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αC helix displacement as a general approach for allosteric modulation of protein kinases

Lorenzo Palmieri and Giulio Rastelli

Department of Life Sciences, University of Modena and Reggio Emilia, Via Campi 183, 41125 Modena, Italy

Owing to their crucial role in the modulation of cell pathways, protein kinases are important targets for several human diseases, including but not limited to cancer. The classic approach of targeting the ATP active site has recently come up against selectivity issues, which can be considerably reduced by following an allosteric modulation approach. Being closely related to protein kinase inactivation, allosteric targeting via displacement of the conserved structural αC helix enables a direct and specific modulation mechanism. A structure-based survey of the allosteric regulation of αC helix conformation in various kinase families is provided, highlighting key allosteric pockets and modulation mechanisms that appear to be more broadly conserved than was previously thought.

Introduction

The human kinome is made up of more than 500 protein kinases [1] and represents a crucial regulatory mechanism of biological processes in cells. Owing to their role in cell regulation, protein kinases have become preferred targets for the treatment of several diseases resulting from their aberrant functioning, including inflammation, diabetes, neurological disorders and cancer. As opposed to protein phosphatases, protein kinases receive phosphorylation signals on specific Thr/Ser and Tyr residues, and retransmit them to downstream substrates to produce a regulatory pathway cascade.

As a result of the huge amount of work that has been carried out on protein kinases by structural biologists there is now an invaluable pool of over 2250 human protein kinase crystal structures (Protein Data Bank (PDB), August 2012 release [2]). By investigating and comparing these crystal structures, we have now reached detailed insights into the most conserved structural elements throughout the kinome, their extremely conserved catalytic mechanisms occurring in the active site and the peculiar features distinguishing kinase families and subtypes.

Several drugs targeting kinases are on the market today. Type I and I½ inhibitors bind at the nucleotide active site and are ATP-competitive (Box 1). Type II inhibitors, such as imatinib, bind at the ATP site and extend into an adjacent site close to the αC helix, and are lined by the conserved DFG (Asp-Phe-Gly) motif in its DFG-out conformation. Novel approaches that target 'truly' allosteric sites (i.e. distinct from the ATP site) are currently gaining a foothold in protein kinase drug discovery. Of these, type III inhibitors bind exclusively to allosteric pockets located in the proximity of the αC helix and the ATP site, whereas type IV inhibitors bind to sites distal from the nucleotide binding pocket.

Allosteric modulators are designed to help avoid off-target inhibition issues ascribed to active-site-targeting molecules. In principle, by developing allosteric inhibitors that target less conserved binding sites, higher selectivity can be obtained. Novel allosteric modulators exploit their high selectivity potential by targeting the inactive conformations of protein kinases. Because the structure does not need to be catalytically competent, each inactive kinase conformation is different from other structures, providing far greater opportunities for selectivity compared with those compounds that target the more conserved active conformation. By contrast, the growing realisation that inactive conformations of protein kinases are somewhat recurrent and that these can be grouped into a relatively small number of clusters [10] suggests that targeting these inactive conformations with allosteric inhibitors could reveal shared mechanisms among the various kinases of the human kinome [11].

Exploiting conformational changes in the 3D structure of protein kinases has recently proven to be invaluable for the development of protein kinase inhibitors [8,11,12]. The αC helix is located

Corresponding author: Rastelli, G. (giulio.rastelli@unimore.it)

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BOX 1

Kinase inhibitor classification

ATP-competitive ligands binding to the ATP-binding region, and usually locking the kinase in an active conformation, are called type I inhibitors. Examples of type I inhibitors are staurosporine and dasatinib.

To improve the inhibitor selectivity, a hydrophobic pocket that opens up between the DFG motif and the αC helix upon DFG-flip to the inactive conformation of the DFG motif is targeted together with the active site. Inhibitors of this type, called type II inhibitors, are for instance imatinib, sorafenib and BIRB796 [3], and 'lock up' the kinase in an inactive conformation.

Recently defined type I½ inhibitors exhibit a hybrid binding mode between types I and II, because they bind to the hinge and extend to the 'back pocket' just above the DFG motif in the DFG-in conformation, for catalytically active and inactive kinase conformations with respect to the αC helix [4]. Binding to the 'back pocket' confers selectivity, as well as binding to the adjacent allosteric pocket extending toward the αC helix.

Type III inhibitors do not compete with ATP and bind solely to purely allosteric pockets. They mainly act either by stabilising the DFG-out conformation or displacing the αC helix from its active conformation. Examples of type III inhibitors are CI1040 [5] and its second-generation derivatives binding to MEKs.

A further class of allosteric inhibitors is represented by ligands binding outside the active site cleft, several Å distal from the active site, named type IV inhibitors. Examples of type IV inhibitors are Akt-I-1 for Akt1 [6] and GNF-2 for Brc-Abl [7]. Types III and IV modulators usually target an inactive conformation of the kinase, exploiting multiple and less-conserved binding sites throughout the kinase families, therefore achieving greater selectivity.

Finally, bisubstrate and bivalent inhibitors are named type V inhibitors. More detailed information about inhibitor classification can be found in recent reviews by Rabiller *et al.* [8] and Lamba and Ghosh [9].

in the N-lobe beside the active site. It is present in all human kinase families and has a recognised role in each of the particular mechanisms that protein kinases adopt for activation and/or inactivation.

In this review, we focus on the role of the αC helix in the activation and/or inactivation mechanisms of protein kinases. We analyse in depth the state of the art human protein kinase allosteric inhibitors that directly or indirectly target the αC helix and we introduce the latest available crystal structures of kinase–modulator complexes targeting the allosteric binding sites proximal to the αC helix. Finally, we highlight the similarities and relationships throughout kinase families that have emerged from the analysis of the latest crystallographic data and molecular regulation mechanisms. This information enabled us to infer that allosteric binding proximal to the αC helix could be common to a broader number of kinases than was previously expected.

Role of the αC helix in the activation and inactivation of kinases

Many key structural elements of kinases, as well as the core catalytic mechanism and the key residues determining the phosphate transfer from ATP molecules to the substrate, are highly conserved. The active site, located between the N- and the C-lobe near the hinge-connecting region, enables the adenosine ATP group to dock and position correctly for the catalytic process by means of H-bonding and hydrophobic interactions. H-bonding is also important to stabilise the ribose group of ATP within the active site, and Mg^{2+} ions are fundamental for coordinating the phosphates during the transfer to the substrate. The phosphate transfer to the substrate is driven by a conserved salt bridge formed between a Lys–Glu pair, which coordinates the α- and β-phosphate groups of the ATP molecule. In fact, the conserved Glu residue is located on the αC helix, and disruption of the salt bridge is a strong indicator of protein kinase inactivity.

Conformation of the DFG motif is another important indicator of activity. When the motif is in a DFG-in conformation the Asp residue is correctly oriented to provide a catalytically competent active conformation. In the DFG-out conformation the Asp residue points away from the active site, and its position is swapped in a crankshaft-like motion also known as DFG-flip with the Phe residue, which in the latter conformation opens a hydrophobic pocket between the active site and the αC helix. This pocket opens when the DFG flips. It has been observed in several human kinase families and is the site where type II (and in some cases type III) inhibitors bind. Leu95 [PKA numbering; PDB ID: 1ATP] of the αC helix belongs to a cluster of hydrophobic residues known as the regulatory spine (R-spine), which is also key for kinase regulation [13]. The R-spine is formed by hydrophobic residues located in the β4 strand, the αC helix and the activation loop. The high mobility of the elements of this spatial motif represents a dynamic activity modulator, because the motif is extremely conserved in active kinases, whereas it is disrupted in inactive conformations [14]. Unlike the conserved active conformation, which is essentially the same for all kinases, there are several types of inactive conformations of the autoregulation mechanisms identified throughout the superfamily. Some kinases adopt an autoinhibited conformation, and turn into their active form upon either binding a partner regulatory entity, such as the cyclin-dependent kinase (CDK)/cyclin protein complex [15] and the epidermal growth factor receptor (EGFR) asymmetric dimer [16], or binding a self-regulatory domain such as in proto-oncogene tyrosine protein kinase Fes [17] and a subset of the AGC kinase family [18]. Others are, by default, inhibited by self-regulatory domains, such as Abl SH2 and SH3 domains [19]. It is important to highlight that, to a certain extent, all these autoregulatory mechanisms involve repositioning of the αC helix. In fact, in the inactive conformation, the αC helix is usually swung outward from the active site, and the catalytic Lys–Glu pair is disrupted, as is the hydrophobic spine in which the αC helix takes part.

Targeting the αC helix with allosteric inhibitors

In the following section we review the most recent approaches used to modulate kinase activity by allosterically acting on the conformation of the αC helix, focusing above all on cancer-related kinases. Type II inhibitors that bind to the hinge and the so-called 'allosteric' DFG-out pocket are beyond the scope of this article, because our main focus concerns purely allosteric inhibitors. The analysis is also limited to kinase targets for which allosteric modulation mechanisms have been proven on structural grounds, on the basis of crystal structures deposited in the PDB. Remarkably, drug discovery research using these structures as guidelines has been yielding important and promising classes of allosteric inhibitors.

MEK1/2

To the best of our knowledge, to date no crystal structures of mitogen-activated protein (MAP) kinase kinases (MEKs) in the apo form, in complex with ATP or phosphorylated have been determined. Consequently, the active form of the kinase has not been clearly defined [20]. By contrast, the inactive conformations of MEKs are available from structural studies of inactive MEKs crystallised with allosteric inhibitors [20–26]. Interestingly, the inactive conformation of MEK1/2 is similar to that of CDK2, because for both of them the αC helix is displaced from its active state position, whereas the DFG segment maintains the DFG-in conformation typical to the active state. Also, the positioning of a short helix in the activation loop, conserved in MEKs and closely equivalent to the αL12 helix present in some CDKs and its homologous helix in c-Met, can be considered as a strong indicator of activity. By analysing the available crystal structures of inactive MEK1 a rigid movement of the αL12 helix of about 2 Å has been highlighted, as opposed to the small 0.9 Å helix αC movement, making the αL12 helix an important element for understanding MEK1 inactivation together with the αC helix [20]. The inactive conformation in these determined complexes can be ascribed to two main factors: first, the large distance between Lys97 and Glu114 (belonging to the αC helix) [20,21]; and second, the partial occlusion of the binding site for the extracellular-signal-regulated kinase (ERK) substrate of MEK1 caused by the positioning of a CDK2-like αL12 helix and to the steric hindrance imposed by type III inhibitors.

All of the inhibitors discovered so far support the hypothesis that inhibition occurs by locking the MEK1 protein kinase in the inactive conformation, rather than inducing a new inactive conformation [20]. A similar mechanism has been hypothesised for MEK2, because of its high structural homology with MEK1 [21]. In addition, the existence of a so-called MEK-pocket (Fig. 1a) capable of hosting allosteric inhibitors in several other protein kinases has also been speculated [24].

An analysis of the crystal structures available shows that the allosteric MEK-pocket is partially superimposed with the one targeted by lapatinib-like compounds. In fact, although the latter compounds bind to the active site, extend above the DFG and displace the αC helix, some of the residues that line the binding

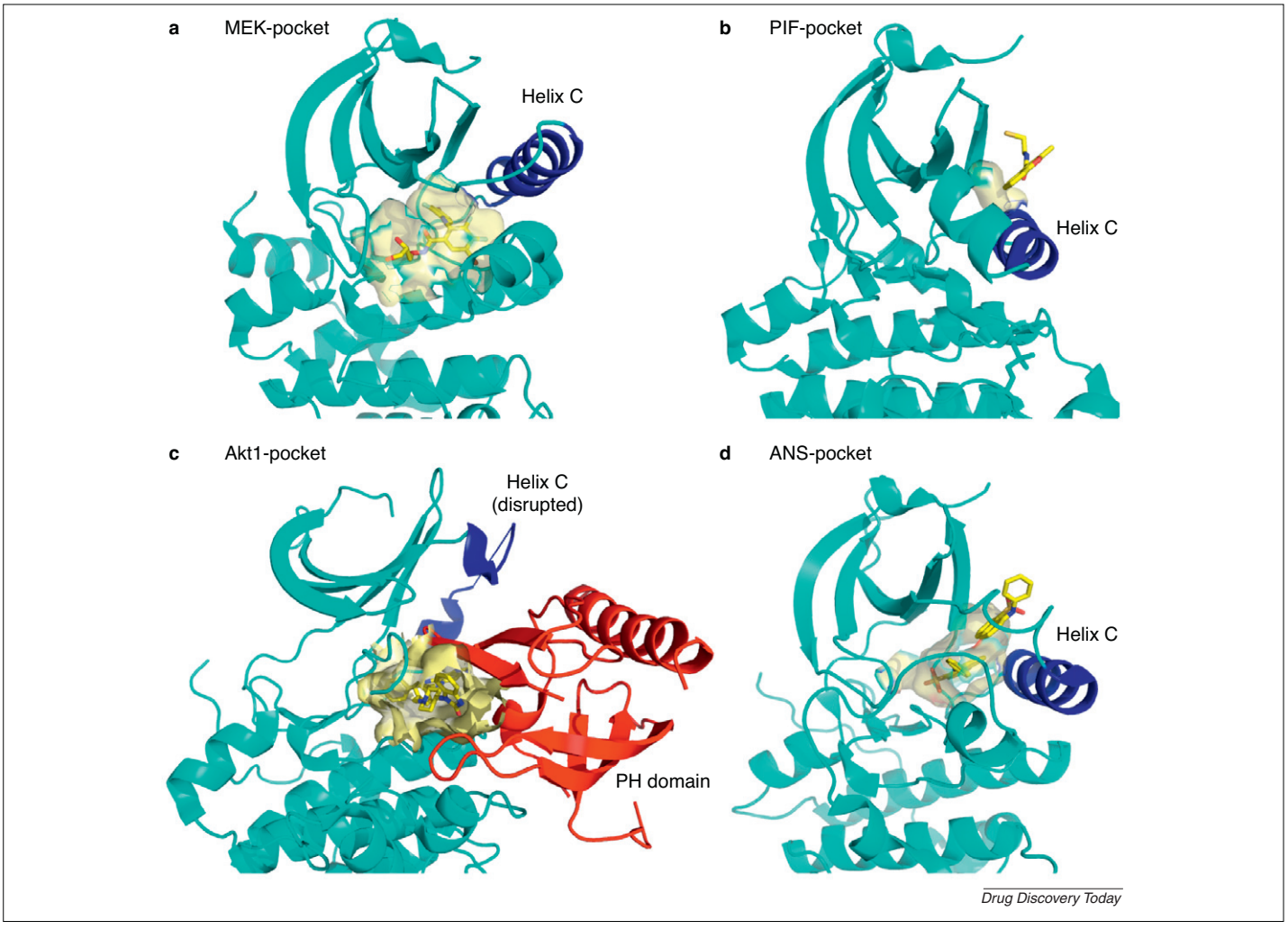

FIGURE 1

Allosteric pockets surrounding the αC helix. The N-lobe of protein kinases is shown as cyan cartoons, kinase inhibitors are represented by sticks, αC helix is in blue and pockets are pale yellow. **(a)** The MEK-pocket of MEKs is located between the DFG-motif and the αC helix (PDB ID: 1S9J). **(b)** The PIF-pocket of PDK1 is located above the αC helix, on the kinase surface (PDB ID: 3ORX). **(c)** The Akt1-pocket is located at the interface between the PH domain (in the 'PH-in' conformation) and the N- and the C-lobes of Akt1 (PDB ID: 3O96). **(d)** The ANS-pocket of CDK2 is located in between the MEK-pocket and the PIF-pocket (PDB ID: 3PXF).

site are common with the allosteric MEK-pocket. Lapatinib and lapatinib-like inhibitors can bind a wide range of kinase families such as: EGFR, human epidermal growth factor receptor (HER)2 and HER4 [27–31] (PDB ID: 3POZ, 1XKK, 3BBT, 3RCD, 3PP0), c-Met [32,33] (PDB ID: 3EFK, 3U6H, 3U6I), B-Raf [34] (PDB ID: 3OG7), Syk [35] (PDB ID: 3TUC) and protein kinase RNA-like endoplasmic reticulum kinase (PERK) [36] (PDB ID: 4G31, 4G34), thus pointing out that there is a shared kinase inhibition mechanism based on αC helix displacement. In these cases, the kinase is in a DFG-in conformation, the Lys–Glu ion pair is broken and the αC helix adopts an outward orientation.

To date, potent and selective type III allosteric inhibitors of MEK1/2 with a known crystal structure include PD318088 and PD334581 [21] (PDB ID: 1S9J, 1S9I), compound 12b [22] (PDB ID: 3EQB), PD325089 [20] (PDB ID: 3EQG), BAY869766 [23] (PDB ID: 3E8N), PD316684 and PF4622664 [24] (PDB ID: 3DY7, 3DV3), G894 and G925 [25] (PDB ID: 3V04, 3V01) and UCB1353770 [26] (PDB ID: 3SLS).

It is interesting to note that all these type III inhibitors crystallise in the presence of ATP or an ATP analogue, and most of them form direct H-bonds with the phosphates of the nucleotide. This is consistent with the synergistic thermodynamics of binding between type III inhibitors and nucleotides observed with isothermal titration calorimetry and temperature-dependent circular dichroism experiments [37]. Type III MEK inhibitors with chemical groups that extend into the ATP site (and thus potentially able to displace ATP) have not been reported. Given the crystal structures available and the synergism in binding observed, it might be challenging to displace ATP while at the same time achieve potent activity, but such a possibility cannot be ruled out. Finally, MEK1 and MEK2 sequences are 85% identical, and residues at a distance $\leq$10 Å from the crystallised allosteric inhibitors (PDB codes: 1S9J and 1S9I, respectively) are also totally identical. For this reason, we believe that it would be an extremely challenging task to design allosteric inhibitors that are selective for one of the two isoforms using this pocket.

First-generation type III inhibitors, such as CI1040 [5], were discontinued from clinical development mainly because of reduced antitumour activity, poor solubility and low bioavailability. By contrast, several second-generation inhibitors with improved pharmacokinetic properties are ongoing in clinical trials for several types of cancers. These inhibitors include such promising molecules as GSK1120212, AZD6244, BAY869766, TAK733 and RO04987655 [38].

PDK1 and PKCζ

Phosphoinositide-dependent kinase (PDK)1 and protein kinase C (PKC)ζ are highly homologous protein kinases belonging to the AGC family. In PDK1 a hydrophobic pocket positioned in the N-lobe of the kinase, termed PDK1-interacting fragment pocket (PIF-pocket), has been identified as a modulation target for kinase activity, because it serves as a binding site for the hydrophobic motif of its substrates [39,40]. The PIF-pocket is 5 Å deep, and is formed by residues belonging to the β5 sheet and αB and αC helices [41], as shown in Fig. 1b. Owing to its central position in the catalytic core and the presence of the PIF-pocket adjacent to it, the αC helix appears to provide a structural link between the regulatory elements of the kinase: the phosphate pocket adjacent to the PIF-pocket, the pSer241 belonging to the activation loop in the C-lobe, and the active site [41]. Binding of the PS48 modulator to the PIF-pocket induces conformational changes to the active site, as well as the activation loop and the αC helix, stabilisation of which is crucial for PDK1 activation [42]. Binding of several modulators to the PIF-pocket shortens the distance between Lys111 in the active site and Glu130 on the αC helix, thus confirming the allosteric nature of activation [42] and that the PIF-pocket of PDK1 is a druggable site [43].

The finding that an ambivalent function of activation and inhibition can be obtained by targeting the PIF-pocket with different ligands [44] has been confirmed by recent PDK1 co-crystallisation with activators and inhibitors [42,45] (PDB ID: 3HRF, 3OTU, 3ORZ). Ultra-HTS and docking approaches have highlighted the differences in binding modes between the two types of modulators [46]. More precisely, activators present a carboxylate group that induces key structural rearrangements leading to kinase activation [42,44], whereas compounds that are lacking this functional group elicit kinase inhibition [46]. It is worth noting that the αC helix positioning swings by up to 5 Å between the activated and inhibited conformations, together with the characterising αB helix of PDK1 (Fig. 2a), whereas in the active conformation the position of the helices is in between the two extremes (PDB ID: 1H1W). Tyr126 on the αC helix has been observed to form a favourable interaction with Asp223 of the DFG motif in cases where the helix is positioned close enough to it [45]. Conversely, Tyr126 lies far from the active site when the kinase is inhibited, and forms a H-bond with the phosphate of pSer241 [45].

In PKCζ there is also a PIF-pocket, which when targeted by PDK1 activators leads to PKCζ inhibition. PKCζ has been exploited to design highly selective allosteric inhibitors. Recently, a family of 4-benzimidazolyl-3-(4-chloro-phenyl)butanoic acid scaffold compounds, which are weak activators of PDK1 (PDB ID: 4A06, 4A07), have also been found to bind to the PIF-pocket of PKCζ and to inhibit its activity by means of a similar modulation mechanism [44,47]. These indirect connections suggest the presence of a PIF-pocket-related modulation mechanism in other members of the family of AGC kinases [47] and, similarly, suggest that opposite modulation effects can be achieved by different compounds targeting the same pocket [46]. Furthermore, the inhibition obtained by targeting the PIF-pocket of PKCζ is highly selective, even toward PKCι – the most closely related isoform of PKCζ [48].

Akt1

Akt1 is another member of the AGC family that is peculiarly regulated by the interaction with its pleckstrin homology (PH) domain. When bound to the cell membrane, the PH domain assumes an open PH-out conformation, and the kinase is ready to be activated by phosphorylation, whereas Akt1 typically assumes an inactive closed PH-in conformation when in its apo form. The PH-in conformation in the cytoplasm is in equilibrium with a PH-out unphosphorylated conformation.

Allosteric inhibitors of Akt1 have been discovered, and the existence of an allosteric binding site only in the presence of the PH-in conformation has been hypothesised [6,49]. Recently, two allosteric inhibitors, inhibitor VIII [50] and 12j [51] (PDB ID:

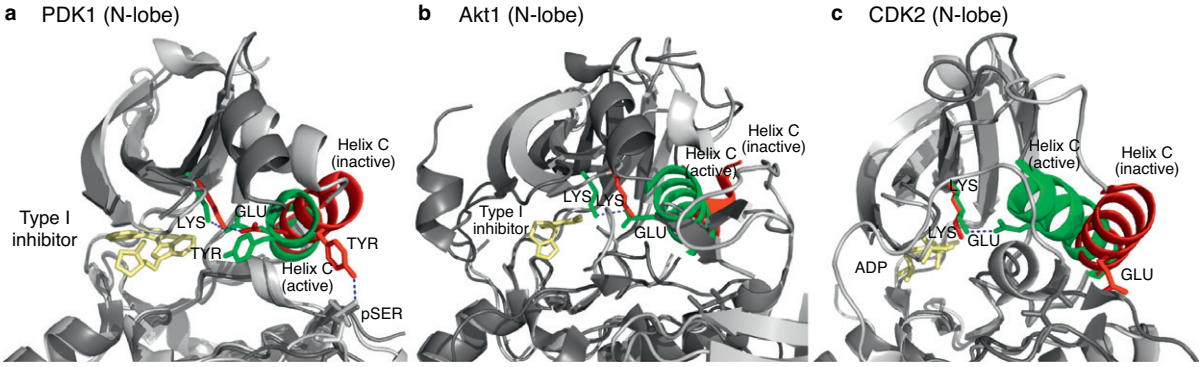

FIGURE 2

αC helix swing between active and inactive conformations. Active kinases are shown in dark grey and the αC helix in green, whereas inactive kinases are shown in light grey and here the αC helix is red. The Lys and Glu residues (ion pair) are shown with the same colour-coding, and interactions are represented by blue dotted lines. Ligands binding in the active site are shown, for reference, as pale yellow sticks. **(a)** Active (PDB ID: 3OTU) versus inactive (PDB ID: 3ORX) conformation of PDK1. Tyr126 and pSer241 are also shown. **(b)** Active (PDB ID: 3CQW) versus inactive (PDB ID: 3O96) conformation of Akt1. **(c)** Active (PDB ID: 3QHW) versus inactive (PDB ID: 3PXF) conformation of CDK2.

3O96, 4EJN), which bind between the N- and C-lobes just below the αC helix and close to the activation loop, have been co-crystallised with Akt1. Allosteric binding to AKt1 provokes dramatic conformational changes to the αC helix. In fact, the PH domain fills part of the space occupied by the αC helix and the activation loop, thereby preventing the kinase domain from attaining an active conformation. In the crystals, the PH domain is pulled up toward the kinase domain, the αC helix is disrupted upon inhibitor binding and the allosteric site lies more than 10 Å apart from the ATP site, at the interface between the PH domain and the N- and C-lobes of the kinase (Fig. 1c). In this way, Akt1 phosphorylation and a correct positioning of the αC helix, which are both needed for an active kinase configuration, are prevented and the complex is locked up in its inactive PH-in conformation (Fig. 2b). By analysing structural superimpositions, the Akt1 allosteric binding site is adjacent to the MEK-pocket found for MEK kinases. However, the formation of the Akt1 allosteric site is strictly dependent on the presence of the PH domain, because it is at the interface with this regulatory domain.

CDKs

CDKs have not been crystallised in a DFG-out conformation as yet, with the noteworthy recent exception of CDK8 in complex with sorafenib, an anticancer drug of clinical relevance [52]. The disruption of the catalytically crucial Lys–Glu ion pair by displacing the αC helix (PSTAIRE helix in CDKs) from its active conformation has recently been pursued to achieve kinase inactivation. Indeed, in CDKs the αC helix is involved in cyclin recruiting to form an active CDK/cyclin complex. By inducing an outward orientation of the αC helix, two effects can be achieved: disruption of the conserved Lys111–Glu130 ion pair; and disruption of the putative interface between CDKs and the conjugated cyclin (Fig. 2c). As a result, the kinase activation is therefore impaired. To date, no inhibitors have been approved for clinical use, mainly because of selectivity issues [53]. Recently, a significant advance in targeting CDKs with small molecules has been provided by the discovery of a completely allosteric pocket located between the αC helix, the β4 and β5 strands of the N-lobe and the active site [54], as shown in Fig. 1d. Two 8-anilino-1-naphthalene sulfonate (ANS) molecules bind to the pocket and induce large conformational changes to the kinase, including a remarkable outward movement of the αC helix, thus inhibiting the kinase activity [54] (PDB ID: 3PXF).

This is the first time that a completely allosteric ligand has been able to displace the αC helix and inactivate a CDK kinase by means of allosteric mechanisms. A new generation of allosteric ligands targeting the so-called ANS pocket is therefore promising because of the selectivity they can add. Furthermore, the entire CDK family might be prone to a similar inhibition mechanism, as a result of the high conservation of residues in the allosteric pocket [54]. Selectivity within the CDK family could be achieved by targeting the allosteric pocket between the αC helix and the active site, by exploiting the characteristic flexibility of the helix as demonstrated for CDK9 in complex with the inhibitor CAN508 [55] (PDB ID: 3TNH). In this case, the higher flexibility of αC induces CDK9-specific conformational changes that could explain CAN508 specificity. It can also be hypothesised that the higher flexibility of the αC helix might favour the opening of the ANS-pocket, so far detected only in CDK2, in some other CDK isoforms but not in the entire family.

αC helix in the EGFR family as a novel target for allosteric drug discovery

Crystallographic and mutational data have recently demonstrated that the EGFR family of kinases are activated by forming asymmetric dimers, in which one kinase plays the part of activator and the other takes on the role of receiver [16,27]. Importantly, the αC helix is located at the dimer interface and is primarily responsible for the interaction of the two monomers. This activation mechanism is closely related to the regulation of the CDK/cyclin family, and might therefore be prone to the same modulation mechanism focused on αC helix repositioning [10]. Crystal structures of lapatinib bound to EGFR [28] and HER4 [29] (PDB ID: 1XKK, 3BBT)

clearly show how it is possible to displace the helix from an active to inactive conformation owing to the bulky aniline group of the inhibitor, thus impairing the formation of an activating kinase complex. Although it is not yet clear whether displacement occurs upon inhibitor binding as a consequence of the bulky 3′-chloro-4′-[(3-fluorobenzyl)oxy]aniline group or whether inhibitor binding stabilises the inactive conformation of the αC helix, the former has been proposed to be more likely [28].

The promising lapatinib-like EGFR/HER2 dual inhibitor TAK285 was recently co-crystallised with EGFR and HER2 [27,30] (PDB ID: 3POZ, 3RCD). In EGFR a notable further rotation of the αC helix by 45° away from the active site, owing to the formation of a short helix in the N-terminal portion of the activation loop, has been noted [27]. The same effect has been achieved with irreversible inhibitors, such as the thienopyrimidine family of compounds [56] (PDB ID: 2R4B) and HKI272 [57] (PDB ID: 2JIV), with the additional advantage of limiting drug resistance [57]. Small but significant differences related to αC helix positioning characterise the autoinhibitory mechanisms throughout the EGFR family. HER3 almost exclusively acts as an activator for the EGFR family because of the lack of catalytically important residues and conformational changes in the αC helix, which is partially unwound, thus distorting and impairing its interface as a receiver kinase [58,59]. The lower intrinsic catalytic activity of HER2 with respect to EGFR is ascribed to the particular higher flexibility of the loop connecting the αC helix and β4 strand (which has a non-conserved glycine-rich pattern in HER2), the latter loop being important for correct positioning of the αC helix for activation [27,60]. The αC helix has a crucial role as a mediating element in EGFR, as well as in other kinases such as Abl [61]. The various features shared between the described mechanisms of activation and signal mediation involving the helix suggest that mastering the allosteric control activity of the EGFR family via interaction with the αC helix will probably provide important classes of new modulators in the future.

Concluding remarks and prospects for future research

Several characteristic allosteric binding sites have been reported in protein kinases. Of these, there is evidence of three main allosteric pockets adjacent to the αC helix that are somewhat recurrent in protein kinases of the human kinome. One is the allosteric pocket located between the αC helix and the active site, just above the DFG motif and named the MEK-pocket in MEKs. Adjacent to the MEK-pocket lies the Akt1-pocket, however this is particular to the Akt family of kinases because it is strictly dependent on the presence of the PH domain.

The second allosteric pocket, named the PIF-pocket and located just above the αC helix and between the αB helix and β4–β5 sheets, was first detected in PDK1 but is also present in other protein kinases. On the basis of accumulated structural and biochemical evidence, the MEK- and PIF-pocket have both been proven to exist in many kinase families and, as suggested, an even wider share of the kinome might be involved [24,47]. For instance, further evidence on the existence of PIF-pocket-analogous binding sites has been proved for glycogen synthase kinase (GSK)-3β, PKB, Aurora A and the cAmp family [62,63].

The third allosteric pocket close to the αC helix, named the ANS-pocket and which was first observed in CDK2, is deeper and spatially distinct from the PIF-pocket, although it partially communicates with it and extends almost up to the MEK-pocket [54].

Although the size and shape of the PIF- and ANS-pockets vary depending on the kinase family and specific native activation and/or inactivation mechanisms, their presence is observed in the active and inactive states. This contrasts with the MEK-pocket, which opens only in correspondence of an active conformation of the DFG motif and an outward movement of the αC helix. There is a tight link between αC helix stabilisation and the spatially conserved pockets around it in many kinase families. These pockets are filled up by several mechanisms to anchor the αC helix in the active conformation. Stabilisation is important for activity, because flexibility generally characterises the αC helix, in some cases to a greater extent than others, such as CDK9 [55] and HER2, often with a negative influence on kinase activity [27]. Investigating and comparing αC mobility among different proteins of the kinome might provide important clues for the design of allosteric inhibitors that bind in proximity to this helix. NMR studies might shed light on protein kinase motions in its active and inactive forms, which could help in understanding whether the opening of allosteric pockets around this helix is a consequence of its mobility or is dependent on inhibitor binding. The integration of X-ray crystallography, NMR studies and computational modelling such as molecular dynamics could provide useful suggestions for the design of truly allosteric inhibitors.

The presence of allosteric pockets around the helix, the mechanisms of αC helix stabilisation and similarities in its role in activation and inactivation throughout the kinase family support the general notion of the αC helix as a central mediator for allosteric inactivation of protein kinases. If computational approaches that can compare protein structures and detect surface pockets [64] were extended to the entire set of kinases with solved crystal structures, this might then reveal unexpected similarities, even within distantly related kinases and kinases with diverse activation mechanisms. Such analyses might provide unprecedented opportunities for the discovery of novel allosteric modulators possessing the desired selectivity profiles, and could also provide important directions for the design of multitarget drugs, for example drugs specifically designed to target more than one kinase in a controlled way.

Some of the features regulating kinase activation, αC helix conformational changes and the formation of crucial networks of residues around this helix are recurrent in the set of kinases for which allosteric mechanisms have so far been brought to light from a structural point of view. This suggests that analogous allosteric modulation mechanisms might be common to a wider range of protein kinases. For instance, EGFR activation by asymmetric dimer formation has strong analogies with the activation mechanism of the CDK2/cyclin A protein complex. This means that it might be possible to design purely allosteric EGFR inhibitors using the ANS allosteric pocket recently detected in CDK2. It will be interesting to see if this approach will provide new drugs for the important EGFR or B-Raf family of targets. A growing amount of evidence suggests that allosteric mechanisms in protein kinases might be more broadly conserved than initially thought. Of these, αC helix displacement is surely one of the most prominent mechanisms that can be exploited for drug discovery. From a structural and mechanistic point of view, it is probable that previous findings might

actually be applicable to other protein kinases as well, without the need to 'reinvent the wheel'. Once a comprehensive and comparative analysis of the information available has been extended to the whole kinome, this should open the way to novel therapeutic opportunities and possibly drugs with better specificity profiles and with fewer off-target effects.

Conflicts of interests

The authors have no conflict of interests to declare.

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