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Autoinhibited Proteins as Promising Drug Targets

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Abstract Current drug discovery efforts generally focus on a limited number of protein classes, typically including proteins with well-defined catalytic active sites (e.g., kinases) or ligand binding sites (e.g., G protein-coupled receptors). Nevertheless, many clinically important pathways are mediated by proteins with no such obvious targets for small molecule inhibitors. Allosteric inhibitors offer an alternative approach to inhibition of protein activities, particularly for proteins that undergo conformational changes as part of their activity cycle. Proteins regulated by autoinhibitory domains represent one broad class of proteins that meets this criterion. In this article, we discuss the potential of autoinhibited proteins as targets for allosteric inhibitors and describe two examples of small molecules that act by stabilizing native autoinhibited conformations of their targets. We propose that proteins regulated by autoinhibition may be generally amenable to allosteric inhibition by small molecules that stabilize the native, autoinhibited fold. J. Cell. Biochem. 93: 68–73, 2004. © 2004 Wiley-Liss, Inc.

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The process of drug discovery typically begins with the selection of a target protein relevant to disease. Since high-throughput screening (HTS) is an expensive and time-consuming process, validation of the target is critical before initiating a screening program. This process begins with the consideration of known proteins mediating the clinically relevant pathway. Selection of a candidate target involves first considering whether the protein is likely to be amenable to small molecule inhibition (i.e., "druggable"). This decision is often made based on: (a) structural similarity to known drug targets or (b) knowledge of endogenous ligands or substrates that define a target site. Having identified a potential target, considerable effort is then expended to validate the target by demonstration that inhibition of the target will produce the desired phenotype, ideally in an animal model of disease, or, alternatively, in a cell or tissue-based model. Only once there is confidence that a target CAN be inhibited by a small molecule AND that this inhibition will produce a therapeutically beneficial response, is the investment in HTS made.

Experimental approaches to target validation have become increasingly available through the use of genetic knockouts and small interfering RNA-mediated knockdown of candidate targets. These approaches are highly effective, and allow ready determination of the biological relevance and significance of most candidate targets for a given disease. The decision about whether a protein is likely to provide a fruitful target for small molecule inhibitors, however, is not straightforward. Historically, proteins with enzymatic activity have been favored as targets, as it is usually simple to develop HTS screens for such proteins based on in vitro measurement of their catalytic activity or ligand binding. The small catalytic pockets of common classes of regulatory enzymes (e.g., kinases) are generally druggable, either by classic suicide substrates or by active site-directed inhibitors. In some cases compound libraries may even be chosen to be biased for structural similarity to endogenous ligands/substrates. While this approach has

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resulted in the identification of a number of clinically effective compounds, it has some drawbacks: most significant is the potential lack of specificity of drugs targeting highly conserved catalytic domains, as discussed below.

Many clinically important signaling pathways are mediated by non-catalytic proteins not known to interact with small molecules or ligands. Instead, these proteins may function as adaptor proteins or scaffolds for multiple interacting partners, not all of which may be known. Non-catalytic or adapter proteins have not typically been considered as highly druggable, causing the drug discovery field to neglect these proteins as targets in favor of more straightforward candidates. Nevertheless, over the past several years, an increasing number of allosteric inhibition and protein interaction targeting strategies have been described (reviewed in [Arkin and Wells, 2004]), suggesting that the fraction of the proteome promising for use in HTS screens is greater than the highly derived subset of catalytic proteins. We propose here that a strategy of targeting proteins that employ auto-interactive, autoinhibitory strategies are particularly attractive targets for further drug investigations, offering opportunities for both allosteric and protein-interaction based modulation of their activity.

Allostery

Allostery refers to the modulation of protein activity by the binding of a regulator to a site distinct from the catalytic active site. The underlying mechanism involves a conformational change between active and inactive states of the protein, with the regulator exhibiting strong preference for one conformation and consequently shifting the equilibrium distribution of the protein toward the regulator-bound state. This regulatory mechanism is frequently used by nature, as illustrated by the classical examples of "product feedback inhibition" in metabolic pathways [Pardee and Reddy, 2003]. From a drug discovery perspective, allosteric inhibitors represent a potentially powerful alternative approach to active site-directed inhibitors. Competitive inhibitors must bind with relatively higher affinity or be present at higher concentrations to effectively compete with the binding of naturally occurring substrates [DeDecker, 2000]. In contrast, a small molecule allosteric inhibitor may recognize a target site bound by no other protein or small molecule, allowing effectiveness at substantially lower concentrations. Furthermore, enzyme active sites are highly conserved across large protein families (e.g., kinases, proteases, or GTPases), rendering the identification of inhibitors specific to a particular family member problematic because of cross-reactivity. In contrast, binding sites for potential allosteric modulators, involving sequences distant from the catalytic site, may vary significantly among the members of a protein family.

Proteins that undergo conformational changes during their activity cycle have been proposed to be particularly amenable to allosteric inhibition [Peterson et al., 2001]. In this view, the conformational plasticity implied by distinct active and inactive states increases the number of conformers to which a drug-like compound could potentially bind. In addition, this flexibility may allow for structural rearrangements in the protein to accommodate compound binding by an induced fit mechanism [Luque and Freire, 2000]. A challenge to exploiting the idea of allostery is the difficulty of predicting in advance which proteins offer particularly promising targets. Indeed, few proteins of therapeutic interest are known to be allosterically regulated in vivo. Nevertheless, several examples exist of HTS identification of allosteric inhibitors of proteins not known in advance to be similarly regulated by endogenous factors [McMillan et al., 2000; Peterson et al., 2001; Arkin and Wells, 2004]. How, then, can targets of HTS screens be chosen to maximize the likelihood of identification of allosteric inhibitors? One possibility is to select protein targets that exhibit conformational changes as part of their functional cycle, for example, proteins that are regulated by autoinhibitory domains.

Autoinhibition

It has been increasingly recognized that a major mechanism for negatively regulating otherwise constitutive protein activities is the presence of cis-acting inhibitory sequence elements [Pufall and Graves, 2002]. The negative regulation of a protein domain by intramolecular interaction with an inhibitory element in the same polypeptide is known as "autoinhibition." The existence of an autoinhibitory domain in a protein of interest is often revealed by studying truncated fragments. The identification of a protein fragment that exhibits greater functional activity than the full-

length protein suggests the presence of an autoinhibitory domain in the full-length protein, and nominates the deleted region of the protein as contributing to that function. During autoinhibition, the inhibitory effect can be mediated by direct masking of an active site or interaction domain: among kinases, the use of "pseudosubstrate" domains as autoinhibitory motifs is well established (recently reviewed in [Lew, 2003]). Alternatively, inhibition can occur by an indirect (allosteric) mechanism. Relief of the autoinhibitory interaction can occur by several mechanisms including interaction with other proteins, covalent modification of the autoinhibited protein, for example by phosphorylation, or by proteolytic removal of the inhibitory domain (Fig. 1). When autoinhibition is relieved by reversible binding of a regulatory protein (case "a" in Fig. 1), modulation of the conformational equilibrium between active and inactive states is analogous to classical allostery.

Autoinhibition has been widely used in nature to repress diverse protein functions, including protein–protein interactions, DNA binding, kinase activity, transcription activation, and protein localization signals (recently reviewed in [Pufall and Graves, 2002]). Examples of proteins bearing autoinhibitory domains are abundant, including, among others, receptor tyrosine kinases (e.g., the epidermal growth factor receptor and platelet-derived growth factor receptor) [Cho and Leahy, 2002; Chiara et al., 2004], cytoplasmic kinases (Pak1, PKA/PKG, Abl, Src), transcription factors (p53, NF-κB), and other non-catalytic signaling proteins (Bid, N-WASP, VAV, ERM proteins)

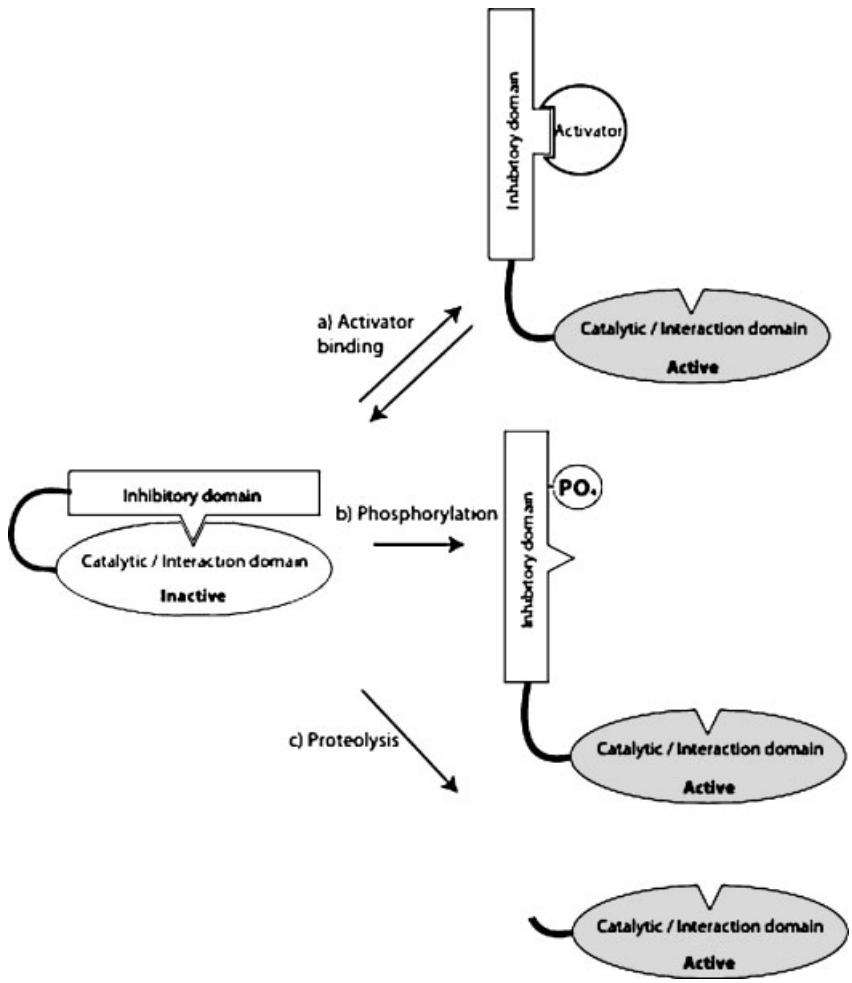

Fig. 1. Regulation by autoinhibition. Inhibition of a catalytic activity or a region mediating protein–protein interactions is caused by an intramolecular interaction with a separate inhibitory domain. Activation can occur by displacing the inhibitory domain by (**a**) reversible binding of a regulatory protein to the inhibitory domain or (**b**) covalent modification of the inhibitory domain (such as phosphorylation). Alternatively, the inhibitory domain can be removed by proteolysis as in (**c**).

[Aghazadeh et al., 2000; Pufall and Graves, 2002]. Many of these targets are highly validated as relevant to cancer development, inflammation, and other disease processes. The fact that autoinhibited proteins undergo a conformational change during activation (relief of the inhibitory interaction) similar to allosterically regulated proteins suggests that they may also be amenable to inhibition by small molecules that perturb the conformational equilibrium. In the past few years, examples of chemical inhibitors have been identified that target autoinhibitory elements of proteins not known to be modulated by endogenous small molecules. Below we describe two examples that illustrate this principle.

Imatinib (Gleevec, STI-571)

The Bcr-Abl fusion protein created by the chromosomal rearrangement known as the Philadelphia chromosome exhibits consitutive tyrosine kinase activity, and is a hallmark of chronic myelogenous leukemia (CML). Imatinib was identified as a selective inhibitor of Bcr-Abl kinase activity and has proven effective for producing remission in CML patients [Roskoski, 2003]. Structural studies of this compound and derivatives bound to an isolated Abl kinase domain have indicated that imatinib binds within the kinase domain in a site overlapping the adenine-binding site [Schindler et al., 2000; Nagar et al., 2002]. However, rather than acting as a simple competitive inhibitor, Abl catalytic activity is prevented, in part, by imatinib-induced displacement of Asp^{381} away from the active site. Asp^{381} is present within the highly conserved Asp-Phe-Gly motif of the activation loop and is critical for coordinating magnesium during catalysis. In addition, the imatinib-Abl structure revealed that the Abl activation loop interacts with the kinase domain in a manner mimicking, and therefore competitive with, substrate binding. The similarity between the conformation of the activation loop in the imatinib-Abl structure and the binding mode of substrate suggested that imatinib bound to a native autoinhibited conformation of Abl.

Recently, a crystal structure of a larger Abl fragment comprising the kinase domain as well as N-terminal regulatory domains known to be important for Abl autoinhibition was reported [Nagar et al., 2003]. The structure of autoinhibited Abl revealed a similar displacement of Asp^{381} out of the active site, supporting the conclusion that the imatinib-bound structure reflects a native conformation. Strikingly, even though the Abl kinase is well conserved with other kinases, such as Src, and Src kinase undergoes similar autoinhibitory folding, specific features of the allosteric folding associated with Abl but not Src make imatinib specific for Abl [Nagar et al., 2003]. Finally, indirect evidence of the importance of the conformational switch to the efficacy of imatinib arises from studies of spontaneous BCR-Abl mutations that cause resistance to this compound class: mutations are divided between the Abl catalytic site, and residues thought to be critical for stabilization of the inactive conformation [Shah et al., 2002].

Wiskostatin

The Wiskott–Aldrich syndrome protein (WASP) family plays central roles in mediating signaling from diverse upstream pathways to the Arp2/3 complex, a seven polypeptide-containing complex that is a major nucleator of actin filaments in eukaryotes (recently reviewed in [Millard et al., 2004]). N-WASP, a ubiquitously expressed member of the family, has multiple sequence elements that interact in a complex way to mediate protein activity. The N-WASP C-terminus is responsible for binding and activating the Arp2/3 complex. Within the regulatory N-terminus are distinct motifs that mediate interactions with diverse activators of N-WASP. These include a stretch of acidic residues that binds phosphatidylinositol 4,5-bisphophate, a GTPase binding domain (GBD) that binds Cdc42, and a proline-rich region that interacts with several SH3 domain-containing proteins including Nck and Grb2 [Millard et al., 2004]. Significantly, the N-WASP GBD engages in an autoinhibitory interaction with the N-WASP C-terminus, preventing activation of the Arp2/3 complex [Panchal et al., 2003]. Binding of Cdc42 to the GBD relieves this autoinhibition, allowing subsequent Arp2/3 complex activation. Indeed, comparative NMR structures of the GBD bound alternatively to Cdc42 or to the N-WASP C-terminus have revealed that the GBD is a conformationally plastic element that adopts at least two distinct conformations, depending on its inter- or intra-molecular binding partner [Abdul-Manan et al., 1999; Kim et al., 2000].

Two chemical inhibitors of N-WASP were identified in a broad screen for inhibitors of a signaling pathway regulating nucleation of

actin filaments in cytoplasmic extracts [Peterson et al., 2001; Peterson et al., 2004]. These compounds, named 187-1 and wiskostatin, had no effect on the ability of the isolated C-terminal fragment of N-WASP to activate the Arp2/3 complex in vitro, but were active in inhibiting Arp2/3 complex activation by full-length N-WASP. This suggested an allosteric inhibitory mechanism. In support of this idea, the 187-1 inhibitor was shown to stabilize the autoinhibitory interaction of N-WASP against activation by Cdc42 [Peterson et al., 2001]. While the structural details of 187-1 binding to N-WASP are as yet unknown, the solution structure of wiskostatin bound to the WASP protein was recently reported [Peterson et al., 2004]. Wiskostatin was found to bind entirely within the GBD of WASP without grossly affecting the overall autoinhibited conformation. This observation suggested that wiskostatin stabilized the autoinhibited fold of the GBD. To test this directly, the authors expressed the GBD as a recombinant polypeptide, and investigated its structure by NMR in the presence and absence of wiskostatin. The isolated GBD was disordered in solution, consistent with the observation that this flexible domain can adopt either a Cdc42-binding (active) or WASP C-terminus-binding (autoinhibited) conformation. Remarkably, wiskostatin induced folding of the GBD into the autoinhibited conformation in the absence of any interacting protein partner, demonstrating that this compound indeed stabilized the native autoinhibited fold of WASP. The consequences of this increased stability were predicted to be inhibition of Cdc42-binding, and direct impedance of the conformational change required to activate the Arp2/3 complex.

Implications for HTS Target Selection and Screening

The above examples illustrate how small molecules can perturb the conformational equilibrium inherent in autoinhibited proteins by stabilizing the native autoinhibited conformation, producing biologically significant inhibition. The identification of wiskostatin and a progenitor of imatinib [Druker and Lydon, 2000] by HTS demonstrates that such screens can be utilized to identify novel allosteric sites of this type, even in proteins not known to be regulated by endogenous small molecules.

We propose that the conformational changes that accompany activation of autoinhibited proteins may render these proteins generally susceptible to inhibition by small molecules. The regulation of protein activity by a conformational change requires a careful thermodynamic balance in the free energies of folding of the active and inactive states such that the affinities and abundance of endogenous modulators of this equilibrium are capable of shifting the position of the equilibrium under physiologically relevant conditions. Since protein activity is determined by the fraction of time spent in the active state, chemical inhibitors that even slightly bias this conformational equilibrium may produce physiologically relevant inhibition. Autoinhibition has been harnessed to regulate many types of protein "activities" including kinase activity (Abl) and protein–protein interactions (N-WASP). Thus targeting autoinhibited proteins should greatly broaden the spectrum of proteins considered as potential drug targets.

Future drug discovery programs are likely to uncover more examples of small molecules that stabilize native autoinhibitory interactions. To tap this potential we must broaden our concept of the types of proteins that are druggable and develop screens that will permit identification of allosteric inhibitors. What steps can be taken to take advantage of this potential opportunity?

(1) If a known autoinhibited protein is present in a pathway of interest, consider it as a potential target in HTS.
(2) When designing screens, use full-length versions of proteins as opposed to fragments containing catalytic domains, since these may have lost domains that contribute to auto-regulation. As a primary or secondary screening strategy, comparatively assess compound inhibition of full-length versus truncated (activated) protein forms.
(3) Where an autoinhibitory interaction is known or likely to exist, screen directly for stabilization of this interaction (e.g., for the intra-domain association of a target protein) rather than activity-based readouts.
(4) Finally, preferentially use unbiased, structurally diverse chemical libraries. While this strategy may involve more screening than the use of designed catalytic site-targeting libraries, it is more likely to

reveal and inhibit as yet uncharacterized allosteric regulatory sites.

In the increasingly distant past, drug discovery efforts identified compounds with some degree of pharmacological efficacy against a disease syndrome, but with no defined mechanism of action, and with some or many nonspecific effects. Given the enormous resources developed over the past two decades addressing proteome content, protein interactions, protein structures, and mechanisms of signaling, the new aspiration is to exploit this information to make drugs that efficiently correct a given syndrome, while limiting any other drug activities. The more efficiently and precisely we can modulate cell signaling, the better the chance of reaching this ideal state. Targeting autoinhibition is one promising strategy.

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