Leading Edge
Review
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Allostery in Disease and in Drug Discovery

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Allostery is largely associated with conformational and functional transitions in individual proteins. This concept can be extended to consider the impact of conformational perturbations on cellular function and disease states. Here, we clarify the concept of allostery and how it controls physiological activities. We focus on the challenging questions of how allostery can both cause disease and contribute to development of new therapeutics. We aim to increase the awareness of the linkage between disease symptoms on the cellular level and specific aberrant allosteric actions on the molecular level and to emphasize the potential of allosteric drugs in innovative therapies.

Introduction

Allostery is regulation at a distance. It is a universal phenomenon whereby a perturbation by an effector at one site of the molecule leads to a functional change at another through alteration of shape and/or dynamics. Allosteric perturbation is common in the cell. It arises from noncovalent events, such as binding of ions, lipids, cAMP, drugs, proteins, RNA, or DNA (Csermely et al., 2010; Cui and Karplus, 2008; Pan et al., 2010); from light absorption (Strickland et al., 2008); and from covalent events, such as phosphorylation, point mutations (Sinha and Nussinov, 2001), or reaction with a small molecule (Figure 1A). Allostery takes place in all dynamic proteins (Lechtenberg et al., 2012; Tsai et al., 2008), single chains, and multimolecular assemblies and in RNA and DNA polymers.

These biomolecules exist in a range of closely related conformational states termed an ensemble. Allostery is a property of this conformational ensemble, as perturbation at any site in the structure leads to a shift in the distribution of the conformational states across the entire population (Fenwick et al., 2011; Kumar et al., 2000). Thus, allosteric structural and/or dynamic perturbations do not create new conformational states; they only change the relative distributions of the states within the ensemble. Interactions at a remote site, like those described above, change the functional site through the propagation of subtle conformational changes through physically contiguous and coevolving amino acids (Reynolds et al., 2011) along pre-existing pathways (del Sol et al., 2009). Evolution has exploited this purely physicochemical phenomenon and has optimized it for function.

Many reviews and research papers have been written on the allosteric effect; the vast majority of these focus on allostery on the protein level (e.g., Cui and Karplus, 2008; del Sol et al., 2009; Endres et al., 2011; Goodey and Benkovic, 2008; Kenakin and Miller, 2010; Kenakin, 2009; Kuriyan and Eisenberg, 2007; Leitner, 2008; Ma et al., 2011; Tsai et al., 2009; Tzeng and Kalodimos, 2011; Whitley and Lee, 2009; Wrabl et al., 2011; Zhuravlev and Papoian, 2010; Zocchi, 2009). However, the fundamental importance of allostery is not in the functional effects on the protein itself but, rather, on the cell (Good et al., 2009; Good et al., 2011) and on the organism as a whole. Cell health and death reflect the functioning of its entire network, and a comprehensive view of the impact of an inactive or partially active protein can only be achieved by connecting molecular causes to system outcomes. At its basic level, allostery is indeed a phenomenon related to proteins (or to other biomacromolecules, such as DNA or RNA); however, to grasp its full biological relevance, we need to consider the effects of the allosteric changes in the protein molecule on its pathway and, because cellular pathways are interconnected, on the entire network (Nussinov et al., 2013).

A cell-wide view may lead to questions like: how would the allosteric change in a single protein propagate to affect other proteins downstream; and in particular, how would it influence the behavior of the cell? What would be the impact of a mutation or of binding of a pathogen protein that disrupts an essential allosteric effect? And related to such questions, can we identify the allosteric cause that leads to the consequent observed disease syndromes? Addressing such questions that attempt to trace a global physiological expression to its molecular root is enormously challenging; however, because they encapsulate the essence of the "biological allosteric effect," they are of overwhelming importance, helping to relate symptoms to specific allosteric effects and effective therapeutics.

Allostery governs regulation and is the means through which environmental signals are communicated (del Sol et al., 2009; Kar et al., 2010; Tsai et al., 2009) within, across, and between cells. This fundamental role of allostery in cellular function underscores its relevance to disease. Pathological orthosteric (at the binding site) and allosteric (elsewhere) events can deregulate a

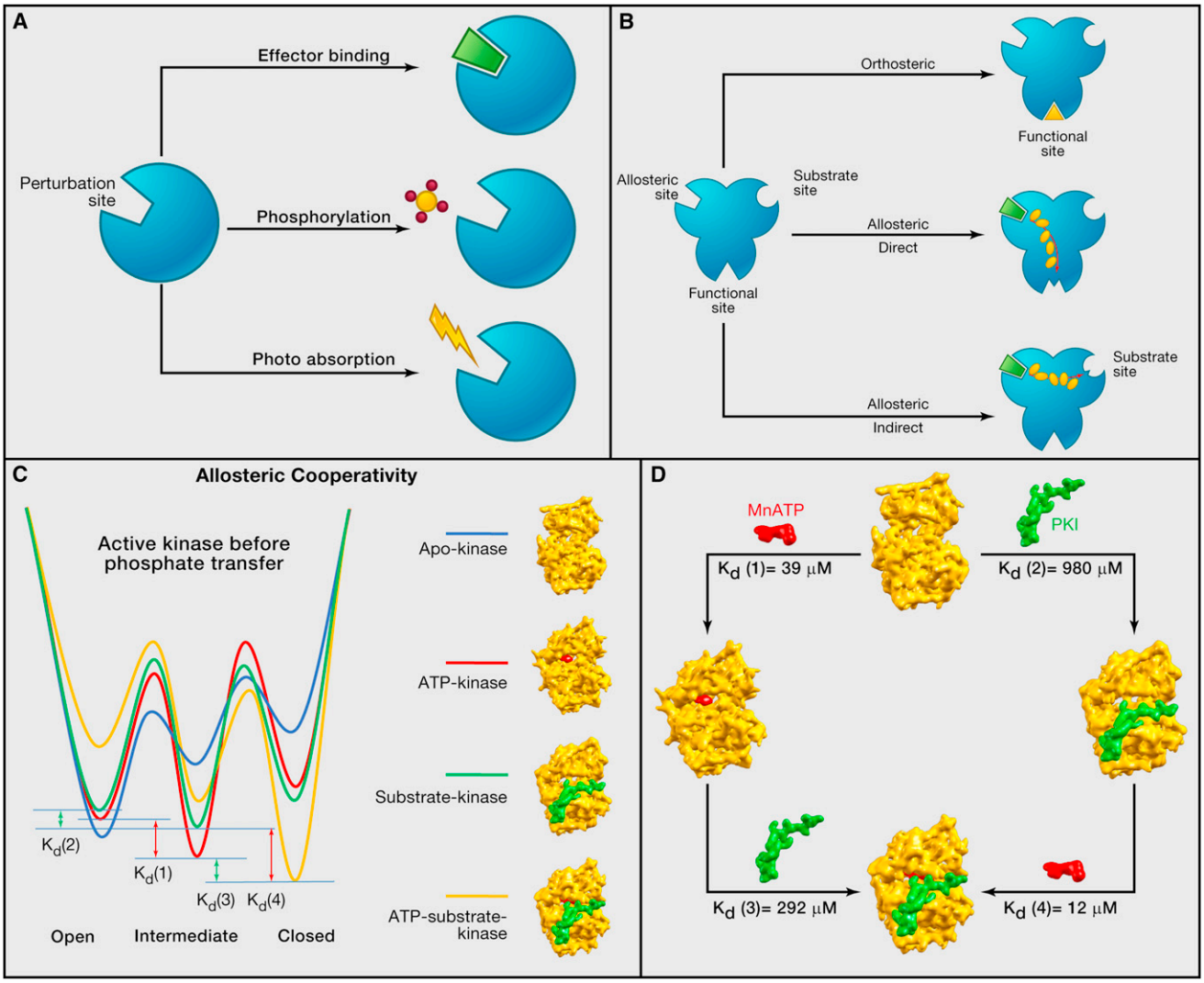

Figure 1. Allostery in Action

(A) Allosteric changes to a biomacromolecule initiate with a localized perturbation such as a binding event, posttranslational modification, mutation (not illustrated), or light absorption.

(B) A cellular target of allosteric regulation is schematically represented by three distantly separated sites: the functional site, the allosteric site, and the substrate site. The cellular perturbation is called orthosteric if it originates exactly at the functional site of a cellular target. On the other hand, if the perturbation is located at a site that is not in the vicinity of the functional site, it is an allosteric event. As shown, a binding event at the allosteric site initiates a local conformational disturbance, which creates strain energy that propagates outward, as depicted by the consecutive interactions. It is termed a direct allosteric event if the primary propagation reaches out to the functional site and directly changes its original state. If the propagation alters the conformational state at the substrate site, which in turn invokes another event that alters the functional state, it is classified as an indirect allosteric event.

(C) Allosteric cooperativity is illustrated through the catalytic reaction of protein kinase A (PKA, yellow) with three conformations (open, intermediate, and closed) and four kinase-binding complexes with combinations of cosubstrate ATP (red) and peptide substrate (green). The structures were derived from PDB entries 1j3h, 1bkx, and 1jlu.

(D) Kinetic framework for substrate binding to PKA. The model invokes experimental kinetic dissociation constants (K_d) for transitions from the apo-kinase (PDB: 1j3h) to the two structures with only the single-substrate bound (PDB: 1bkx and 1jlu) and then to the double-substrate bound kinase (PDB: 1atp). Allosteric cooperativity is clearly seen in the 3-fold ($K_d(1)$ versus $K_d(4)$) or 4-fold ($K_d(2)$ versus $K_d(3)$) improvements in the binding to the second substrate (the transition from intermediate to closed) as compared to the first substrate affinity (open to intermediate transition). The free-energy landscape in Figure 1C relates the magnitudes of the binding affinity given in Figure 1D to the relative stability for the four binding states as a function of three conformations (see text for detailed description). PKI is cAMP-dependent protein kinase peptide inhibitor.

protein, trapping it in either an active or inactive conformation. Uncontrolled protein activity typically leads to disease. When the protein is trapped in a single state, the signal transmitted is always switched ON (or OFF), keeping the proteins downstream in the signaling pathway activated (or inactivated). Because there is crosstalk between pathways, this dysregulation can elicit multiple disease consequences. From the biological standpoint, the key problem is whether we can predict the consequences of a pathological allosteric event to the entire cellular network and the organism?

We define ''allosteric disease'' as a disease that can be the result of a combination of events; however, at least one of these is the outcome of an allosteric effect. More classically, allosteric drugs work on the principle that binding of a molecule to a site remote from the enzyme active site influences the enzyme's function. Allosteric diseases and allosteric drugs are rooted in the same principles and work via the same mechanisms. Allosteric drug regimes can integrate allosteric and orthosteric drugs. However, their successes (and failures) must be assessed based on the entire system.

In this Review, we first define and explain the basic concept of allostery in terms of a dynamic free-energy landscape and in vivo events. From this, we can classify allosteric mechanisms involved in disease and in allosteric modulator (drug) actions. We proceed to focus on allostery from the functional standpoint: how allostery controls cellular function, how it plays a role in disease, and how it can be harnessed by drugs. Kinases provide apt examples for detailed mechanistic illustrations to show how allosteric mutations can cause disease and to conceptually clarify allostery from a ''systems biology'' perspective. Protein kinases are key regulators of cell function and are one of the largest and most functionally diverse gene families. They direct the activity, localization, and overall function of a large number of proteins and orchestrate almost all cellular processes (Huse and Kuriyan, 2002; Kim et al., 2006; Taylor et al., 2005; Taylor and Kornev, 2011). Throughout the Review, we attempt to cast the pathological and therapeutic allosteric effects into the framework of the cell, drawing on examples from the kinase literature. Overall, the Review aims to draw increased attention to the possible linkage between specific deleterious allosteric (often gain-of-function) events on the molecular level and distinct disease syndromes at the cellular and organism levels. It further highlights the potential impact of recent advances on allosteric drug discovery. We make the case that the much-needed new drug classes are likely to come from allosteric strategies rather than modifications of existing drug compounds.

The Basic Concept of Allostery

Traditionally, allostery was proposed to operate on the single-protein level, where binding of an effector molecule at one (allosteric) site on the protein surface would change the conformation of another (the active or binding) site and, in this way, regulate protein activity (Changeux, 2012; Cui and Karplus, 2008; Goodey and Benkovic, 2008; Tsai et al., 2009) (Figure 1B). Allostery was formally described in the hemoglobin oligomer as a cooperative phenomenon with oxygen binding to one monomer enhancing the affinity of a second oxygen molecule binding to a neighboring monomer (Monod et al., 1965).

The paramount advantage of allosteric cooperatively can be witnessed by the diversity of evolved targets of protein kinases (Figures 1C and 1D). The phosphorylation reaction occurs as the free enzyme binds its two substrates, ATP and a polypeptide, and catalyzes phosphoryl transfer. Activated kinases have been captured in crystal structures with three distinct conformations along the reaction coordinates: open, intermediate, and closed. The open structure refers to the apo-kinase. The intermediate conformation can reflect kinases with either the ATP cosubstrate or the peptide substrate bound. Binding to both substrates shifts the protein to the closed structure.

The kinetic scheme for moving from the apo-enzyme to the fully bound or closed conformation involves four experimental binding constants (Masterson et al., 2008): $K_d(1)$, $K_d(2)$, $K_d(3)$, and $K_d(4)$ (Figure 1D). Allosteric cooperativity appears clearly with a 3- to 4-fold enhancement upon binding of the second substrate (compare $K_d(1)$ to $K_d(4)$) or ($K_d(2)$ to $K_d(3)$), reflecting a conformational transition from an intermediate to a closed state, as compared to binding of the first substrate (open to intermediate transition). Figure 1C illustrates the allosteric cooperativity for the three conformations. The relative stability of each of the four binding states related to these indicates that it can reflect the magnitude of the affinity.

Formal descriptions of allostery span energetic coupling between the two (allosteric and active site) binding events and the conformational and dynamic changes observed at the active site following the perturbation by an effector at the allosteric site (Cui and Karplus, 2008; Fuxreiter, 2012; Hilser et al., 2012; Liu et al., 2009; Popovych et al., 2006; Tsai et al., 2008; Weinkam et al., 2012; Wrabl et al., 2011). Formal models of allostery have also been developed in pharmacology (Maksay, 2011). All can be clarified by the basic physical fact that biomacromolecules consist of ensembles of conformations with a certain distribution, which can be described by their free-energy landscape (Figure 2A) (Boehr et al., 2009; Dill and Chan, 1997; Frauenfelder et al., 1991). An energy landscape is a mapping of all possible conformations of the protein (or the spatial positions of the interacting molecules in a system), as a function of their corresponding energy levels, on a two- or three-dimensional Cartesian coordinate system. This map encompasses the native conformation as well as any nonnative conformations, which can be sampled during folding or catalysis. Such a landscape description is useful in that it serves as the physical basis for portraying the ensemble around the native state of the protein; however, from the biological standpoint, the landscape is limited, as it provides a static view of the ensemble under a certain set of conditions, and thus it is unable to explain molecular cooperativity.

To understand how allostery can both render a molecule functional and conversely dysregulate it to cause disease, we have to consider changes in the free-energy landscape (Gunasekaran et al., 2004; Ma et al., 1999; Tsai et al., 1999a; Tsai et al., 1999b). Changes take place because the relative stabilities of the conformations change following allosteric events (Figure 2B). For example, allosteric mutations can redistribute the ensemble to favor the ON state, making the protein constitutively active (Sinha and Nussinov, 2001). Figure 2 illustrates the principles of the redistribution of the ensemble when there is an allosteric mutation (e.g., gain-of-function) or following the binding of a drug at an allosteric site. As shown, the conformational ensemble of the regulatory subunit (RA) of protein kinase A (PKA) undergoes redistribution upon binding to PKA.

Allostery initiates when the protein undergoes some structural disturbance. This perturbation can be the result of a change in the physical environment (Figure 1A), including exposure to light (Strickland et al., 2008); irradiation; change in pH (thus, protonation states; Martí et al., 2009); and concentration. It can also

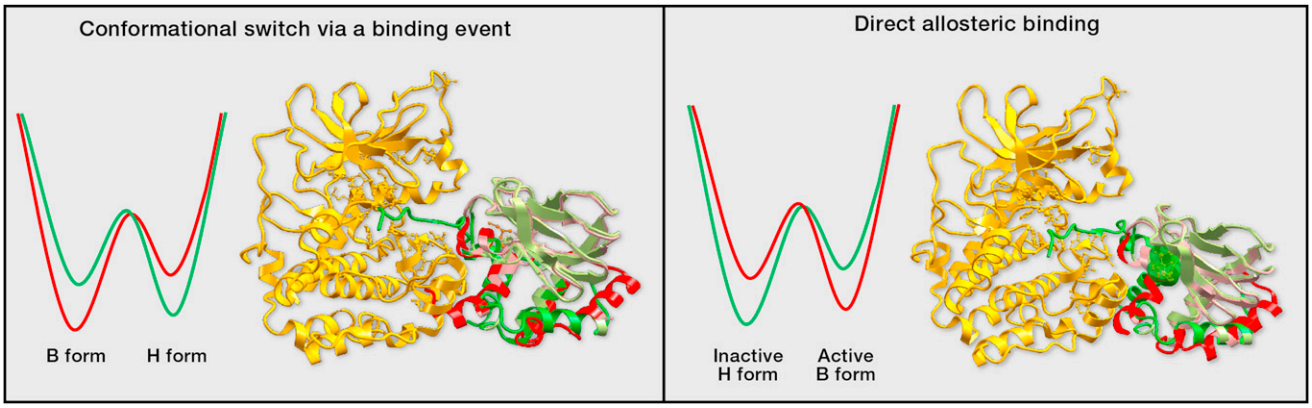

Figure 2. Linking the Free-Energy Landscape to Allosteric Conformational Switches

The regulatory subunit (RA) of protein kinase A (PKA) exists as an ensemble in two distinct dominant conformations: the B form (either in apo or cAMP-bound) and the H form (PKA-bound).

(Left) The free energy shifts from the favorable B form in apo-RA (red) to the H form following binding to PKA (green). The accompanying conformational change in the regulatory subunit due to the binding to PKA (yellow ribbon) is highlighted by two superimposed RAs with the apo B form (PDB: 3iia) in red ribbon and the PKA-bound H form (PDB: 3fhi) in green.

(Right) The energy landscape shifts from the H form (green) back to the favorable B form (red) after the cAMP (a ball-and-stick model enclosed in transparent green) preferentially binds the B form of RA (PDB: 3pna) in the red ribbon. The steric hindrance between B form of RA and PKA due to the conformational changes caused by the allosteric cAMP binding will result in PKA activation by releasing RA.

stem from noncovalent binding or a change in a covalent linkage, such as that taking place during a mutational event or when adding or removing a posttranslational modification (PTM) (Nussinov et al., 2012). To optimize the newly formed interactions, atoms within the protein move and reorient. This creates strain energy (or frustration), which forces the next layer of atoms to also move, which in turn affects the next layer, etc. In this way, the perturbation travels across the structure through both major and minor pathways (del Sol et al., 2009) to reach another site and change its conformation and/or dynamics (Figure 1B). Thus, allostery works via a "population shift" (Kar et al., 2010); that is, the distributions of the populations of the conformers in the ensemble shift, or change, as the allosteric "wave" propagates (Figure 1C, left). The observed cooperativity between the allosteric and active site is the outcome of such a population shift. To switch from one conformation to another requires climbing over an energetic barrier. The timescales for the overall conformational change are determined by the barrier heights between the different conformations; the higher the barrier, the slower the change.

The description above emphasizes two key attributes of the allosteric phenomenon: (1) a static effect, which is reflected by the final conformational change and (2) a dynamic effect, which reflects the changes that take place during the propagation of the allosteric "wave." Mutations can lead to disease via both mechanisms. In the first case, mutations may stabilize (or destabilize) the final state, resulting in an ON (or OFF) conformation. Alternatively, a final change in the structure of the active site can lead to altered ligand selection. For the second case, allostery acts by changing the relative stabilities along the propagation pathways in the structure. Such changes can abolish or create new interatom and intermolecule interactions. Simply put, the final state, or conformational selection, does not care about the dynamics leading to it; the dynamic propagation does not care about the outcome.

If the cross-correlation of the motions (e.g., as obtained from molecular dynamics simulations) between the conformation of an allosteric site and an active (or functional) site is always "perfect" (100%), a mutation that causes a constitutive activation can be fully described as either stabilizing the active conformation or disrupting the favorable interactions of an inactive conformation. Nonetheless, the dynamic propagation is important: even though allostery can be conveniently explained by a population shift, propagation clarifies how the change at the allosteric site modifies a distal active site. A binding event or a mutation can enhance or restrict allosteric propagation, or it can act via both propagation and changes in the relative stabilities of the active versus inactive states. Below, we describe allosteric disease mechanisms from this standpoint.

How Allostery Controls Physiological Activities

On a single-protein level, the outcome of allosteric propagation is a highly specific active site conformation. This architectural specification helps the protein to choose a particular ligand among the many possible. A slight change in the perturbation at the allosteric site can lead to different active site conformations. A regulated protein within a signaling pathway is, in reality, a population of molecules shaped by allosteric events in which individual molecules can be differently modified (e.g., phosphorylated) or bound to cofactors or ligands. These events produce combinatorial effects within the population that define specific structures and dynamic changes that influence downstream signaling (Deribe et al., 2010; Nussinov et al., 2012). This immense complexity is advantageous because it allows the same protein to bind specifically to different partners, thus extending functional diversity (Figure 3).

Allostery is not the sole means for conformational selectivity in the cell. Concentration, availability (Segal and Widom, 2009), micro-organization (Ferrai et al., 2010; Gavin et al., 2002; Mitchell and Fraser, 2008; Osborne et al., 2004; Shopland et al., 2003;

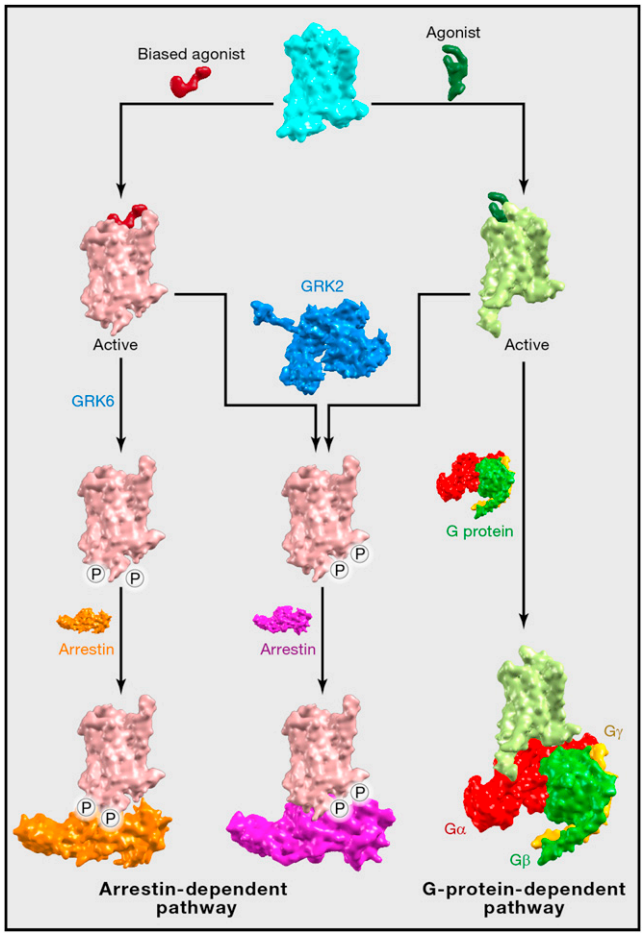

Figure 3. Allostery Can Diversify Cellular Signaling Pathways through a Single Receptor

G-protein-coupled receptors use conformational selection to shape signaling. Two (different) conformations of GPCR bind two (different) agonists. Binding stabilizes these two activated conformations, which branch into two pathways. In the agonist (G-protein-dependent) pathway, the activated GPCR either activates the heterotrimeric G proteins, which then promote the consequent signaling through a second messenger such as cyclic AMP, or recruits the GPCR kinases (GRKs) to phosphorylate Ser/Thr in the cytoplasmic loops and tail of the GPCR. In turn, the phosphorylation enables the recruitment of β-arrestins to mediate receptor desensitization and internalization. In the biased agonist (arrestin-dependent) pathway, distinct active GPCR conformations recruit a different set of GRKs. These kinases create distinct phosphorylation patterns on the GPCR. These patterns impart distinct conformations. Via conformational selection, each pattern of modifications recruits a specific conformation of the arrestin (illustrated in different colors) either through orthosteric or allosteric interactions. Because the resulting conformation is different, each complex mediates different signaling pathways such as the ERK 1/2 activation. The illustration is adapted from Figure 5 in Nussinov et al. (2013), with permission. The illustrated structures are at the following PDB codes: GPCR, 3ny8 (cyan), 4amj (pink), 3sn6 (light green); ligands, 3qak (green) and 4amj (red); GRK, 3nyn (blue); arrestins, 3gd1 (orange) and 3p2d (magenta); G protein, 3sn6 (red, green, and yellow).

Sutherland and Bickmore, 2009), recognition domains (Pawson, 2007), etc. are all fundamental parts of the cellular machinery. However, allostery plays key roles in regulation via molecular cooperativity and recognition specificity. Because allostery propagates across molecular interfaces, cooperativity, ligand selection, and specificity affect consecutive components in cellular pathways.

A prime example of this functional diversification comes from G-protein-coupled receptor (GPCR) kinases (GRKs) (Figure 3) (Liggett, 2011). GPCRs exist in an ensemble of conformations. Different agonists selectively bind distinct GPCR-active conformations and allosterically shift the GPCR population toward these active states. Distinct GRKs recognize these activated conformations and phosphorylate them at different serine/threonine residues. The specific arrangements of the phosphorylated residues on the GPCR serve as blueprints for selective recruitment of specific β-arrestin conformations, which via their allosteric scaffolding, stimulate diverse downstream signaling pathways such as ERK 1/2 activation. Alternatively, distinct activated GPCR conformations either activate the associated heterotrimeric G proteins, which then promote the consequent signaling of a second messenger such as cyclic AMP, or recruit the GRKs with subsequent selective arrestin binding. Arrestin binding partially quenches the recruitment of G proteins to GPCRs, leading to desensitization and internalization.

Allosteric Mutations and Disease

The kinase catalytic core domain contains two lobes connected by a hinge linker. The active site is located in the deep cleft between the two lobes, where one cosubstrate, ATP, binds mostly to the N lobe and the peptide substrate binds to the C lobe. The catalytic reaction involves a cycle of ATP and peptide/protein substrate binding, phosphate transfer from ATP to a phosphate receptor residue, and release of ADP and the phosphorylated substrate. Kinetic studies have indicated that ADP release is the rate-determining process of the overall catalytic reaction (Grant and Adams, 1996), implying that transient substrate binding and kinase flexibility are important for its function. As we discuss below, deprivation of flexibility is utilized for regulation.

The crucial function of an activated kinase requires a populated conformation that is not only able to bind both ATP and substrate, but is also able to orient precisely the γ-phosphate of the bound ATP and the hydroxyl group of the phosphate receptor and to surround them with appropriate catalytic residues to facilitate the phosphate transfer reaction. Thus, it is not surprising that all activated conformations of the different kinase families (as captured in crystals) share a strikingly similar structure (Huse and Kuriyan, 2002; Nolen et al., 2004) in contrast to the variety of inactivated states awaiting regulation.

The conserved structural elements in the active conformation are coordinated by the unique hydrophobic and specific electrostatic interactions in order to achieve the precise positioning required for function. Specific orientation of the key structural elements, including the αC helix, Gly-rich loop, catalytic loop, and activation segment (Mg-binding loop, activation loop, and P+1 loop), are coordinated by the αF-helix via two conserved hydrophobic interactions—the regulatory spine (R-spine) and the catalytic spine (C spine) (Taylor and Kornev, 2011) —as well as some specific electrostatic interactions. Here, we elaborate on the allosteric regulation of the conformational switch from the inactive αC-helix-out (also referred to as DFG-out) conformation to the active αC-helix-in (also referred to as

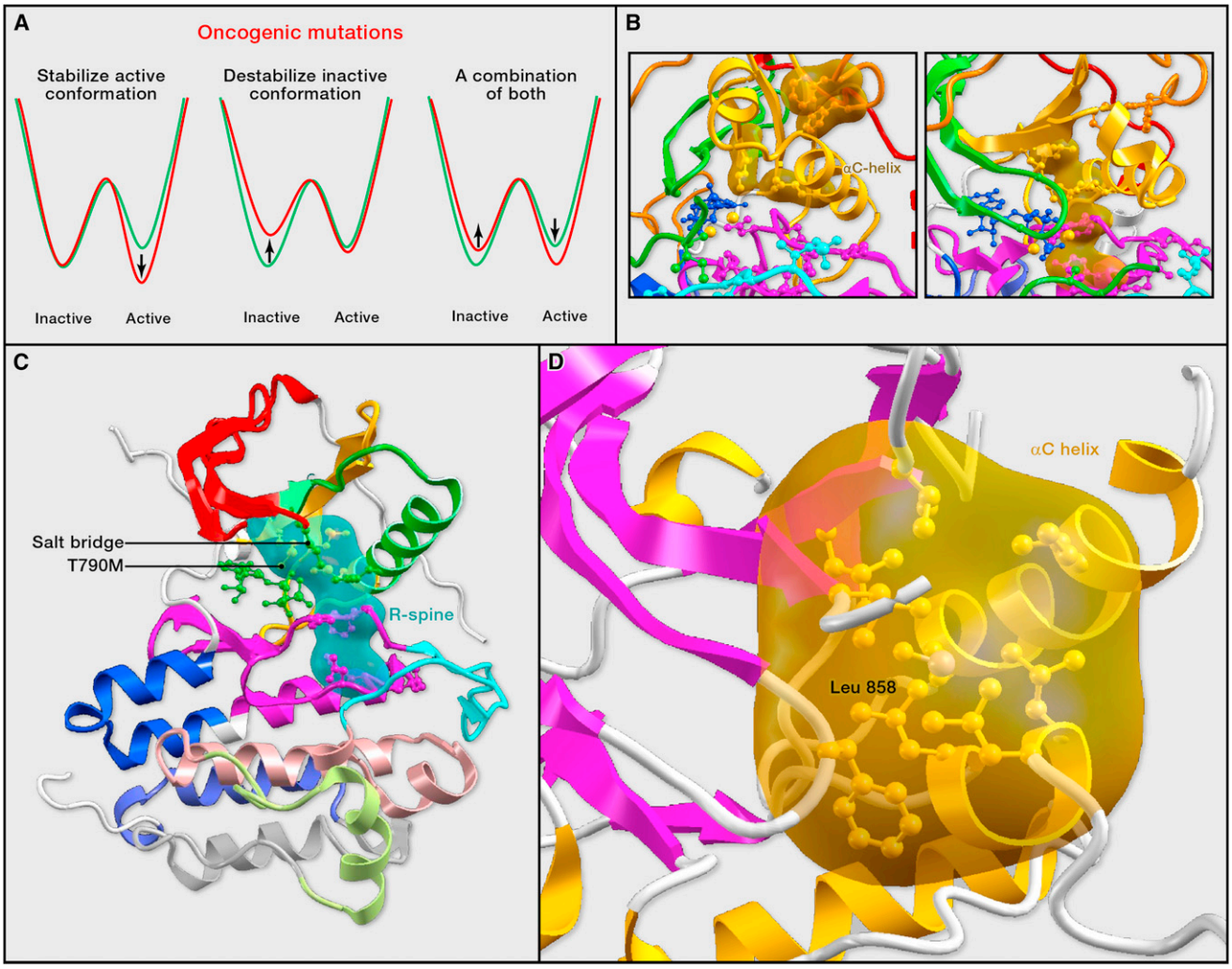

Figure 4. Impact of Mutations on Kinase Conformation

(A) Protein kinases are usually held in an inactive state by regulatory interactions. Oncogenic mutations, however, bypass the autoinhibited state and result in aberrant constitutive kinase activation. There are three general mechanisms for shifting a population from a favored inactive to an active state by mutations via either stabilizing an active conformation or disrupting critical interactions in an inactive conformation (or a combination of both).

(B) Many kinase families use the allosteric switch from an inactive αC-helix-out to an active αC-helix-in conformation.

(C) Hallmarks of activated kinases (illustrated here for PKA PDB: 1atp) include the presence of a salt-bridge between the β3-lysine and the αC-glutamate (left) and the formation of regulatory spine (right). The T790M mutation in EGFR (PDB: 3vjn) promotes the assembly of an enzymatically active kinase conformation by stabilizing the hydrophobic R spine.

(D) Leu858 sits in the middle of a hydrophobic core of inactive EGFR (PDB: 1xkk), suggesting that critical interactions will be disrupted by the L858R mutation, leading to the shift of the population toward the active conformation. A recent study (Shan et al., 2012b) further indicated that the L858R mutation also stabilizes the active αC-helix-in conformation from an intrinsically disordered structure through heterodimerization without the help of ligand-induced receptor dimerization.

DFG-in) conformation as examples for how allosteric mutations can cause disease.

As a transducer in signaling pathways, a kinase is usually regulated when in an inactive autoinhibited state, waiting to be stimulated into a partially active and/or fully active conformation via autophosphorylation. Although there are several mechanisms of activation, many kinase families use an allosteric switch from the inactive αC-helix-out state to the active αC-helix-in conformation through a rotation and shift movement of the αC helix. The presence of a salt bridge between the β3 lysine and the αC glutamate (Figure 4C) and the formation of R spine (Taylor and Kornev, 2011) are the two hallmarks of activated αC-helix-in conformation, distinguishing it from the αC-helix-out state. Prior to the engagement of the αC-helix patch, the kinase catalytic core domain is populated mostly in an inactive αC-helix-out conformation (Jura et al., 2011). Different proteins drive the αC helix in the activated state through different mechanisms; for instance, PKA uses its intramolecular C-terminal tail, RET engages its juxtamembrane segment, the Fes employs an SH2 domain, and EGFR via dimerization. However, oncogenic mutations in the kinase catalytic core domain, which either stabilize the active αC-helix-in conformation or disrupt interactions

critical for maintaining the inactive αC-helix-out conformation (or do a combination of both), shift the kinase conformation toward an active population, resulting in constitutively active aberrant kinase (Figure 4A). The oncogenic L834R (or L858R in an alternative numbering of the human EGFR; Figure 4D), accounting for 41% of EGFR mutations in lung cancer patients, belongs to the latter case. Recent molecular dynamic simulations and NMR H/D exchange experiments have clarified the mechanism of this mutation, showing that it works by stabilizing the intrinsically disordered active αC-helix-in conformation via heterodimerization, even in the absence of a prior ligand-promoted receptor dimerization (Shan et al., 2012b). On the other hand, the T790M mutation in EGFR, T315I in BCR-ABL, T334I in c-ABL, T341I in Src, T670I in KIT, and T674I in PDGFRA promote the assembly of an enzymatically active kinase conformation by stabilizing the hydrophobic R spine. These kinase examples illustrate how allosteric mutations can exploit different mechanisms, even within a single protein family, to reach similar outcomes, underscoring the importance of in-depth detailed mechanistic understanding.

Basis for Allosteric Disease Mechanisms

Although many of the disease-causing mutations in proteins that have been characterized are in binding sites, genetic studies suggest that, in fact, the majority of these mutations occur elsewhere in proteins. Similarly, binding of pathogen proteins (e.g., oncogenic proteins E6 and E7 of human papillomavirus (Chi et al., 2011) and poliovirus (Autret et al., 2007) and apamin, a neurotoxin in apitoxin [bee venom]), as well as the sun and UV irradiation, may all interfere with signaling via allosteric mechanisms. As allosteric mechanisms can be traced to some (often subtle) changes in the relative stabilities of the conformational states, the consequent disease can take place either (1) because the final shift in the populations of the ensemble (i.e., the free-energy landscape) leads to a higher population of ON (as in the kinases example above) or OFF states or leads to a change in the active (binding) site shape and dynamics or (2) because of the dynamic redistribution of the propagation pathways in the protein structure.

Allosteric mutations can cause disease by either one of the mechanisms above or by abolishing or creating sites for allosteric posttranslational modifications, which can also lead to similar outcomes. The effects of shifting the relative ON/OFF populations are described above. Here, we focus on mutations that can act by shifting major allosteric pathways (Nussinov, 2012; Shan et al., 2012a) or alter patterns of posttranslational modifications (Deng et al., 2009). Allosteric mutations can uncouple the distinct conformational changes that normally take place in an active site upon agonist binding and thus impact the cellular response. Uncoupling typically occurs by modulating a major allosteric propagation pathway between two binding sites. The mutations can be on or in the microenvironment of the pathway. For example, disruption of glucocorticoid steroid signaling plays a role in diverse disease states, including depression, leukemia, and asthma. Hormones and coregulators bind at distinct sites in the GR-ligand-binding domain. Mutation of Met752 to Ile strengthens the GR-peptide interaction while dramatically slowing hormone association with peptide-bound GR. The mutation disrupts intraprotein communication and compromises GR signaling by effectively eliminating hormone binding as a prerequisite for receptor function (Pfaff and Fletterick, 2010). More generally, a disease may also be caused by mutations located on different pathways or by shifting the ensemble toward conformations with altered (lower) stability, as in familial amyotrophic lateral sclerosis (fALS) motor neuron disease (Pfaff and Fletterick, 2010).

Mutations can deregulate function by affecting posttranslational modifications. Coming back to the kinases, their extended regulatory spine, which transmits signals from the regulatory to the catalytic domain, contains conserved residues, including in the linker between the two lobes. Mutation of the gatekeeper residue at the edge of the regulatory spine stabilizes the regulatory spine, resulting in a constitutively active kinase domain (Figure 4), rendering phosphorylation on the activation loop unnecessary for its activity (Joseph et al., 2010). Thus, allosteric disease-causing mutations are common and can act through diverse mechanisms, depending on the protein function, conformation, their location in the conformation, and whether the residue is a target for posttranslational modification.

Allosteric Diseases versus Allosteric Drugs

Drugs also act via cellular effects. Such networks may vary across a patient population because of external conditions and genetics; thus, drug regimes that work for one patient may not be successful in another even though the symptoms appear similar.

Nuclear factor (erythroid-derived 2)-like 2 (NRF2) is a tumor suppressor that controls cell fate through transcriptional upregulation of antioxidant response element-bearing genes and provides an example of allosteric impact with complex network effects. NRF2 knockout mice under calorie restriction afford reduced protection from tumorigenesis (Martín-Montalvo et al., 2011). Oxidative stress, or compounds that inhibit the Keap1-Cul3-Rbx1 E3 ubiquitin ligase, upregulate NRF2 levels and lead to activation of its downstream target genes. NRF2 activator drugs such as sulforaphane and *tert*-butylhydroquinone that modify Keap1 C151 apparently cause a conformational change in the Keap1-Cul3 E3 complex, which switches from catalyzing NRF2 ubiquitination to autoubiquitination of Keap1. Subtoxic doses of activator drugs, to counter the environmental effects, are a cancer prevention protocol. At the same time, overexpressed NRF2 is responsible for acquired chemoresistance in tumor cells, which requires suppression of the NRF2 pathway (Lau et al., 2008). This example highlights the delicate balance in the cellular network, here relating to cancer prevention versus cellular resistance.

Mechanisms for Allosteric Drugs

Similar to orthosteric drugs, allosteric drugs can be classified as either noncovalent or covalent. Low-dosage allosteric drug regimes using noncovalent binders are likely to be effective if the protein displays a "conformational switching" mechanism between the active and inactive conformations. Drug binding would lead to a shift in the free-energy landscape toward the inactive conformation. Later, in the absence of the drug, if the

energy barriers between the states are high, switching back to the active conformation may require long timescales, which may be beneficial in leading to lower drug dosages. Covalent allosteric drugs are, by contrast, more likely to display irreversible action even though this reversible/irreversible distinction is not absolute.

Noncovalent

The vast majority of the currently available allosteric drugs are noncovalent. Examples include valium and the benzodiazepines, which target the ionotropic GABA receptor, positive allosteric modulators of mGluRs (Wood et al., 2011), and positive and negative modulators of GPCRs (Conn et al., 2009). GPCR modulators include cinacalcet as a positive regulator at the Ca^{2+}-sensing receptor and maraviroc as a negative modulator of the chemokine CCR5 (Smith and Milligan, 2010). In another example, interdomain communication in the hepatitis C virus polymerase was abolished by indole-based allosteric inhibitors binding on the surface of the thumb domain (Di Marco et al., 2005). We have also described some allosteric drugs, including anticancer and HIV-1 (Nussinov et al., 2011), and the recently expanded diversity of the allosteric Bcr-Abl inhibitors provides additional important examples (Deng et al., 2010). Drugs binding to RNA can also work via conformational change (Paulsen et al., 2010).

Covalent Allosteric Drugs

Covalent drugs work in a way similar to that of PTMs: in the binding site (orthosteric PTMs) or elsewhere (allosteric PTMs; Nussinov et al., 2012). There are many examples of covalent orthosteric drugs, including aspirin, although their development has received mixed reactions because of toxicity concerns (Singh et al., 2011). Unlike covalent orthosteric drugs, covalent allosteric drugs are a nascent area. The successful tethering of allosteric small-molecule inhibitors in the caspases, a class of cysteine-dependent aspartate-specific proteases, can be viewed as a first-generation covalent allosteric drug design. This advance was made possible through the identification of an allosteric site at the dimer interface of some caspases and disulfide trapping. Among the trapped thiol-containing fragments, a naphthyl-thiazole-containing molecule selectively labeled the allosteric cysteine in the p10 subunit of caspase-5 and inhibited it but caused minor inhibition or labeling of caspase-1. Of interest, some allosteric tethered-compounds to caspase-5 did not inhibit its enzymatic activity, suggesting that thiol labeling is not sufficient to drive inhibition and that other inhibitor-protein interactions also play a role, emphasizing the challenge in allosteric designs (Gao and Wells, 2012).

Collectively, allostery works similarly in disease and in response to drugs. Allosteric drugs bind away from the active site (Figure 1) and, like PTMs, may form a covalent bond with a reactive residue (Singh et al., 2011). In disease and for allosteric modulators, the effects—harmful or beneficial—propagate in the cellular network and may present complex patterns because of the heterogeneity of the population. Moreover, it is challenging to predict whether the modulator will enhance (agonist) or diminish (antagonist) the activity, and even small differences, the presence of an additional chemical group, or interaction can lead to different—even opposite—modulation effects (Sadowsky et al., 2011).

Advantages and Hurdles Facing Allosteric Drugs

Advantages

Allosteric drugs present several key advantages over orthosteric drugs that target a protein's functional site. They are highly specific because they do not bind in active sites which tend to be highly conserved in protein families. Their beneficial effect is compounded in combinatorial strategies, where lower chances of side effects may be particularly advantageous. They allow modulation of the protein activity rather than completely eliminating it. Moreover, allosteric drugs generally work when the endogenous ligand is bound; that is, they work when the cell needs the protein to work. It is important to note that both small molecules and biological macromolecules can serve as allosteric drugs.

Importantly, allosteric drugs can activate a target protein not only by directly binding to it, but also by indirect allosteric effects (Nussinov et al., 2011). For example, when multiple receptors are integrated into one signaling unit, drug binding to one receptor molecule can allosterically modulate the response of another to a ligand and thereby create a mechanism of tissue-specific fine-tuning (Schelshorn et al., 2012). Such indirect allosteric modulation takes place through protein-protein interfaces (Lee et al., 2008). This avenue increases the potential repertoire of allosteric drugs and can further fine-tune modulation.

GPCRs illustrate the concept of indirect allosteric modulation. Binding of different ligands to distinct GPCR conformations can activate distinct downstream cascades with the transmembrane or the extracellular domains communicating the signal across monomers and higher-order complexes. Allostery in GPCR heteromers may allow receptor subtype selectivity and tissue specificity (Smith and Milligan, 2010). For example, in the glucagon receptor family (GPCR class B), interactions of some of the receptor combinations decrease upon ligand binding (Smith and Milligan, 2010). However, an increase in interactions between receptors was exclusively observed between the gastric-inhibitory-peptide-receptor (GIPR) and the glucagon-like-peptide-1-receptor (GLP 1R) upon binding of GLP-1. Addition of gastric-inhibitory-peptide (GIP) to the GIPR-GLP 1R heteromer reversed this effect, showing a specific pharmacology for GLP-1-induced β-arrestin recruitment and calcium flux, which suggests allosteric regulation between these functionally related and physiologically coexpressed receptors. GIP rescued the normal GLP-1 pharmacology and restored GLP-1R response when expressed alone (Schelshorn et al., 2012).

Allosteric propagation may also be mediated by molecules other than proteins, which further expands the landscape of molecular targets. One recent example relates to cholesterol. Cholesterol has a role in the signaling of endogenous β_2-adrenergic receptor (β_2AR), as it improves β_2AR stability and may mediate receptor-receptor interactions. Remarkably, the crystal structure of β_2AR contains six cholesterol and two palmitic acid molecules, forming a 2-fold symmetric sheet between the receptors (Cherezov et al., 2007), which raises the question of whether signaling can be helped by molecules such as cholesterol. This idea has recently been reinforced by the discovery that oxysterols, which are endogenous signaling molecules, can function in leukocyte chemotaxis by acting on GPCRs and can also activate the Hedgehog-signaling pathway by binding allosterically to the

seven-pass transmembrane protein Smoothened (Smo), an oncology target similar to GPCRs (Nachtergaele et al., 2012). Inhibition of Smo abrogates the effects of oxysterols on Hedgehog signaling. This principle of propagation across a signaling unit may apply across multimolecular complexes as well, where allosteric drugs could exploit it (Nussinov et al., 2011). To date, most allosteric modulators that have been developed target the protein of interest directly rather than via its neighbors in the functional module, suggesting much room remaining for growth.

The fast growth in the repertoire of allosteric modulators is testament to their advantages. At the same time, allosteric drugs pose major hurdles, some of which may be alleviated by effective allosteric/orthosteric drug combinations.

Hurdles

Allosteric drug discovery is challenging. Unlike orthosteric drugs that dock into a known active site, allosteric sites are often unknown and the drug modulatory effects are difficult to predict. Moreover, slightly different inhibitors or subtly altered inhibitor interactions may lead to different downstream effects (Sadowsky et al., 2011). Allosteric drugs also suffer from the same hurdles faced by orthosteric drugs—in particular, the emergence of drug-resistant mutations. These typically lead to alternate pathways that upregulate activation, as observed for example in the case of COT-expressing B-RAF(V600E) cell lines, which exhibit resistance to allosteric MEK inhibitors (Johannessen et al., 2010). In this case, melanoma cells acquire resistance to B-RAF(V600E) inhibition by upregulating receptor tyrosine kinases (RTKs) or N-RAS. Similarly, mutations at positions Thr315 and Glu255 in Bcr-Abl confer resistance to imatinib (Adrián et al., 2006).

Though many allosteric effects can be characterized individually, drug development targets populations of patients. In this context, similarity in disease symptoms and in protein levels does not necessarily imply identical preferred propagation pathways because the metabolic and genetic conditions of patients may vary. In a related vein, network regulation and feedback loop effects may hinder drug treatments, as in the case of rapamycin and its derivatives temsirolimus and everolimus (rapalogs), which are allosteric inhibitors. Rapamycin binds to the cytosolic protein FKBP12 with subsequent binding of the complex to the FK-rapamycin-binding domain of mTOR and selective disruption of mTORC1 assembly. This association decreases phosphorylation of mTORC1 substrates. However, while cell survival diminishes, its extent depends on additional factors such as Akt activation, which relates to its phosphorylation on Ser473, the outcome of inhibition of negative feedback loops. Prolonged rapalog treatment can also decrease mTORC2-induced Akt activation (Gupta et al., 2012). As mTOR is highly regulated by pathways reflecting individual age and metabolic status, patient diversity is also relevant to the robustness of the control loops.

Hurdles can also arise from the higher divergence rate of allosteric sites in species homologs through evolution as compared to orthosteric sites, which can make translation from initial pharmacological studies on a heterologously expressed human receptor family to animal models of disease even more challenging (Smith and Milligan, 2010). Finally, there can be toxicity effects. Toxicity often relates to dosage, as high doses can lead to binding to additional, lower-affinity proteins or to formation of reactive species by metabolizing enzymes such as p450; or, they may upset the fine-tuned network balance. Even though the effective concentration of an allosteric drug can generally be lower than an active site inhibitor, this pattern is a trend and not a rule. Specific dosing requirements may depend on the affinity, as shown by chloroquine, an antimalarial drug that inhibits the enzymatic activity of the 20S archaeal proteasome. The low affinity requires high concentrations, which humans can sustain (Ruschak et al., 2011).

Development of allosteric inhibitors is often pursued for overcoming clinically acquired resistance mutations to the first generation of competitive inhibitors, as in the case of ATP-competitive inhibitors toward Bcr-Abl (Hassan et al., 2010). Nonetheless, allosteric drugs are not always superior to orthosteric drugs, as shown by the rapamycin (mTOR) example above (Gupta et al., 2012).

Allosteric and Orthosteric Collaboration

One avenue for therapeutic regimes to combat drug-resistant mutations arising against allosteric drugs is to combine them with orthosteric drugs. For example, the T315I mutation in the Bcr-Abl fusion tyrosine kinase is resistant to all ATP-competitive drugs because it stabilizes the active Abl conformation (Azam et al., 2008; Medves and Demoulin, 2012; Yun et al., 2007). GNF-2 is a selective allosteric Bcr-Abl inhibitor. GNF-2 binds to the hydrophobic myristate-binding site of Abl, mimicking the myristoyl group. This allosterically leads to changes in the conformational dynamics of the ATP-binding site and increases the population of the Bcr-Abl inactive conformation. In combination with ATP-competitive inhibitors imatinib or nilotinib of Bcr-Abl, GNF-5, an improved analog of GNF-2, showed better pharmacology: it suppressed the emergence of resistance mutations and displayed additive inhibitory activity against the human T315I mutant in vitro the murine bone marrow transplantation model in vivo. Together with these ATP-competitive inhibitors, GNF-5 was also used to target other imatinib-resistant Bcr-abl mutants (Adrián et al., 2006). These results suggest that combining allosteric and ATP-competitive inhibitors can synergistically help to overcome resistance to either agent alone (Zhang et al., 2010). However, multiple mutations may arise, and combination therapies may work only with moderate success, as in the case of imatinib/GNF-2. Identification of the alternate cellular pathways and selection of the additional proteins to target can also be difficult. Complications may also arise because the alternative pathways may not be identical across the patient population.

Such drug combinations do not necessarily increase the risk of toxicity. Examples include allosteric mTOR inhibitors (rapamycin and RAD001), which were used in combination with dual PI3K/mTOR kinase inhibitor (PI-103). The combination was more effective in mutant human ovarian and prostate cancer cells. The combined inhibition affected Akt phosphorylation and activation that takes place after treatment with rapamycin. The combination also inhibited the expression of PI3K/Akt/mTOR downstream proteins better than either agent alone, leading to increasing amounts of hypophosphorylated 4EBP1 and selective inhibition of CAP-dependent translation of c-Myc. Network

analysis indicated that transcription of all 11 downregulated proteins identified as affected by the combination was regulated by c-Myc, and the shortest-path algorithm showed that c-Myc interacts directly with 12 proteins whose abundance was significantly reduced by rapamycin. The greater activity of the drug combination was obtained without an increase in toxicity as compared to either drug alone (Mazzoletti et al., 2011).

Combinatorial drug regimes are promising. Nonetheless, as the examples above illustrate, they too may encounter hurdles, mostly the result of persistent drug resistant mutations. A database containing multiple combinations encompassing orthosteric/allosteric drugs targeting the same protein, as well as combinations targeting parallel pathways may ease the problem and at the same time help to address patient diversity. Within such combinatorial framework, the pluses of allosteric drugs can be expected to make them major players.

Characteristics in Allosteric Regulation

We classify allosteric inhibition based on three useful characteristics: selectivity (or specificity), potency, and effectiveness. Binding sites away from the orthosteric active site are usually considered much more diversified, as they did not sustain direct evolutionary pressure to preserve key functional residues (Capra et al., 2009). Therefore, a first characteristic to be specified in allosteric inhibitor design is the extent of diversification of the residues involved in binding among the protein family members. The fact that higher diversity is more tolerant (or less prone) to network perturbation under high-dose treatment justifies this condition. A simple genomic survey based on both sequence and structure alignment should provide the distinct features that may help to define the selectivity of an allosteric inhibitor. Second, the potency of an inhibitor depends on the intrinsic affinity of the inhibitor (the dissociation constant, K_i) and the competition from the cosubstrate ([ATP] and $K_{m,ATP}$ in the case of protein kinases; Knight and Shokat, 2005). Though an orthosteric inhibitor is by definition competitive with substrate binding at the active site, allosteric inhibitors are not necessarily substrate competition free. Thus, whether an allosteric drug is substrate competitive or not should be specified to reflect the actual potency of the inhibition. Because the cosubstrate's K_m displays large differences with respect to distinct enzyme conformations, the state of the enzyme to which the inhibitor binds also needs to be specified. For example, as described above, the protein kinase core domain is believed to largely populate two states, an active αC-helix-in conformation and an inactive αC-helix-Out conformation, with the inactive state being much less favorable to ATP binding. Figure 5 provides examples of complexes of protein kinases with inhibitors, with the classification described here.

Conclusions and Perspectives

Proteins function through highly interconnected cellular pathway linkages; thus, changes in their conformations affect the cell. Yet, to date, studies of allostery have largely focused on effects in single proteins and their immediate surroundings. Here, our central thesis is that allostery needs to be tackled from a "systems biology" perspective and that this would help to link aberrant gain-of-function allosteric events on the single-molecule level

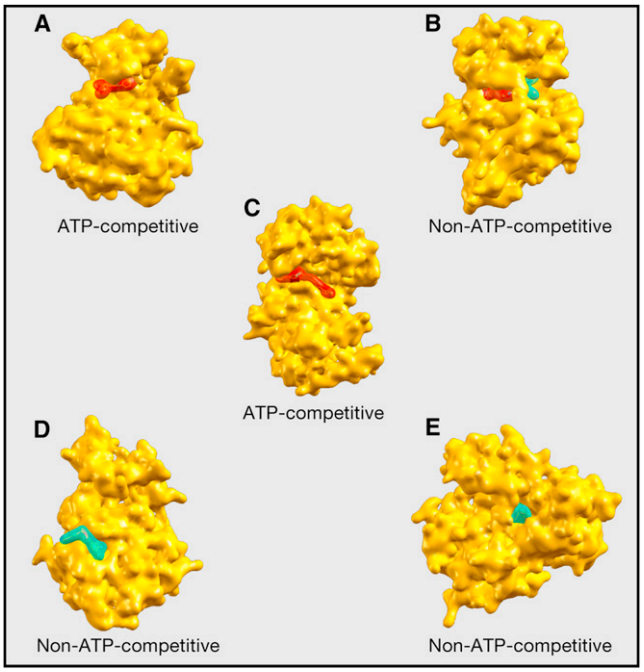

Figure 5. Allosteric Drugs for Kinase Inhibition

The figure provides a few examples of protein kinase complexes with various allosteric inhibitors.

(A) Orthosteric ATP-competitive inhibitor Gefitinib (red) in complex with active EGFR kinase (PDB: 3ug2).

(B) Allosteric non-ATP-competitive inhibitor PD318088 (cyan) in complex with inactive MEK1 kinase and ATP (red) (PDB: 1s9i).

(C) ATP-competitive inhibitor Imatinib in complex with inactive P38 αC-helix-out conformation (PDB: 3hec).

(D) Allosteric non-ATP-competitive inhibitor bound to C lobe of CHK1 kinase (PDB: 3jvr).

(E) Allosteric non-ATP-competitive inhibitor bound at the interface between the PH domain and catalytic core domain of AKT1 (PDB: 3o96).

to disease syndromes on the cellular (and organism) level. These insights will foster an understanding of possible effects of drug regimens and will aid in drug discovery. The example conceptualized in Figure 3 for G protein signaling clearly argues for such a view.

Combined with earlier observations, the Review leads to several major conclusions. (1) In its strictest definition, allostery takes place in a single protein. But to understand its effects, it must be put in the framework of the cell (Nussinov et al., 2013). (2) Allosteric effects propagate across the protein borders to their partners in complex assemblies (Lee et al., 2008). As a consequence, combinatorial allosteric effects are likely to be pronounced in multiprotein complexes, which are shared by several pathways. This may explain why pathological mutations in proteins clustered in the same complex, or module, can lead to the same disease. (3) Not all pathological allosteric mutations (or negative regulatory events, like pathogen protein binding) relate to long-range allosteric propagation in a protein. Short-range propagation or shifting of the free-energy landscape by stabilizing active (or inactive) conformations can also impair native function. Mutations in the kinases can act in this way. Mutations that shift the equilibrium toward a dimerization-favored state, as

recently observed by long timescale simulations of the epidermal growth factor receptor (EGFR), also appear to follow such a mechanism (Shan et al., 2012b).

The number of possible combinations of allosteric effects is staggering, making it extremely challenging to predict disease effects and the outcome of allosteric therapy. Nevertheless, we believe that the landscape of new classes of drug therapies will come from allosteric drugs. The diversity of the mechanisms through which allosteric modulators can act, as illustrated here through many examples, further emphasizes their potential heterogeneity. We end on an optimistic note. While drug discovery is challenging and has encountered a meager handful of successes and many highly costly failures, the allosteric drug space has barely been explored. The fact that many proteins are considered ''undruggable'' does not imply that this is indeed the case. Transient allosteric pockets in these or in proteins with which they interact, directly or indirectly (Nussinov et al., 2011), can and we expect will lead to therapeutic advances. Lastly, a combinatorial allosteric drug regime may show its mettle particularly in combating intractable, tenacious drug-resistant mutations in which the lower chances of side effects may allow more extensive collective therapy.

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