed state, the binding of either PIP_2 or Cdc42 (red), individually, to the B or GBD domains is unfavorable; however, once one input is prebound and the closed state is disrupted, binding of the subsequent ligand is more favorable, by the cooperativity factor, c. Thus, the two ligands cooperate to stabilize the open state. Note that cooperativity is indirect – it does not involve direct interactions between Cdc42 and PIP_2. (c) Experimental signal integration of N-WASP fits the above model. Curves indicate the concentrations of the two inputs required to achieve 50% activation, simulated using the above model and a c value of 1 (no cooperativity; dotted line) or 350 (solid line). The experimental behavior of a minimal construct of N-WASP (circles) fits this model with a high cooperativity value (31). (d) Cooperativity provides a mechanism for signal integration. The top graph shows the hypothetical spatially overlapping concentration gradients of activated Cdc42 (dotted line) and PIP_2 (solid line). The bottom graph shows the calculated response of N-WASP (fraction maximal activity) assuming the model shown in (b) and a cooperativity of either 1 or 350. With high cooperativity, coincident signals are integrated and amplified.

Multi-input switches: signal integration through interdomain linkage

One of the most striking properties of these switch proteins is that they often do not display simple additive responses to activating inputs. Whereas individual inputs may be weak activators, specific combinations can yield synergistic activation. Here, we define synergistic activation to mean nonadditive activation: when the effect of the input combinations exceeds the sum of the effects of the individual inputs. Such nonadditive effects are observed with combinations of multiple ligand species, as well as with multivalent composite ligands. This type of combinatorial control yields much higher signaling specificity. How do these allosteric switch proteins integrate the effects of multiple inputs when each binding event occurs at a distinct domain? In principle, some form of coupling between domains is required.

One simple mechanism that allows composite ligands to activate more potently is the higher affinity with which they

Figure 4



Interdomain coupling in Src family kinases. (a) Thermodynamic cycle model of interdomain energetics and conformational coupling. The relative size of reaction arrows reflects the favorability of the reaction. Displacement of the individual SH domains or shift of the kinase to the active conformation (via autophosphorylation) are energetically unfavorable; however, cooperative displacement of both SH domains allows stabilization of the active state (followed by the positive feedback effect of autophosphorylation). Unstable intermediate states: i, SH3 release; ii, SH2 release; iii, kinase autophosphorylation/relaxation. (b) Uncoupling mutants are defined as mutations that yield a constitutively active kinase, although they do not disrupt the autoinhibitory interactions. Most such mutations are found in interdomain linker regions. Positions in Src kinases where uncoupling mutations have been found are colored in purple.

bind tandem interaction domains. The tandem SH2 domains of SHP-2 bind with extremely high specificity to matched bisphosphotyrosine motifs, presumably because of avidity effects [41,42]. Correspondingly, such multivalent ligands are far more potent activators of the phosphatase than single ligands [15]. Thus, even though only one SH2 domain (N-terminal) is directly involved in autoinhibition (Figure 1b), the second SH2 domain indirectly provides additional energy for disrupting the autoinhibitory interaction and imposes higher input selectivity [26].

A somewhat more sophisticated mechanism of thermodynamic linkage allows N-WASP to integrate the effects of inputs that bind to ostensibly independent binding domains (Figure 3a). The activating ligands Cdc42 and PIP_2 bind to the GBD and B modules, respectively. Although the binding of Cdc42 and PIP_2 to the isolated B–GBD fragment is noncooperative, binding to an autoinhibited form of N-WASP is highly cooperative [31••]. The cooperative behavior can be explained by the model shown in Figure 3b. In the repressed state, N-WASP is held in a closed conformation by the autoinhibitory interactions of the B and GBD modules. Cdc42 and PIP_2 can individually disrupt these interactions and activate the complex; however, because their binding sites are masked by competing autoinhibitory interactions, the binding of either input alone is relatively weak. In contrast, if one input is prebound, the closed state is already destabilized and the binding of the second input is significantly enhanced. Cdc42 and PIP_2 therefore mutually enhance each other's binding to N-WASP by cooperating to stabilize the open state. This simple two-state model, in which the accessibility of the B and GBD domains to external ligand binding is coupled to the activation state of the output domain, can quantitatively describe the experimentally observed synergistic activation of N-WASP (Figure 3c) [31••] This model, in turn, also provides a basis for the potent integration of multiple signals (Figure 3d).

Tight structural and thermodynamic coupling between domains is also observed in the Src family kinases (Figure 4a). Experimental evidence indicates that the conformation of the kinase domain (active versus inactive) is coupled to the accessibility of the SH2 and SH3 domains to external ligand binding [43,44]. Moreover, targeted molecular dynamics simulations show that forcing the kinase domain into its active conformation results in the simultaneous displacement of the SH2 domain [45]. Such simulations also reveal that the motions of the SH2 and SH3 domains are highly correlated in the repressed state. Together, these studies yield a model in which the SH2 and SH3 domains function cooperatively to lock the kinase in a closed,

repressed state (Figure 4a). Although unexplored at present, such a model would predict highly cooperative activation by the combination of SH2 and SH3 ligands.

How is coupling between distinct domains achieved? Mutational studies have revealed that conformational properties of interdomain linker regions are critical for effective coupling. A mutation in the Src SH2–SH3 interdomain linker disrupts the interdomain coupling observed in simulations and correspondingly yields a constitutively active kinase (Figure 4b). Recent structural studies of the isolated SH2–SH3 segment from the Src kinase Fyn support the model in which the linker has unique properties: even without the kinase, the two regulatory domains and the linker maintain a disposition similar to that observed in the repressed kinases [46]. It is possible that linker sequence differences among the family members may help tune the degree of interdomain cooperativity.

Other uncoupling mutations in Src kinases are found in the linker between the SH3 docking site and the kinase domain (Figure 4b) [47,48]. These mutations lead to the activation of the kinase even though the intramolecular SH3 interaction is maintained. Similarly, in N-WASP, cleavage of the linker between the B and GBD motifs disrupts repression of the output domain even though the individual autoinhibitory interactions are maintained [31••]. Thus, the precise manner in which domains are linked is required for proper coupling and regulation.

Conclusions

Much of the complex behavior of these switch proteins can be understood within the framework of their modular construction. The allosteric systems described in this review could, in principle, have evolved from subtle shuffling and recombination of catalytic domains, interaction domains and internal ligand motifs [49].

The three modular switch proteins discussed here share the mechanism of using multiple binding domains to effect cooperative repression and, reciprocally, cooperative activation of a core catalytic function. This type of mechanism has several advantages for signaling switches. First, by functioning together, multiple weak interactions can yield more complete repression of the switch's output activity. Second, positive cooperative activation by combinations of input ligands allows such switches to display higher input specificity and sensitivity. Third, because these activating ligands serve to properly localize the activated switch (a property requiring the covalent tethering of the regulatory and catalytic domains), activation and localization are intrinsically coupled, leading to tight spatial control.

It is interesting to note several parallel features of these modular switches and classical allosteric switches such as hemoglobin [50]. First, both types of switches have at their core an unregulated output activity that is repressed by additional interactions. Homologs of hemoglobin that are monomeric display unregulated but high-affinity oxygen binding; this activity is repressed in the hemoglobin tetramer by subunit interactions that stabilize the low-affinity tense conformation. In modular switches, sets of intramolecular domain interactions force an unregulated output domain into an inactive state. Second, both types of switches utilize conformational changes to mediate linkage between distant binding and catalytic sites. In hemoglobin, changes in quaternary structure — the relationship between monomers — mediate this communication. In modular switches, communication is mediated by changes in the structural relationships between multiple domains within the same polypeptide.

These simple conclusions have been drawn from studies of only a handful of modular signaling switches and studies of many other signaling systems will be required to determine their generality. Nonetheless, several basic principles emerge. In the future, a true measure of our understanding will be whether we are able to predict the input/output behavior of functionally uncharacterized signaling proteins purely on the basis of recognizing modular domains within their sequence. An additional challenge will be to apply our understanding of these systems by designing engineered protein switches. Such designer switches, such as engineered transcriptional elements, could be powerful tools for the study of signaling pathways and might eventually have therapeutic applications.

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- •• of outstanding interest

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