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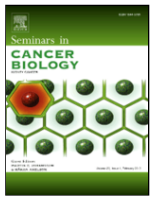

Review

AGC kinases, mechanisms of regulation and innovative drug development

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ABSTRACT

The group of AGC kinases consists of 63 evolutionarily related serine/threonine protein kinases comprising PDK1, PKB/Akt, SGK, PKC, PRK/PKN, MSK, RSK, S6K, PKA, PKG, DMPK, MRCK, ROCK, NDR, LATS, CRIK, MAST, GRK, Sgk494, and YANK, while two other families, Aurora and PLK, are the most closely related to the group. Eight of these families are physiologically activated downstream of growth factor signalling, while other AGC kinases are downstream effectors of a wide range of signals. The different AGC kinase families share aspects of their mechanisms of inhibition and activation. In the present review, we update the knowledge of the mechanisms of regulation of different AGC kinases. The conformation of the catalytic domain of many AGC kinases is regulated allosterically through the modulation of the conformation of a regulatory site on the small lobe of the kinase domain, the PIF-pocket. The PIF-pocket acts like an ON-OFF switch in AGC kinases with different modes of regulation, *i.e.* PDK1, PKB/Akt, LATS and Aurora kinases. In this review, we make emphasis on how the knowledge of the molecular mechanisms of regulation can guide the discovery and development of small allosteric modulators. Molecular probes stabilizing the PIF-pocket in the active conformation are activators, while compounds stabilizing the disrupted site are allosteric inhibitors. One challenge for the rational development of allosteric modulators is the lack of complete structural information of the inhibited forms of full-length AGC kinases. On the other hand, we suggest that the available information derived from molecular biology and biochemical studies can already guide screening strategies for the identification of innovative mode of action molecular probes and the development of selective allosteric drugs for the treatment of human diseases.

1. AGC protein kinases

Protein phosphorylation is a molecular mechanism that serves to transfer information within a cell to regulate cellular and organism-wide functions and to adequately respond to extracellular and intracellular signals [1]. The human kinome consists of over 500 protein kinases [2] that participate in a complex network that can specifically modify a large number of cellular proteins as a response to different stimuli in a time-dependent fashion. Based on the evolutionary relationships between their catalytic domains, the protein kinases are clustered into seven major groups: the tyrosine kinase (TK), tyrosine kinase–like kinase (TKL), homologues of yeast Sterile 7, Sterile 11, and Sterile 20 (STE), cyclin-dependent kinase (CDK), map kinase (MAPK), glycogen synthase kinase 3 (GSK3) and CDK2-like kinase (CLK) (CMGC), casein kinase 1 (CK1), calcium/calmodulin-dependent protein kinase (CAMK), and AGC kinase groups (Fig. 1) [2]. The latter group includes the kinases related to cAMP-dependent protein kinase 1 (PKA), cGMP-dependent protein kinase (PKG) and protein kinase C

Abbreviations: AGC kinase, group of kinases related to PKA PKG and PKC; PKA, protein kinase A; PKC, protein kinase C; PKG, protein kinase G; PRK, protein kinase C related kinase (also termed PKN protein kinase N); PDK1, 3-phosphoinositide-dependent protein kinase 1; PKB, protein kinase B (also termed Akt); SGK, serum and glucocorticoid-induced kinase; MSK, mitogen- and stress-activated protein kinase; RSK, p90-ribosomal S6-kinase; S6K, p70-ribosomal S6-kinase; GRK, G protein-coupled receptor kinase; NDR, nuclear dbf2-related kinase; LATS, large tumour suppressor kinase; MAST, microtubule-associated protein kinase; MASTL, MAST-like kinase; DMPK, myotonic dystrophy protein kinase; MRCK, myotonic dystrophy kinase-related CDC42-binding kinase; YANK, yet another kinase; CRICK, Citron Rho-interacting kinase; ROCK, Rho-associated protein kinase; SgK494, uncharacterized serine/threonine-protein kinase SgK494; Aurora, aurora kinase; PLK, polo-like kinase; PIF, PDK1-interacting fragment; HM, hydrophobic motif; MD, molecular dynamics.

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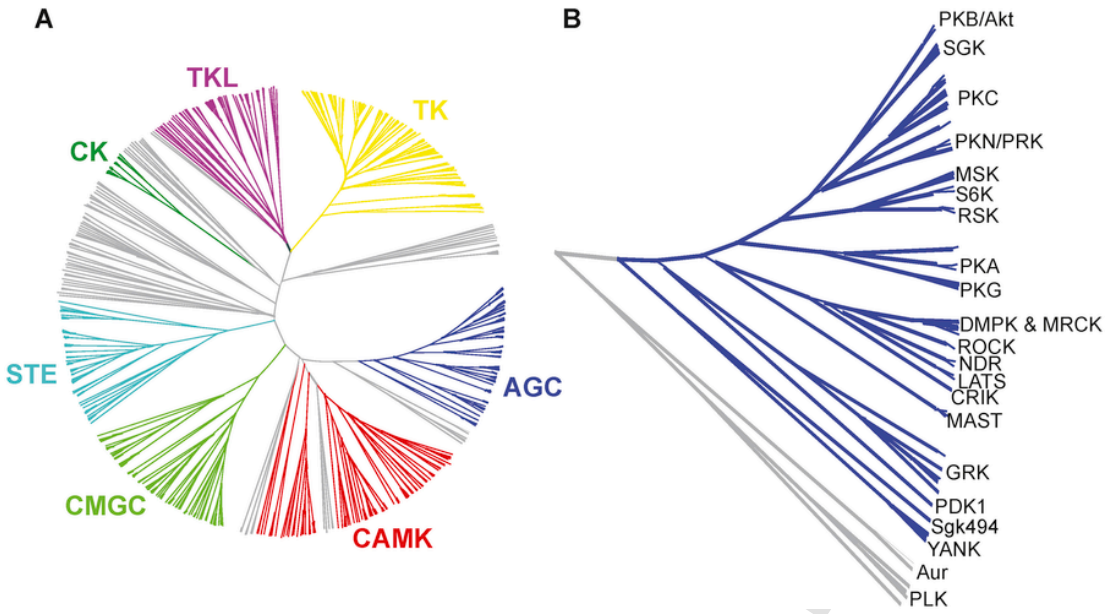


Fig. 1. AGC kinase group within the human kinome. A, Representation of the evolutionary relation of the catalytic domains between the AGC kinases and other human protein kinases. B, Evolutionary relationships within the AGC kinase group. The mayor families and subfamilies of AGC kinases are represented. PLKs and Aurora kinases are not considered AGC kinases; Aurora kinases share aspects of the molecular mechanism of regulation mediated by the PIF-pocket.

(PKC). Members of this group are widely present throughout distantly related life forms [3,4]. In the human genome, there are 63 genes coding for AGC kinases that are divided into 14 families and 21 subfamilies (Fig. 1) [2]. Within those families, there are two pseudokinases, defined based on the prediction that they lack features necessary for catalytic activity. In addition, grouped within the AGC kinases, there are 6 pseudogenes that code for nonfunctional copies of genes, which are not expressed or encode severely truncated proteins.

To achieve their functions, the protein kinases have common ancestral structural features in the catalytic domain and have also independently evolved sophisticated mechanisms of regulation. Alterations of protein kinase activity have been linked to a large variety of human diseases. Hence, protein kinases became a major drug target for the pharmaceutical industry, totalling approximately 30% of drug development programmes over the last 15 years [5]. Members of the AGC kinase group are involved in different pathologies, affecting human health issues including, among others, cancer, metabolic disorders, cardiovascular disease, immunological disorders, muscular dystrophies, and neurological disorders [3,6–10]. Most of the drug development efforts in the protein kinase field have been focused on inhibitors targeting the ATP-binding site. The high degree of conservation of this site across the human kinome proved to be a real challenge for the development of compounds that selectively inhibit one target kinase without affecting multiple other kinases. On the other hand, a few examples, including the allosteric PKB/Akt protein kinase inhibitor MK2206 [11], provide evidence that molecules targeting the regulatory mechanisms of the kinases can be very efficient inhibitors and more selective agents with fewer side effects, more suitable for combination therapies. In view of the prominent roles of AGC kinases in cancer and other human diseases, it has been our interest to understand the molecular mechanisms of the regulation of AGC kinases and consider how they could be exploited for drug discovery and the drug development of innovative medicines.

Previous publications have provided descriptions of the roles of different AGC kinases in plants, in animal health and in disease states and described the mechanisms of regulation within the different families of AGC kinases [3,4,6]. In this review, we summarize aspects of the mechanisms of allosteric regulation of different AGC protein kinases with emphasis on recent knowledge and analyse the potential use of this information for the discovery and development of a new generation of protein kinase inhibitors.

2. Common and specific structural features of AGC protein kinases

The first protein kinase to be crystallized was the catalytic domain of PKA, an AGC kinase that served as a model to understand the common structural features of the whole superfamily [12,13]. The AGC group members share the folding of the catalytic core as a common feature with the rest of the protein kinase superfamily. The catalytic domain consists of a small N-terminal lobe and a large C-terminal lobe. The small lobe consists of a 5-stranded β-sheet and a helix, αC, while the large lobe is mostly alpha-helical. AGC kinases often also present a second helix, αB, contiguous to the αC helix. Between the two lobes, in a deep pocket with contacts on both lobes, sits the ATP-binding site. The peptide-substrate binds in an extended conformation on the surface between the two lobes [14]. When we observe the active site of protein kinases with the small lobe on the top, the extended peptide-substrate/pseudosubstrate sits on the cleft with the amino acids N-terminal to the phosphorylation site sitting to the left (see Fig. 2). As a whole, one should visualize protein kinases as dynamic proteins, with major flexibility, for example, with hinge and twist motions related to the movement between the lobes, and in many AGC kinases, the dynamic nature of the αB and αC helices, whose modulation through activating or inhibitory regulatory mechanisms can mediate allosteric effects on the active site [15–17]. Like numerous protein kinases from other groups, many AGC kinases are also activated by phosphorylation [6,18,19] (see below).

As part of their substrate recognition, most AGC kinases are basophilic, interacting with positively charged amino acids at positions −2 to −5 N-terminal to the phosphorylation site [6,14,20–22]. These charged residues make specific interactions with Asp and Glu residues in the small lobe and the large lobe. Therefore, some substrates could, upon binding, stabilize the active-closed conformation of the kinase and release inhibitory domains.

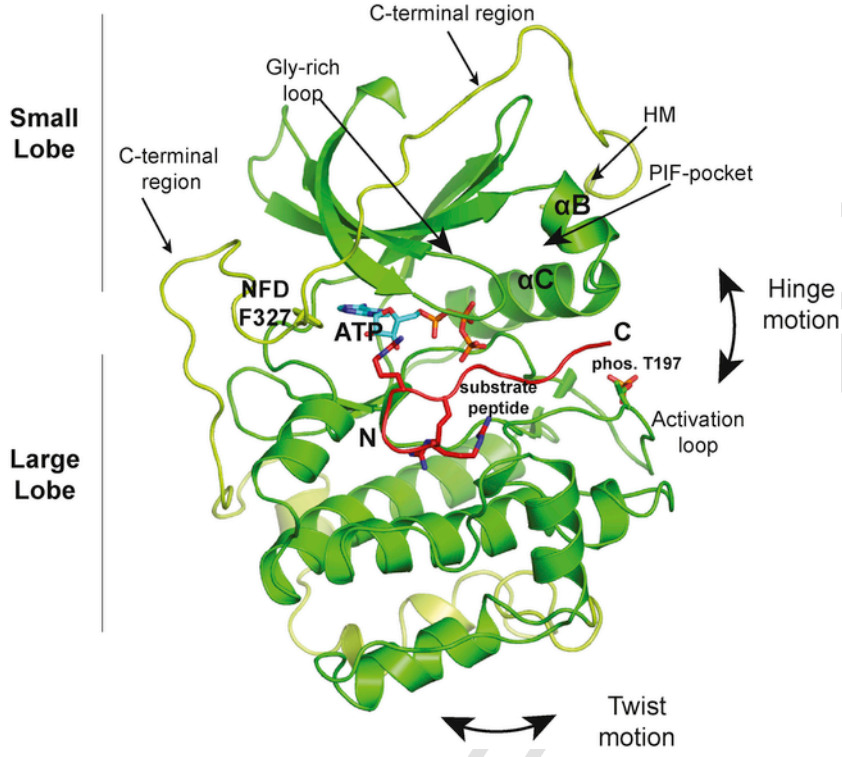


Fig. 2. Structural features of protein kinases and AGC kinases. Shown is the crystal structure of PKA (PDB code 1ATP), highlighting common features of protein kinases like small lobe, large lobe, ATP-binding site, peptide substrate binding, activation loop and glycine (Gly)-rich loop. Key specific features present in most AGC kinases are the C-terminal extension (shown in light green), comprising the NFD motif that interacts with the adenosine moiety of ATP and a hydrophobic motif (HM) that binds the PIF-pocket. Key to the allosteric regulation of many AGC kinases by the PIF-pocket is the flexibility of the helices αB and αC and the dynamics between both lobes, characterized by hinge and twist motions.

A peculiarity within the AGC kinase group is the presence of a hydrophobic motif (HM) sequence 50–60 amino acids C-terminal to the conserved catalytic core. This HM, consisting of the general motif Phe/Tyr-Xaa-Xaa-Phe/Tyr-Ser/Thr/Glu/Asp-Phe/Tyr, docks to a co-evolved hydrophobic site in the small lobe of the protein kinase core, termed the PIF-pocket (Fig. 2) [23,24]. In addition, distinctly from other groups of kinases, most AGC kinases – excluding PDK1, GRKs 1,4-7 and MASTL – have a Phe residue within a conserved NFD motif at the C-terminal extension (approximately 25 amino acids before the HM; Phe327 in PKA) that docks into the catalytic domain and interacts with the adenine of ATP.

In addition to these widely conserved specific motifs, AGC kinases from different families have evolved other family-specific regulatory mechanisms mediated by a variety of regulatory domains that mediate the interaction with cellular membranes or other interacting proteins (Table 1). Currently, a number of crystal structures of the catalytic domains from different AGC kinase families have been solved to high resolution, *i.e.* PKA, PDK1, PKB/Akt, PKC, SGK, S6K, RSK, MSK, GRK, NDR/LATS, PRK, MASTL, YANK, MRCK, ROCK, and DMPK, while its closely related families, Aurora and PLK kinases, are also available. In comparison to this vast structural knowledge, the different mechanisms of regulation are more widely deduced from biochemical experiments.

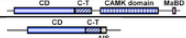
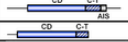
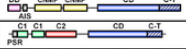
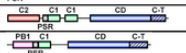
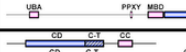
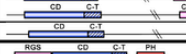
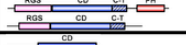
2.1. Regulation of AGC kinases

Since protein kinases are intracellular mediators of signals and information, they have evolved multiple mechanisms to efficiently phosphorylate specific substrates at the time and location needed. In a general manner, protein kinases of the AGC group are regulated by interacting with inhibitory domains or inhibitory regulatory subunits, by modulating their localization, by modulating their ability to interact with substrates and by modulating the conformation of the kinase domain. The protein kinase catalytic domain has made it possible to regulate its specific activity by enabling regulated conformational changes; for example, stabilizing the active conformation of the kinase by phosphorylation results in the activation of catalytic activity in many members of the AGC kinase group. Interestingly, independently of the catalytic activity, the ability to modulate the conformation of the kinase could also lead to specific signals in pseudokinases.

2.2. Regulation by phosphorylation

Phosphorylation is a widely described mechanism of regulation of the whole field of protein kinases and plays an important role in the regulation of different families within the AGC kinase group. In addition to the activation loop, common to different groups of kinases, many AGC kinases also possess two additional regulatory phosphorylation sites, the HM and the turn motif/zipper phosphorylation sites. The three phosphorylation sites are located within protein regions with extended conformations. Phosphorylation then triggers specific interactions – intramolecular or intermolecular – that ultimately regulate the catalytic activity. The actual mechanism by which phosphorylation activates AGC kinases has been depicted for all three of the most conserved phosphorylation sites [19,25,26]. The phosphorylation at the activation loop makes it possible to fix the activation loop to the small lobe and the large lobe [26]; for example, in PDK1, the phosphate makes specific interactions with Arg129 from helix αC and Arg204 from the large lobe [24], affecting the interlobe dynamics, stabilizing to some degree helix αC and the closed-active conformation of the kinase. Moreover, the phosphate also positions the activation loop in relation to the kinase domain, which may be relevant for substrate recognition in protein kinases such as PDK1 whose polypeptide-substrate recognition motif is located C-terminal to the phosphorylation site, in a region where the activation loop sits.

Table 1
Domain organization within AGC kinases [176–182].

Kinase	Isoforms	Domain organization[a]	HM sequence[b]	Phospho A-loop [c]	Membrane Targeting	Pr-Pr interaction	Ref.
PDK1	-		No HM	C	PH	-	[23, 24]
PKB/Akt	1 - 3		F-P-Q-F-**S**-Y	D	PH	-	[80]
SGK	1 & 2 3		F-L/V-G-F-**S**-Y	D	- PX	-	[18]
RSK	1 - 4		F-K/R-G-F-**S**-F	D	-	-	[116, 176]
MSK	1 & 2		F-Q-G-Y-**S**-F	D	-	-	[176]
S6K	S6K, S6Kβ		F-L-G-F-**T**-Y	D	-	-	[18]
PKA	α, β, γ		F-S/G-E-F	C	-	-	[12, 177]
PKG	1 & 2		W-D-I/K-D-F	C	-	DD	[178]
PKC	α, β, γ		F-X-G/N-F-**S/T**-Y/F	C	C1,C2	C1	[19, 32, 33, 91, 95, 105, 112, 179]
	δ, ε, θ, η			C	C1,C2	C1	
	ι, ζ		F-E-G-F-**D**-Y	C	C1	C1, PB1	
PKN/PRK	1-3		F-L/R-D-F-**D**-F/Y	D	C2	HR1, PKL	
NDR	1 & 2		F-I/L-N-Y-**T**-Y	D	-	MBD	[7, 146, 180]
LATS	1 & 2		F-Y-E-F-**T**-F	D	-	UBA, PPXY ,MBD	[7, 142][180]
DMPK	1 2		F-V-G-Y/F-S/T-Y	A	- C1, PH	CC, C1, CNH	[151]
ROCK	1 & 2		F-V/I-G-F-T-Y	A	PH, C1	HR1, C1	[149, 152]
MRCK	α β		F-V/I-G-F-T-Y/F	A	C1, PH	CC, C1, CNH, PBD	[150]
CRIK	-		F-V-G-F-S-Y	A	C1, PH	C1, CNH	[6]
GRK	2 & 3 1,4 - 7		L/F-F/Y-X-X-F	A	PH -	RGS	[3, 6]
Aurora	A-C		No HM	A	-	-	[126, 181]
PLK	1-4		No HM	A	-	PoBD	[182]
RSLK	1 2		No HM	A	PX -	MIT	[6]

[a]Schematic representation of each polypeptide including the relevant domains and motifs shown in different colors: protein–protein interaction, membrane targeting and catalytic domains are depicted in pink, red, and blue, respectively, while the inhibitory motifs (AIS, PSR and PKL), C1 and CNMP domains are colored in black, green and yellow, respectively. The size of the bar is proportional to the amino acid sequence length and cutting lines symbolize parts of the sequences that were removed to allow proper representation.
[c]The hydrophobic motif (HM) sequence is shown and the phosphorylatable or phosphomimetic residue is presented in bold red lettering.
[c]Phosphorylation of the activation loop (A-loop): D, dynamic; C, constitutive; A, absent.
Abbreviations: AIS, autoinhibitory segment; C1, PKC conserved region 1; C2, PKC conserved region 2; CC, DMPK coiled coil domain; CD, catalytic core domain; CNH, citron-NIK homology domain; CNMP, cyclic nucleotide-monophosphate binding domain; C-T, C-terminal extension of catalytic core; DD, PKG dimerization domain; HR1, PKC-related kinase homology region 1; MaBD, MAPK binding domain; MBD, MOB-binding domain; MIT, microtubule interacting and trafficking molecule domain; PB1, Phox and Bem1 domain; PBD, p21 activated kinase binding domain; pH , pleckstrin homology domain; PKL, PRK2 autoinhibitory and oligomerization region; PoBD, Polo box binding domain; PPXY, proline tyrosine motif; PSR, pseudosubstrate region; PX, Phox homology domain; RBD, putative Rho-binding domain; RGS, regulator of G protein signalling domain; UBA, ubiquitin-associated domain.

In addition, the specific activity of many AGC kinases is also modulated by the two other conserved phosphorylation sites, the HM and the zipper/turn-motif phosphorylation sites, located on a region C-terminal to the catalytic core. The mechanism by which these phosphorylations promote the activity of AGC kinases is related to the enhancement of the interaction between the HM and the PIF-pocket regulatory site (described below in more detail), which includes the helix αC. *Via* this important helix, the three phosphorylation sites can act in concert to stabilize the active form of the kinase. The turn-motif/zipper site is present in the PKA, PKG, Akt, S6K, SGK, RSK, MSK, PKC and PRK families, while the PKA, PKG, PRK and aPKC families have an HM but not the associated HM phosphorylation site. Thus, although the turn-motif/zipper phosphorylation site and HM phosphorylation site are widely present in AGC kinases, there are numerous variations on the theme. Notably, in PKA and PKG, the HM is truncated and does not have an HM-associated phosphorylation site; PKA and PKG are thus examples that evolved not to be regulated by the C-terminal region. The HM phosphorylation only increases the specific activity of PKB/Akt, but it is necessary for the activity of S6K, RSK and MSK. PRKs and aPKCs lack an HM phosphorylation site but have Asp or Glu instead of the HM phosphorylation site. The "turn-motif" phosphorylation was first described in PKA. However, in PKA, the phosphate does not make direct contact with the catalytic core (*i.e.*, PDB code 1ATP). In other crystallized AGC kinases, the phosphate makes direct interactions with residues at the top of the small lobe, where one of the interacting residues is from the Gly-rich loop, which forms the cap of the ATP-binding site (*i.e.*, PRK2, PDB code 4CRS; PKCι, PDB code 3A8W). The phosphorylation of the turn-motif/zipper site triggers the interaction of the C-terminal region with the catalytic core, supporting the binding of the HM to the PIF-pocket. For this reason, the site, which plays a different role from the "turn-motif" site on PKA, was also termed the "zipper" phosphorylation site [25]. It remains to be established if, in addition, the interaction of this phosphate with the Gly-rich loop also participates directly in the activation mechanism. Further variations on the role of the turn-motif/zipper phosphorylation have also been described. In PRK2, the phosphorylation of the turn-motif/zipper site not only participates as a zipper, bringing the HM to the PIF-pocket in the activation of PRK2, but also participates in the release of the docking interaction with its upstream kinase PDK1 [27].

Although widely present in AGC kinases, the GRK, MAST, CRIK and ROCK families do not require activation loop phosphorylation for activity [6]. In these cases, it is presumed that the kinase core is stabilized in the active conformation by other constitutive or regulatory mechanisms.

2.3. The PIF-pocket regulatory site

The PIF-pocket was first shown in the crystal structure of PKA as the site where the Phe residues within the truncated HM Phe-Thr-Glu-Phe-COOH dock (PDB code 1ATP). The PIF-pocket is bounded by the αB and αC helices and the beta sheet, on the small lobe of the kinase catalytic core. While the β-sheet is rigid, the αB and αC helices are highly dynamic and are at the core of the PIF-pocket regulatory mechanisms. Examples of the dynamic nature of the αB and αC helices are described below for the PDK1 [17], PKB/Akt [20,28], and aPKC [29] families. The interaction of the HM with the PIF-pocket allosterically affects the ATP-binding site and the peptide substrate binding site, as described for PKB/Akt [20,28], PDK1 [17,30,31], aPKC [29,32] and PRK2 [33]. Although the terminology "PIF-pocket" is applied to AGC protein kinases, the helix αC and the equivalent sites are also regula-

tory sites in other protein kinases, such as CDKs [34], and even evolutionary more distant Tyr-kinases, such as EGFR [35,36].

The PIF-pocket ability to regulate the activity of AGC kinases was first demonstrated in PDK1, which does not have an HM at its C-terminus. PDK1 binds with high affinity to a polypeptide derived from the HM of PRK2, termed PDK1 Interacting Fragment (PIF). It was shown that the polypeptide PIFtide (REPRILSEEEQEMFRDFDYIADWC) interacted with the PIF-pocket and activated PDK1 *in vitro* to phosphorylate a polypeptide substrate [23,37]. Similarly, PIFtide and other HM-derived polypeptides activated in trans representative family members of kinases PKB/Akt, S6K, SGK, aPKC, RSK, and MSK *in vitro* [28,38]. Interestingly, replacing the C-terminal region of PKB/Akt with the PIF sequence (PKB/Akt-PIF) rendered a constitutively active kinase [39]; PKB/Akt-PIF was later crystallized, showing that the PIFtide sequence stabilized the active conformation of the kinase domain by docking directly into the PIF-pocket [20]. The fusion to PIF also rendered other AGC kinases constitutively active, even a member from a more distant AGC kinase family, NDR/LATS [40–42].

In this line, the crystal structures of diverse active AGC kinases show the HM bound in the PIF-pocket. HM polypeptides that activate AGC kinases, *e.g.*, the 24 amino acid peptide PIFtide, bind to the PIF-pocket but also to regions surrounding the PIF-pocket. Mutations within the PIF-pocket of PDK1 or covalent modifications on residues forming part of the PIF-pocket can increase or decrease the catalytic activity of PDK1, indicating that the PIF-pocket itself can mediate the stabilization of more active forms or the de-stabilization of the regulatory site, leading to more inactive conformations of the kinase [23,43]. The mechanism of activation and allosteric inhibition has been investigated using small compounds that bind precisely to the PIF-pocket [17,31,32,43–52]. Small compounds binding to the PIF-pocket of PDK1 were able to mimic the *in vitro* activation of the kinase domain, mimicking the phosphorylation-dependent activation of the kinase. Thus, polypeptides binding to the PIF-pocket, mutagenesis of the PIF-pocket, covalent modification of the residues forming the PIF-pocket and the development of small compounds that bind to the PIF-pocket, allosteric activators and allosteric inhibitors, together, provided evidence that the PIF-pocket is a central and common ON-OFF switch in a broad range of AGC kinases. Although there is considerable more information about the structure of the overall active ON forms of the kinases and the mechanism of activation, there is also key knowledge on the structural aspects of the inhibited form of selected AGC kinases.

In short, in a basic model, the PIF-pocket can be visualized as an ON-OFF switch, a regulatory site that has an active conformation, featured in the active structures with the HM bound to the PIF-pocket, and an inactive conformation, which in principle can have different forms depending on the kinase. Information on structural aspects of the OFF switch stabilized by additional domains is summarized below for PKB/Akt, (*i.e.* PDB code 3O96), aPKCs and NDR/LATS (*i.e.* PDB code 4LQQ).

3. Regulation of PDK1, the upstream kinase phosphorylating the activation loop of PKB/Akt, SGK, S6K, PKC and RSK families

PDK1 occurs widely throughout evolution, consisting of an N-terminal kinase domain followed by a linker region without a noticeable HM and a C-terminal pH domain. PDK1 is a master kinase that phosphorylates the activation loop site and is required for the activity of at least 23 other AGC kinases. It is a key mediator of the phosphoinositide 3-kinase (PI3K) pathway and therefore plays an important role in downstream growth factor signalling [3,6,53–55]. PDK1 and PKB/Akt bind the second messenger PI(3,4,5)P3 with high affinity, which localizes PDK1 with PKB/Akt at the membrane when PI3K is activated by growth factors [56]. Binding to PI(3,4,5)P3 does not affect the specific activity of mammalian PDK1. The ability of the pH domain of PDK1 to interact with the second messenger PI(3,4,5)P3 is necessary for the phosphorylation of PKB/Akt but not for the phosphorylation of any other substrates of PDK1, as determined by elegant knock-in mouse model systems [57–59].

More recently, it was also established that PDK1 binds to phosphatidylserine and that this interaction is essential for membrane binding [60]. Interestingly, a wide screening study established that the binding specificity of the pH domain of PDK1 and PKB/Akt can be modulated cooperatively by the presence of combinations of specific lipids. For example, the pH domain of PKB/Akt binds with low affinity to PI(4,5)P2, yet the presence of long chain bases (LCBs, *i.e.*, dihydrosphingosine-1P) induces a selective 5-fold increase in the affinity for liposomes containing PI(4,5)P2 [61]. Since PDK1 binds with relatively high affinity to PI(4,5)P2, this mechanism could explain the activation of PKB/Akt by LCBs [62]. In the same screening by Vonkoba et al., the isolated pH domain of PDK1 showed negative cooperativity for binding to PI(3,4,5)P3 in the presence of several LCBs, *e.g.*, dihydrosphingosine-1P [61]. The findings could help to unveil new ways of modulating the signals mediated by PDK1 and PKB/Akt. Interestingly, the PDK1 orthologue from yeasts (Pkh2) is also regulated by the PIF-pocket, but its specific activity is regulated by lipids, where, for example, the activity of Pkh is inhibited by phosphatidylserine, while the activity is released with dihydrosphingosine [63,64]. PDK1 has also been reported to form dimers in cells, although not constitutively. The mechanism of dimerization and its physiological significance are not clear [65–67].

3.1. The PIF-pocket as a docking site for a subset of PDK1 substrates

In addition to the role of the PIF-pocket of PDK1 in the intrinsic activity of the catalytic domain, it also plays a fundamental role in the docking interaction with substrates [39,54,68,69]. Indeed, the phosphorylation of up to 20 AGC kinases by PDK1 may require the docking interaction of the HM from substrates with the PIF-pocket of PDK1 [3,39,54]. The PIF-pocket of PDK1 is the docking pocket where HM from substrates can dock. Some substrates such as SGK, S6K, and RSK appear to require the previous phosphorylation of the HM to increase the interaction with PDK1 [37,39,68]. Other kinases do not require previous phosphorylation at the HM for efficient phosphorylation by PDK1. That case holds for PKC isoforms that become phosphorylated by PDK1 immediately after synthesis and are not stable without this phosphorylation [70–72]. Interestingly, while the PIF-pocket docking interaction is required for the phosphorylation of S6K, in parallel experiments it was demonstrated that PKB/Akt interaction with PDK1 and its phosphorylation in the presence of PI(3,4,5)P3 do not require the PIF-pocket of PDK1 *in vitro* [39] or in mouse knock-in models where PDK1 is replaced by PDK1 [Leu155Glu], a mutant of PDK1 with disruption of the PIF-pocket [58,69,73,74]. Nevertheless, even if the phosphorylation of PKB/Akt does not rely on the interaction with the PIF-pocket, the phosphorylated HM of PKB/Akt does interact with the PIF-pocket of PDK1, as revealed in a recent crystal structure determination of the complex (PDB code 5LVP) [48]. Interestingly, several pieces of research suggest that the activation of PKB/Akt induced by other stimuli may be dependent on the PIF-pocket, *i.e.* [75].

3.2. Structure, dynamics and chemical biology studies on the PIF-pocket of PDK1

Studies of PDK1 in solution showed that the PIF-pocket is not as stable as might be presumed from the crystal structures of PDK1. Indeed, after one minute in a deuterated solution, almost all exchangeable hydrogens from αB and αC helices are exchanged for deuterium, indicating that helices αB and αC of PDK1 are highly mobile [17]. Molecular dynamics simulations of PDK1 further described the mobility of the helix αB within the microsecond timescale [48]. Small compounds that

bind to the PIF-pocket of PDK1 have been identified by *in silico* approaches, scaffold hopping, and the screening of compound libraries [31,43,45–52]. The crystal structure shows the binding of compounds mimicking the Phe interactions while a carboxylate interacted with residues Arg131 and Gln150, a site that was termed "phosphate-binding site" (*i.e.* PDB codes 3HRF, 5AW1, 5ACK, 5LVO). In the crystal structure of PDK1 bound to the phosphorylated HM of PKB/Akt, the HM phosphate interacts only with Gln150 (PDB code 5LVP) [48].

3.3. Allosteric communication between the PIF-pocket and the ATP-binding site of PDK1

The *in vitro* intrinsic activity of PDK1 is increased by 3- to 4-fold by PIFtide and phosphorylated HM-polypeptides [23,37,39]. Hydrogen/deuterium exchange experiments can be very helpful to understand the dynamics of a protein in solution. This methodology also helps to identify regions that are affected, exposed or protected, by compounds, interacting partners, and so on. Hydrogen/deuterium exchange experiments show that the binding of the activating compounds to the PIF-pocket stabilize the polypeptides comprising the binding site and produce allosteric effects on the Gly-rich loop (the cap of the ATP-binding site) and on a polypeptide encompassing the activation loop and the DFG motif [45] (Fig. 3). The effects on the ATP-binding site induced by the binding of small compounds to the PIF-pocket were confirmed by a fluorescent ATP-analogue probe (TNP-ATP) [30,45] and defined at a molecular level in the crystal structure of the complex between PDK1 and PS210 (PDB code 4AW1), a compound that increases the *in vitro* activity of PDK1 6- to 8-fold (twice as much as PIFtide and other small compounds) [31] (Fig. 3; B,C,E,F). In the complex with PS210, the binding induced the complete closure of the kinase domain, to appear exactly as previously described for the active-closed structure of PKA (PDB code 1ATP). More recent work has described the dynamics of the catalytic domain of PDK1 and the binding of compounds to the PIF-pocket using unbiased molecular dynamics simulations (up to 100 ns) and long multiple-replica parallel tempering simulations in the well-tempered ensemble (PT-WTE), which makes it possible to analyse μs to ms dynamic simulations focusing on the communication between the PIF-pocket and the ATP-binding site. The PT-WTE simulation identified the two movements, hinge and twist motions between the small lobe and the large lobe, and identified variations in the length of helix αB that affect the conformation of the PIF-pocket. The binding of PS210 stabilized the PIF-pocket and the closure between the lobes; in this conformation, the interactions between the small lobe and the large lobe are enhanced and can transmit information allosterically.

3.4. Reverse allosteric modulation: from the ATP-binding site to the regulatory site

Defining a regulatory allosteric communication between the PIF-pocket and the ATP-binding site has far-reaching consequences. By definition, allostery implies a bi-directional communication between the orthosteric site and the allosteric site. As a consequence, the ATP-binding site should have the potential to regulate the interaction of PDK1 with substrates mediated by the PIF-pocket, by the "reverse allosteric" path. In addition, by analogy, one could speculate that the mechanism of regulation of protein kinases that affect the activity of the kinase through modulation of the conformation of the active site could also be modulated by reverse allostery, by compounds binding to the ATP-binding site. In this line, recent work shows that different compounds binding to the ATP-binding site of PDK1 can inhibit the binding of PDK1 to PIFtide (*e.g.*, PS653), enhance its binding (*e.g.*, adenosine) or be apparently neutral (*e.g.*, adenine) [48]. Molecular dynamics simulations indicated that the binding of PS653 at the ATP-binding site inhibits the interaction with PIFtide due to the stabilization of the open form of PDK1, even if the αB and αC helices of the PIF-pocket are actually rigidified. In contrast, adenosine balances the hinge motion equilibrium towards a closed form, enhancing the allosteric coupling between the two lobes and affecting the binding of PIFtide to the PIF-pocket by increasing the flexibility of the αB region [48]. The "reverse allosteric" effect on the PIF-pocket by the compound PS653 binding to the ATP-binding site is represented in Fig. 3D,G.

Notably, distinct high affinity small compounds that have been developed to bind at the ATP-binding site can produce different reverse allosteric effects at the PIF-pocket. Thus, while UCN01 does not produce a significant reverse allosteric effect on the PIF-pocket, the selective PDK1 inhibitor GSK2334470 [76]. very potently displaced the interaction of PIFtide with the PIF-pocket of PDK1 [48]. This reverse allosteric effect could explain why GSK2334470 more potently inhibits

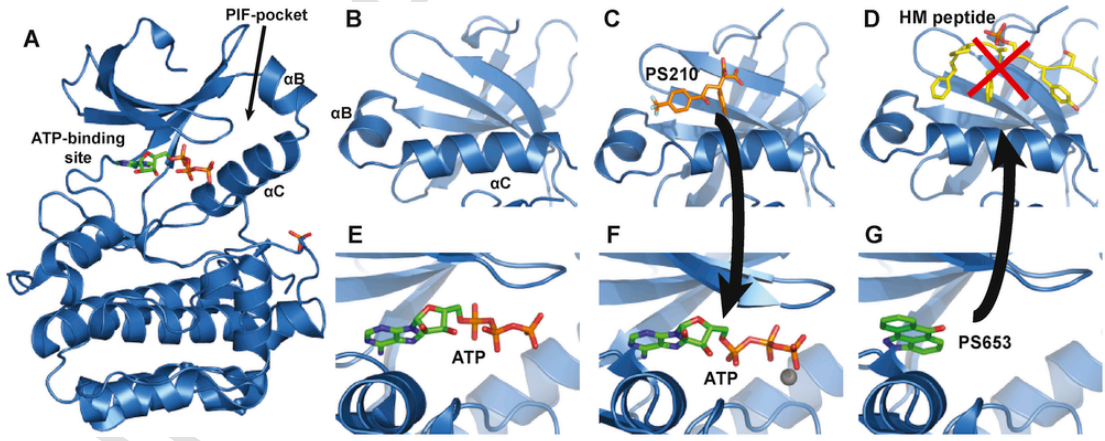


Fig. 3. Bi-directional allosteric modulation of the catalytic domain of PDK1. Allostery is a phenomenon that supports the function of ON-OFF switches in proteins where binding of a partner at one location of the target protein leads to specific perturbations at a distant site, not in direct contact with the region where the binding occurs. The active site conformation of AGC kinases – and their enzymatic activity – is often modulated by the conformation of the αB and αC helices (PIF-pocket regulatory site). The binding of the C-terminal HM to the PIF-pocket is central to the activation in many AGC kinases. A, Crystal structure of the catalytic domain of PDK1 in complex with ATP; B and E, enlarged representations of the PIF-pocket and the ATP-binding site. C and F, binding of PS210 (a small compound activator of PDK1) produces local changes at the PIF-pocket and allosteric changes at the ATP-binding site. Note the different conformation of ATP in E and F. The conformation of ATP in F is similar to the one in the active-closed structure of PKA (PDB code 1ATP). The reverse direction of the allosteric modulation is observed by compounds that bind to the ATP-binding site and affect the binding of HM polypeptides at the PIF-pocket [48]. D, The crystal structure of the PIF-pocket of PDK1 in complex with the HM of PKB/Akt; G, The interaction shown in D is displaced by the binding of small compound PS653 at the ATP-binding site and the resulting "reverse allosteric" effect on the PIF-pocket conformation.

S6 K phosphorylation, which requires docking of its HM to the PIF-pocket, than PKB/Akt phosphorylation.

4. Regulation of PKB/Akt

Protein kinase B (PKB/Akt) is involved in cellular processes downstream of PI3K, and its dysregulation is linked to the development of a variety of pathologies including diabetes, neurological disorders, cardiovascular diseases and cancer [3,6,77,78]. In particular, PKB/Akt is activated in tumours harbouring PTEN mutations that ultimately increase the levels of the PKB/Akt stimulatory second messenger PI(3,4,5)P3. Since the three isoforms are overexpressed in cancer, they have been extensively targeted by drug development programmes [78]. PKB/Akt is constitutively phosphorylated at the turn-motif site and acquires phosphorylation at the activation loop by PDK1 and HM phosphorylation by mTOR or DNA-PK upon PI3K activation or DNA-damage response, respectively [79], producing a fully active conformation. What is the inactive conformation of PKB/Akt, and what is the process of activation? In the absence of signal, the N-terminal pH domain binds intramolecularly with the kinase catalytic domain, stabilizing the kinase in an inactive conformation that furthermore cannot become phosphorylated by its upstream kinase PDK1. The binding of PI(3,4,5)P3 to the pH domain of PKB/Akt releases the intramolecular interaction and helps to colocalize it with PI(3,4,5)P3-rich membranes and PDK1, enabling its phosphorylation. A new generation of allosteric drugs, led by MK2206, exploit the mechanism of inhibition of PKB/Akt, and its studies have helped to understand the molecular details of PKB/Akt regulation. The molecular aspects of the mechanisms involved are described below.

4.1. The PIF-pocket as an ON-OFF switch in PKB/Akt

Whereas other substrates of PDK1 require a docking interaction of the HM with the PIF-pocket for efficient phosphorylation, *in vitro* and *in vivo* experiments show that the growth factor activation of PKB/Akt does not rely on the HM-docking interaction with the PIF-pocket of PDK1. Even if the HM of PKB/Akt does not require binding to the PIF-pocket of PDK1 upon growth factor activation, this interaction can occur, as revealed in the crystal structure of the complex. ΔpH -PKB/Akt is inefficiently phosphorylated by PDK1 *in vitro*; domain swapping between PKB/Akt and SGK revealed that additional epitopes, excluding the HM, account for the differences in the interaction and activation mechanisms of PKB/Akt and SGK by PDK1. In contrast, replacing the C-terminal region of PKB/Akt with the corresponding region from PRK2 (PIF) (PKB/Akt-CT-PIF) enhanced its phosphorylation by PDK1 and the *in vitro* kinase activity. The crystal structure of PKB/Akt-CT-PIF shows that the HM within the PIF-sequence interacts with and stabilizes the PIF-pocket intramolecularly. Overall, the PIF-pocket is a central mediator of the activation of PKB/Akt, where, upon activation loop phosphorylation, the binding of its HM to the PIF-pocket switches the activity ON.

In the crystal structure of the inactive form of PKB/Akt, helices αB and αC, which define the PIF-pocket, are not resolved and apparently flexible [28]. More recently, the crystal structure of full-length PKB/Akt in complex with Akti 1/2 showed that the pH domain interacts intramolecularly with the small lobe of the kinase domain, where the residues constituting helices αB and αC in the active structure are positioned in an elongated conformation against the interacting pH domain (PDB, 3O96) [80]. Thus, the OFF switch of PKB/Akt consists of a PIF-pocket completely disturbed by intramolecular interaction with the pH domain (Fig. 4).

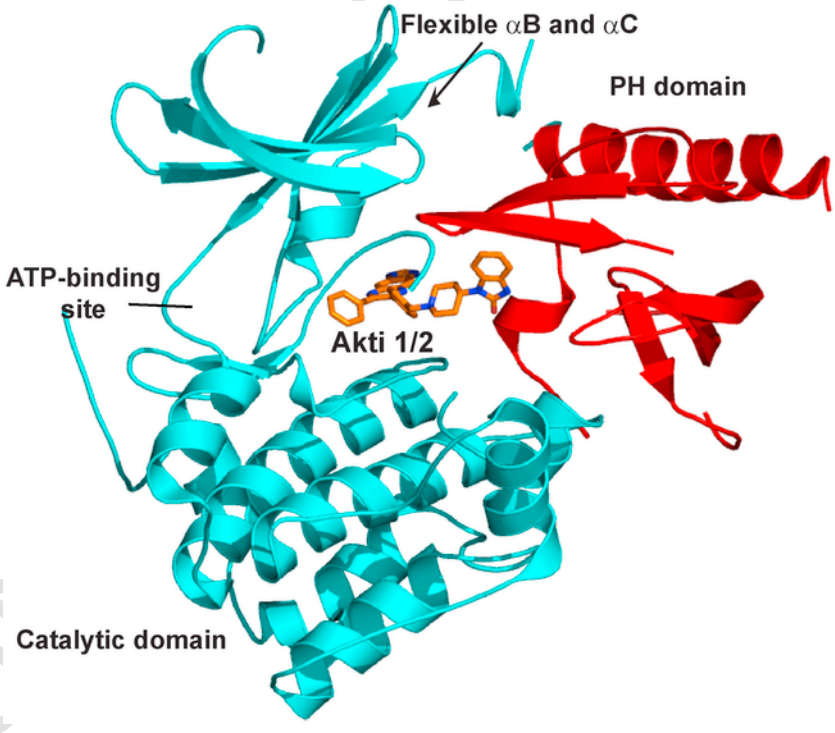


Fig. 4. The structure of the inhibited form of full-length PKB/Akt in complex with allosteric inhibitor Akti 1/2. The inhibitor Akti1/2 led to the development of MK2206, presently in numerous clinical trials for the treatment of diverse types of cancers. The allosteric inhibitor stabilizes the autoinhibited form of PKB/Akt, where the pH domain (shown in red) intramolecularly interacts with the small lobe of the kinase domain (cyan), stabilizing the inactive structure of the PIF-pocket, where αB and αC helices are flexible and unresolved. Upon the binding of PI(3,4,5)P3 to the pH domain, the release of the pH domain interaction with the small lobe of the kinase enables the stabilization of the αB and αC helices and their further stabilization by the binding of the HM.

The above model of intramolecular inhibition by the pH domain implies that the binding of PI(3,4,5)P3 to a pre-phosphorylated molecule of PKB/Akt should release the inhibition and therefore activate the kinase *in vitro*. Although this effect had not been observed, a recent report describes the expected *in vitro* activating effect of PI(3,4,5)P3 on PKB/Akt and suggests that the high concentrations of Mg^{2+} frequently employed in kinase assays inhibit the pH domain − PI(3,4,5)P3 interaction [81].

4.2. Approaches for drugs targeting PKB/Akt

In addition to the ATP-binding site, an initial approach has been to target the PI(3,4,5)P3 binding site on the pH domain of PKB/Akt [82,83]. The rationale is to block the translocation of PKB/Akt to the membrane, avoiding co-localization and activation by PDK1. Since over 259 human proteins contain pH domains, there is still a challenge for the selectivity for such drugs.

One breakthrough in the development of allosteric drugs for protein kinases was the identification of a highly selective allosteric inhibitor of PKB/Akt, Akti1/2 [84], and its further development into MK-2206, which is currently in numerous clinical trials for diverse types of cancers. The crystal structure of Akti1/2 in complex with full-length PKB/Akt shows that the compound sits like the ham of the sandwich between the small lobe of the kinase domain and the pH domain. The inactive form of PKB/Akt is thereby stabilized, inhibiting the interactions with PI(3,4,5)P3 and with PDK1 and inhibiting the intramolecular interaction of the C-terminal HM with the active form of the PIF-pocket. Based on existing knowledge about its mechanism of action, new screenings systems have been published [85] and follow-up drugs are being developed, *e.g.*, BAY 1125976 and ARQ 092 (PDB 5KCV [86]). The mechanism of action resembles PD 098059 [87], which binds to the inactive form of the kinase and inhibits the activation of mitogen-activated kinase–kinase (MKK) [88]. Interestingly, compounds stabilizing this inhibited form of the PKB/Akt would have inhibited the *in vitro* interaction of PKB/Akt with PIFtide, as depicted in patent applications filed in 1999 [89], more than ten years before the crystal structure of full-length PKB/Akt was solved in complex with Akti1/2. This example highlights the importance of the screening approach taking into consideration the known mechanisms of regulation, even in the absence of crystal structure information. Indeed, it is the existence of the allosteric compounds that may pave the way to obtain the crystal structure of full-length protein kinases and more fully understand the mechanism of regulation of the target kinase, as in the case of Akti1/2.

Altogether, PKB/Akt appears as a very flexible protein, with an allosteric mechanism of action that carries information from the PIF-pocket ON-OFF switch to the conformation of the ATP-binding site. The reverse allosteric effects from the ATP-binding site affecting the HM are therefore expected and have been observed to affect the stability of PKB/Akt phosphorylations [90].

4.3. Regulation of PKCs by their N-terminal domains

The PKC family of protein kinases is the largest within the AGC kinase group, comprising three major subfamilies, conventional, novel and atypical PKCs, which differ in the composition of their N-terminal domains (Table 1) [19,91]. The phorbol ester induction of cancer is mediated in part by the activation of members of the PKC family; therefore, PKCs have been of early interest for the development of inhibitors for the treatment of cancer [92]. However, loss of function mutations of PKCs were recently associated with cancer [93,94], and the paradigm may yet change. There is currently still interest in the inhibition and activation of selected isoforms of PKCs for the treatment of diverse human pathologies, from cancer to Alzheimer's disease [95,96]. The mechanism of regulation of PKCs has been a matter of reviews [97–99]. All PKCs are inhibited by their N-terminal domains. The only feature conserved in all N-terminal domains is a pseudosubstrate region contiguous to a C1 domain. Here, we will focus on the mechanism of regulation of atypical PKCs by pseudosubstrate and C1 domains and on the evidence that the pseudosubstrate-C1 domain combination could be the core of the mechanism of inhibition for the whole family.

4.4. Inhibition of atypical PKCs is mediated by the concerted action of the pseudosubstrate region and C1 domain

Atypical PKCs (aPKCs) have been described as oncogenes [100,101] and play roles in cell polarization, migration [91] and insulin signalling [102]. The N-terminal region of aPKCs comprise a PB1 domain, a pseudosubstrate region and a C1 domain, while at the C-terminal region, the HM has a Glu instead of a phosphorylatable residue. The aPKCs can be activated *in vitro* with a phospholipid mix. A construct lacking the PB1 domain maintains the inhibition and the activation by lipids. A construct lacking the pseudosubstrate region in addition is still inhibited but is no longer activated by lipids. The further deletion of the C1 domain produces a PKC construct with a strong increase in basal activity that is not further activated by lipids [32]. Hydrogen/deuterium exchange experiments were performed on the full-length PKCι and compared to the hydrogen/deuterium exchange of the catalytic domain. The N-terminal domains protected the region corresponding to the substrate binding site and a second region, corresponding to the αB and αC helices and the PIF-pocket [29]. In addition, resembling the situation in PDK1 [17] and PKB [28], hydrogen/deuterium exchange experiments showed that in solution, the αB and αC helices are highly flexible in the isolated catalytic domain. In parallel assays, the pseudosubstrate polypeptide protected only the substrate binding site [29]. Together, the research on PKCι accounts for the pseudosubstrate playing a major role in the process of activation by lipids, but where the C1 domain allosterically inhibits the activity by stabilizing the αB and αC helices. *In vitro*, a pseudosubstrate-C1 domain construct interacts with the catalytic domain of PKCι. Interestingly, the HM polypeptide displaces the pseudosubstrate-C1 domain interaction with the catalytic domain, indicating the regulatory allosteric communication between the PIF-pocket and the pseudosubstrate binding. Together, the model shows that in PKCι, the pseudosubstrate and C1 domains participate in the inhibition of the catalytic domain, blocking the substrate-binding site and stabilizing an inhibited conformation of the PIF-pocket, respectively, while the pseudosubstrate is needed for the *in vitro* activation by lipids. In addition, the HM supports the activation by interacting with the PIF-pocket and allosterically releasing the pseudosubstrate binding. The model does not only validate PKCι previous knowledge regarding the role of the pseudosubstrate in the inhibition and interaction with substrates but indicates central roles for the C1 domain and the PIF-pocket in the regulation [103,104]. A model for the location of the pseudosubstrate region and C1 domain with respect to the catalytic domain of aPKCs is presented in Fig. 5A.

4.5. Structural information of inactive PKCs

A new era of knowledge about the regulation of classical PKCs emerged with the crystal structure of a full-length form of PKCβ II, showing the catalytic domain, the C2 domain and the C1B domain, and the low-resolution solution structure of the closed-inactive form of PKCβ derived from small-angle X-ray scattering (SAXS). In the crystal structure, the C1B domain forms a novel interaction with the Phe residue from the NFD motif [105]. This interaction displaces the Phe629 residue (equivalent to Phe327 in PKA) from its position neighbouring ATP, possibly enhancing the dynamics of the hinge motion (Fig. 5B,C). The crystal structure appears to shed light on a mechanism

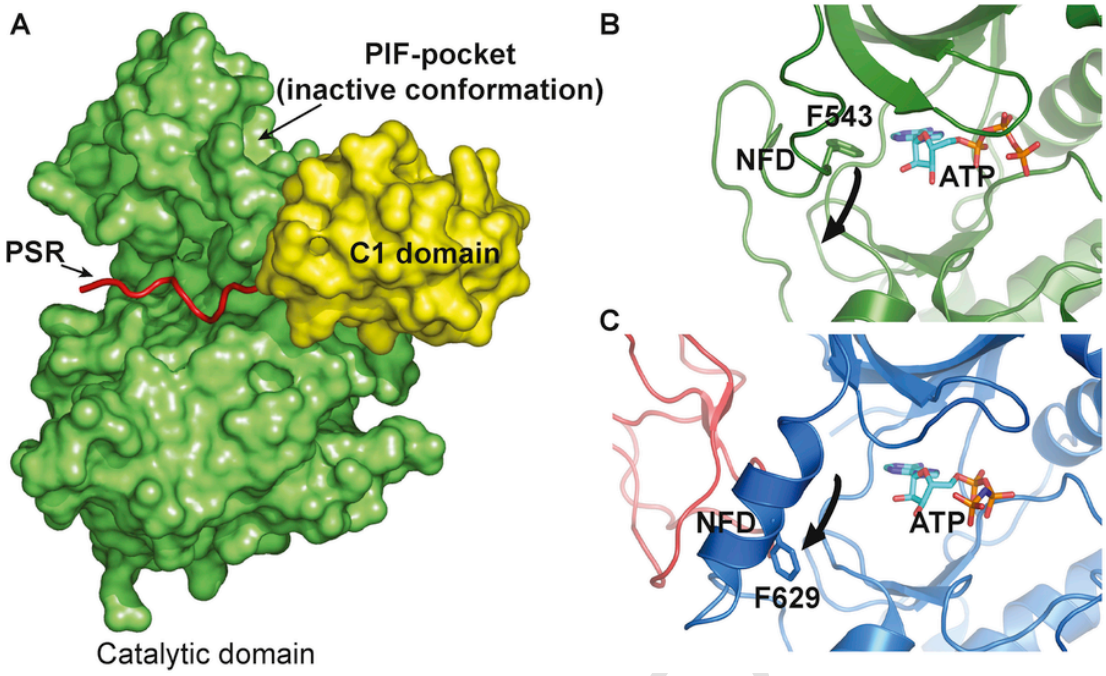


Fig. 5. PKC inhibition by the pseudosubstrate-C1 domain. PKC isoforms are inhibited by their N-terminal region. The only N-terminal feature that is conserved within all PKC isoforms is the presence of a pseudosubstrate segment that is directly contiguous to a C1 domain (C1A domain in classical and novel PKCs). Data from atypical PKCs (aPKCs) and classical PKCs indicate that the C1 domain plays a fundamental role in the inhibition of activity. A, Model of pseudosubstrate-C1 domain interaction with the catalytic domain. Binding of a HM polypeptide to aPKC releases the pseudosubstrate-C1 interaction indicating that in the inhibited form the PIF-pocket is stabilized in an inactive conformation. B, aPKC structure depicting Phe543 of the NFD motif in the active form of the kinase. The NFD motif within the C-terminal extension of the catalytic domain makes a direct interaction with adenosine. C, The crystal structure of PKCβII shows the C1B domain (shown in pink) in direct interaction with NFD motif Phe626, thus displacing it from its position sandwiching the small lobe and the large lobe. The position of the NFD motif within the catalytic domain of AGC kinases is depicted in Fig. 2.

present in classical and novel PKCs, where the C1B domain interaction with the NFD motif could modulate the activation of the kinase domain [106]. In turn, affecting the intramolecular interaction can modulate the interaction with cellular membranes.

Based on the analysis of the crystal structure and on SAXS, the authors describe a model of the inhibited form of PKCβII where the inhibited form has the pseudosubstrate occupying the substrate binding site, followed by the C1A domain interacting with the αC helix on the small lobe [105].

More recently, Antal et al. reanalysed the structural data from Leonard et al. on the location of the C1 and the C2 domains [107]. Docking experiments by Antal et al. suggested that the C2 domain interacts with the catalytic domain next to the pseudosubstrate domain, instead of the position ascribed by Leonard et al. to the C1A domain. Antal et al. mutated the catalytic domain at the predicted C2 binding site and obtained increased translocation of the mutant to the membrane; when the predicted interaction in the C2 domain was also mutated, the ability of the double mutant to translocate to the membrane was inhibited to wild-type levels. This finding was interpreted by the authors as corroboration that the C2 domain occupied the site in contact with the αC helix. However, the mutagenesis results could have also been obtained if the site presumed to bind C2 was actually binding the C1A domain, while the reversal (inhibition of the increased translocation) by additionally mutating the C2 domain could have been obtained if the mutated site also participates in the binding to the membrane.

The most recent research on PKCα investigated the role of the different N-terminal domains in the interaction with the catalytic domain [103]. Interestingly, similarly to the data described above for PKCι, the authors found that the C1 domain participated in both the interaction with the catalytic domain and the inhibition of the catalytic activity. The results are in agreement with older research that had established the C1A domain to have an important inhibitory role in the absence of a pseudosubstrate [104]. Together with the SAXS models by Leonard et al. and the related work on PKCι, we are tempted to suggest that the inhibition by the conserved pseudosubstrate and its contiguous C1 domain module, interacting, respectively, with the substrate binding site and the PIF-pocket helix αC, could be essential features for the inhibition of all PKC isoforms. Together, our analysis supports the model in which the pseudosubstrate-C1A domain in conventional and novel PKCs could have a similar mechanism of inhibition to the one described for aPKCs (Fig. 5A).

4.6. Modulation of PKC isoforms by new mode-of-action allosteric compounds

For many years, the modulation of the activity of PKC isoforms has been achieved by compounds binding to the C1 domain, such as phorbol esters, which activate PKC isoforms [92], and by small molecules binding to the ATP-binding site, although with little selectivity. Exploratory compounds have been reported to bind to the PIF-pocket of atypical PKCs and allosterically inhibit the kinase activity [29,32,44]. Those compounds were identified in a focused library synthesized based on variants of PDK1 activators [32]. The mechanism of action of the allosteric inhibitor PS315 was investigated using hydrogen/deuterium exchange and crystallography. PS315 binds to the PIF-pocket, without affecting the overall active conformation of the kinase but producing subtle effects on the active site Lys (Lys72 in PKA) that, in the active structures, forms a salt bridge with a Glu from helix αC (Glu91 in PKA) and positions the β- and γ- phosphates of ATP [29]. Whereas the activators of PDK1 occupy a rather shallow pocket, PS315 also occupies a deep tunnel formed between helix αC and the β-sheet [29]. When targeting such allosteric ON-OFF regulatory sites, one risks identifying activators rather than inhibitors. To follow up with allosteric inhibitors of aPKCs, compounds predicted to occupy the deep tunnel can be selected [108]. Interestingly, research on aPKCs indicates that the

binding of HM polypeptides allosterically displaces a pseudosubstrate polypeptide that binds to the substrate binding site [32]. One can predict that compounds binding to the PIF-pocket of PKCs could therefore release the N-terminal inhibition and serve as activators of PKCs. Ultimately, chemical biology studies shed light on the dynamics of the PIF-pocket and its allosteric effects.

The understanding of the molecular mechanism of inhibition of PKCs by the C1 domain resembles the case of PKB/Akt, where the intramolecular interaction of the pH domain with the catalytic domain is stabilized by Akti1/2. In the case of PKCs, it should be possible to pursue the identification of compounds that stabilize the inhibitory interaction between the C1/C1A domain and the catalytic domain.

5. Regulation of PRKs/PKNs

PRK1-3, also termed PKN1-3, are related to PKCs and are sometimes included within the PKC family. PRKs signalling is described to regulate epigenetics and be involved in prostate cancer [109]. PRKs are inhibited by the ATP-competitive compound Y 27632 [110], mediate physiological effects downstream of Rho, such as Rho-dependent cell migration [111], and may mediate effects that were previously ascribed to Rho kinase based on Y 27632 sensitivity. PRK

Like PKCs, PRKs require phosphorylation for activity and are inhibited by N-terminal domains, while the C-terminal region also modulates activity. The N-terminal region of PRKs contains three Rho-binding (Hr1) domains, a C2-like domain followed by a pseudosubstrate region (termed PRL in PRK1 [112] and PKL in PRK2 [33]; see Table 1). However, in contrast to PKCs, the PKL inhibition is not intramolecular but supports the formation of inactive dimers [33]. The C-terminal segment of PRK2 corresponds to PIF, the HM polypeptide that interacts with high affinity with PDK1 [68]. The regulated docking interaction with PDK1 is mediated partly by the N-terminal region of PRKs/PKNs, which inhibits the docking interaction with PDK1 [33]. As in atypical PKCs, the HM phosphorylation site is replaced by a negatively charged amino acid, Asp, in PRKs/PKNs. Phosphorylation of the turn-motif/zipper site supports the intramolecular interaction with the turn-motif/zipper phosphate binding site (PDB codes 4CRS, 4OTD), supporting the interaction of PIF with the PIF-pocket of PRK2 and increasing the specific activity of the kinase domain [25]. In addition, the turn-motif/zipper phosphorylation also plays a second role, supporting the release from its docking interaction with PDK1 [27].

In vitro, PRKs can be activated by arachidonic acid and diverse lipids [113]. In cultured cells, the interaction of the N-terminal Rho-binding domains with GTP-Rho stimulates the regulated docking interaction with PDK1 and its subsequent activation [114]. Antibodies that bind to the C-terminal PIF region of PRK2 inhibit kinase activity in the full-length protein and the isolated kinase domain, indicating that the C-terminal region stabilizes the active form of the kinase [33]. In addition, the HM polypeptide PIFtide allosterically displaces PKLtide from the peptide-substrate binding site [33], suggesting that the HM does not bind to the PIF-pocket when the PKL-mediated dimer is stabilized.

Small compounds from a focused library directed to the PIF-pocket of AGC kinases were found to bind to the PIF-pocket and activate PRK2 by allosterically displacing the PKL inhibitory segment from the substrate binding site. In addition, some compounds were identified as allosteric inhibitors (manuscript in preparation). Although structural data on the inhibited dimer/oligomer of PRKs is not available, the rich biochemical information on the regulation of PRKs provides clues for the screening approaches to identify selective inhibitors of PRKs. For example, dimer/oligomer interaction assays as described [33] could be used to identify compounds that stabilize dimer/oligomeric inactive forms.

5.1. Regulation of SGK, S6K, RSK and MSK

Specific mechanisms for the regulation of S6K, SGK and RSK require the phosphorylation of the HM for the regulated phosphorylation-dependent docking interaction with their upstream kinase PDK1, which in turn phosphorylates the activation loop [18,39]. However, the order of phosphorylation of the HM and activation loop has been contested for S6K [115]. Once phosphorylated by PDK1, the HM and turn-motif/zipper promote the intramolecular interaction of the C-terminal segment with the PIF-pocket, and together with the activation loop phosphorylation, these kinases are stabilized in active conformations and are active in cells. In these cases, the phosphorylation at the activation loop and the activation of the kinase may occur after they have been phosphorylated at the HM. However, depending on the kinase, the phosphorylation requirement for interaction with the PIF-pocket of PDK1 may be bypassed by overexpression, which may occur in experimental set-ups and in cancer.

The regulation of RSK has been extensively studied by Frödin's laboratory [37,116,117]. In short, RSK consists of two kinase domains, where the N-terminal kinase is an AGC kinase, and the C-terminal kinase is related to the MAPK group (Table 1). Activation of the C-terminal kinase leads to autophosphorylation at the HM of the N-terminal kinase [118], which in turn triggers the docking interaction with PDK1 and activation mediated by the PIF-pocket. MSKs have a similar disposition to RSK but are not downstream targets of PDK1 [118].

The regulation of S6K also relies on the regulation of the docking interaction with PDK1. Upon synthesis, S6K cannot interact with PDK1, since the C-terminal "autoinhibitory" segment inhibits the interaction [39,119] (Table 1). Once the autoinhibitory segment is polyphosphorylated, S6K acquires the ability to interact with PDK1, which is enhanced by the phosphorylation of the HM by mTOR [120]. As a variation within the group, the activity of S6K requires phosphorylation both at the activation loop and at the HM sites.

In vitro, SGK has overlapping substrate specificity with PKB/Akt [121] and is also stimulated downstream of PI3 kinase signalling [122,123]. Recently, it was found that resistance to pharmacological PKB/Akt inhibition was due to SGK overexpression [124]. The regulation of SGK1 is given first by increased expression, which is induced by glucocorticoids and numerous stimuli, and by degradation mediated by the proteasome [123]. SGKs are constitutively phosphorylated at the turn-motif site and requires phosphorylation at the activation loop for activity, while the activity is maximal when it is also phosphorylated at the HM. Phosphorylation at the HM by TORC2 enhances the HM docking interaction with the PIF-pocket of PDK1, and hence activation loop phosphorylation and activation [39]. The Na(+)/H(+) exchange regulatory factor 2 can also mediate the interaction of SGK and PDK1 and enable activation loop phosphorylation [125]. One can speculate that the phosphorylation-dependent interaction of PDK1 with substrates could be by-passed by the overexpression of the kinase substrates of PDK1; however, kinases such as S6K, RSK and PKB/Akt have a second lock that must be unlocked before the kinases acquire the ability to interact with PDK1 (*i.e.*, S6K has a C-terminal autoinhibitory motif that needs to be phosphorylated by MAP kinases; the RSK N-terminal kinase domain must be activated by the C-terminal kinase; PKB/Akt must interact with PI(3,4,5)P3). In comparison, SGK3, also known as SGKL, has a PX domain (Table 1) that binds PI(3,4,5)P3, although it is not known to block the interaction with PDK1 in the absence of PI(3,4,5)P3. Interestingly, SGK1-2 do not have a known second "lock" to inhibit the interaction with PDK1. Therefore, one can speculate that the overexpression of SGK isoforms alone could trigger the interaction with PDK1 and their activation upon synthesis in the absence of HM phosphorylation.

5.2. Aurora kinases and Polo-Like Kinases (PLKs), close relatives of AGC kinases

The Aurora kinases and PLKs are mitotic kinases. These two families were not listed as AGC kinases by Manning et al. [2] but are discussed here because they are closely evolutionarily related by sequence (Fig. 1). In addition Aurora kinases and AGC kinases are known to share important aspects of the mechanism of regulation.

Aurora kinases do not have N- or C-terminal extensions to the catalytic core (Table 1). However, the site equivalent to the PIF-pocket is also a regulatory site, the site where the regulatory protein TPX2 binds on Aurora A and where INCENP binds on Aurora B [126,127]. TPX2 and INCENP interaction activate Aurora kinase *in vitro*, increasing its specific activity towards MBP, which resembles the activation of other AGC kinases by PIFtide and other HM polypeptides. It has been reported that Aurora mediates the stabilization of the oncogene N-myc [128]. Allosteric inhibitors displacing the Aurora A TPX2 interaction may support anti-cancer treatments in a dual manner, inhibiting Aurora kinase activity and decreasing the levels of N-myc oncogenes [128–131]. The interaction between N-myc and Aurora kinase has recently been identified [132]. The portion of N-myc (61–89) crystallized in complex with Aurora A contacts the small lobe and the large lobe of the kinase domain by sitting in an extended conformation below the αB helix and αC helices and above the activation loop, with a C-terminal helix interacting with the αH helix of Aurora. Aurora A also interacts with heterogeneous nuclear ribonucleoprotein K (hnRNPK) in the nucleus and acts as a transcription factor, enhancing the transcription of C-myc [133]. Low molecular weight allosteric inhibitors binding to the myc-binding site or the PIF-pocket of Aurora kinases have not been described. A nanobody has been found to bind to Aurora kinases and inhibit the kinase activity [134]. Since TPX2, INCENP and the nanobody vNAR-D01 not only interact with the site equivalent to the PIF-pocket but also in neighbouring regions, it remains to be shown that interaction with the PIF-pocket by small compounds alone can displace them.

PLKs(1–4) are also mitotic kinases and have been in the focus of drug development [135]. A compound that bind to the ATP-binding site, BI6727, has already been approved for the treatment of patients suffering from acute myeloid leukemia (AML) and other ATP-competitors are advancing in clinical trials for treatment of cancer. However, there is evidence that higher selectivity and isoform-selective inhibitors may be required for their wider use [136]. Like Aurora kinases, PLKs do not possess an NFD motif, a turn-motif/zipper phosphorylation site or an HM, the characteristic features that AGC kinases possess at the C-terminal extension of the catalytic core (Table 1). PLKs have C-terminal polo-box domains (PBDs) that bind to specific sequences when they are phosphorylated [137] and regulate PLK activity and the localization of the kinase. PLK constructs lacking PBDs have higher specific activities. Phosphopeptides that bind with high affinity to the PBD activate PLK1, increasing the velocity of phosphorylation of a peptide substrate [138]. From this molecular biology and biochemical data it could be concluded that the PBD stabilizes the catalytic domain in an inactive conformation. Recent work provided structural data on the full-length PLK1, depicting the site of interaction between the PBD and the catalytic domain in proximity to the site where the C1 domain interacts with the NFD motif in PKCs [139]. Also recent work shows that dimerization of PLK1 mediated by the PBD participates in its activation [140]. Together, the mechanism of regulation of PLKs resemble PKB/Akt and PKCs in that an inhibitory domain intramolecularly interacts with the kinase core, inhibiting the kinase. While the PIF-pocket is not involved and the actual mechanism of regulation appears to be different, the approach for drug discovery appears equivalent.

5.3. Allosteric activators and allosteric inhibitors of Aurora kinase

The interaction of TPX2 and INCENP with the regulatory site allosterically affects the ATP-binding site. The reverse allosteric effect described in detail for PDK1 was also shown to occur in Aurora kinase. Interestingly, MLN8237 binds to the ATP-binding site and displaces TPX2 binding from Aurora A [48]. In addition, MLN8037 also displaces the Aurora interaction with N-myc [131], promoting its degradation. It is suggested that MLN8037 would promote conformational changes at the activation loop that are incompatible with the binding of N-myc$_{61\text{-}89}$ [132]. Thus, small compounds targeting the ATP-binding site of Aurora A can have "reverse allosteric" effects on the PIF-pocket and activation loop and produce additive anti-cancer effects by inhibiting Aurora kinase activity, disrupting the interaction with TPX2, destabilizing N-myc and decreasing the transcription of C-myc. In addition, it is possible that inhibitors binding to the ATP-binding site, if they have reverse allosteric effects that displace the interaction with key partners, could delocalize the kinase from its cellular substrates and inhibit the activity of the kinase in cells, even after the compound is dissociated.

5.4. Regulation of LATS and NDR

NDR and LATS are closely related kinases downstream of the Hippo signalling, whose major role in mammals is to inhibit cell proliferation and survival [141]. Widely conserved throughout evolution, NDR/LATS family members (Warts, Wts in Drosophila) require for their activity the interaction with the co-activator Mps1-one binder (MOB) and the phosphorylation of the HM, typically by Ste20-like kinases (MST1-2, termed Hippo, Hpo in Drosophila) [7,141].

The current model of activation of LATS requires its interaction with the adaptor protein MOB. MOB binds to both, LATS and to its upstream kinase MST. MST phosphorylates the HM of LATS and also phosphorylates MOB. Phosphorylation of MOB by MST both enhances the interaction of MOB with LATS and decreases its affinity for MST [42,141–145]. Phosphorylation at the HM enhances the activity of LATS, which can then autophosphorylate at the activation loop.

5.5. The role of the PIF-pocket in the activation of NDR/LATS

The crystal structures of the yeast homologue Cbk1 and HM mutants were solved in complex with Mob2 in three distinct crystal packings [146] (PDBs 4LQP, 4LQQ, 4LQS). In all cases the kinase domain is in an inactive form, where the helix αC is not visible (see Fig. 6), similarly to previous observations in the crystal structure of the catalytic domain of PKB/Akt (PDB, 1GZK) [28]. NDR/LATS family members have a conserved N-terminal helix, termed α-Mob, that participates in the interaction with MOB co-activator. The α-Mob helix sits on top of the small lobe and has a large interaction region with MOB.

Most interestingly, the non-phosphorylated HM is indeed depicted in the Cbk1-Mob2 inactive structures in the absence of helix αC (where the PIF-pocket is completely distorted). The structure of the non-phosphorylated HM of Cbk1 shows the HM sitting in the vicinity of the PIF-pocket, interacting with α-Mob helix and MOB, to stabilize the inactive structure of Cbk1-Mob2. In addition, modelling of the active structure suggested that the phosphorylated HM would indeed occupy the PIF-pocket [146]. Additional work showed that a fusion protein comprising the catalytic domain of NDR or LATS with PIF rendered constitutively active kinases [40,41,147], as also described previously for the PKB/Akt fused to PIF [20,39]. Therefore, both lines of evidence suggest that the active form of NDR/LATS would require the docking of the phosphorylated HM to the PIF-pocket, as observed in other AGC kinases. Thus, the overall model suggests that the HM would have two different

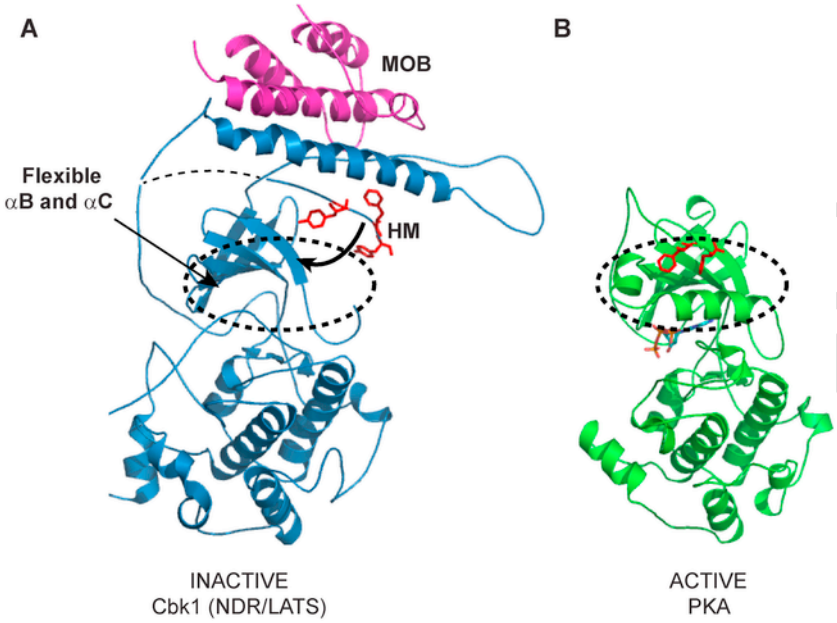


Fig. 6. The inactive structure of Cbk1, the yeast homologue of NDR/LATS. A In the inactive crystal structure of Cbk1 in complex with co-activator MOB the HM (Tyr-X-X-Phe-Thr-Tyr) is not binding in the PIF-pocket but located at the interface, while the helices αB and αC are mobile and not resolved. However, LATS can be activated by the HM peptide PIF, indicating that the stabilization of the αB and αC helices at the PIF-pocket activates LATS. B Phosphorylation of the HM must therefore trigger the relocalization of the HM from the observed location to at the PIF-pocket like in active AGC kinases such as PKA depicted here.

binding sites, a first binding site for the non-phosphorylated form stabilizing the inactive structure [146] and a second binding site within the PIF-pocket as described in other active AGC kinases, which preferentially binds the phosphorylated HM.

Gogl et al. further found that a region from the large lobe comprising the αE and αD helices provides a docking interaction with docking motifs in substrates [146]. In contrast to the docking of substrates to the PIF-pocket of PDK1 or GRKs, the docking interaction of substrates with Cbk1 does not modulate the conformation and intrinsic kinase activity of Cbk1-Mob2.

6. Regulation of ROCK

Rho-associated coiled-coil containing kinases (ROCKs) promote actin–myosin-mediated contractile force generation through the phosphorylation of numerous proteins. They play roles in proliferation, differentiation, apoptosis, and oncogenic transformation and are considered potential targets for the treatment of a wide range of pathological conditions including cancer [148]. ROCKs have an N-terminal kinase domain followed by a very large coiled-coil region, a C1 and a split pH domain at the C-terminus (see Table 1). Like MRCKs and DMPKs, ROCKs are dimers, and their kinase domains are active in the absence of phosphorylation [149–151]. It was reported that the C1 and pH domains regulate the activity of ROCKs; similarly, it has been described that the binding of Rho regulates the activity of the kinase domains. However, a recent work depicting the low resolution structure of a full-length ROCK obtained using electron microscopy has introduced doubts and initiated a debate into the mechanism of regulation of ROCKs [152,153]. The structure shows that this ROCK is a 120 nm long dimer with the kinase domains at one extreme and the C1 and pH domains that bind negatively charged lipids in the other. The crystal structure of the isolated catalytic domain forming dimmers had previously been described (PDB, 2F2U), as well as the putative Rho binding domain–crystallized in complex with Rho (PDB, 1S1C), and the pH (PDB, 2ROV) and C1 domains (PDB, 2ROW). The Rho binding domain lies 90 nm from the kinase domain. It is unclear how the C1 and pH domain or the Rho binding could modulate the activity of the kinase at this distance. In light of the recent work where the authors were unable to replicate Rho binding or modulation by the C1 and pH domains, the authors describe ROCK as a constitutively active kinase that would phosphorylate substrates when they are present at a given distance from the cell membrane [152,153]. This model resembles the model for PDK1 regulation, where the catalytic domain has a constitutively overall active conformation, and the phosphorylation of its substrates is regulated by the ability of the substrates to interact with PDK1.

ROCKs and the related kinases MRCKs and DMPKs are active as dimers. However, dimerization has not been described as a regulatory process. It was shown in DMPK that the coiled-coil region enhances activity by promoting dimerization [154]. In these families, the HM binds to the PIF-pocket and is sandwiched in the dimeric interface (*i.e.*, PDB 3TKU). Interestingly, PDK1 was found to bind to ROCK and MRCK and modulate their activities by modulating their plasma membrane localization, in a pH -dependent but kinase-independent manner [155,156]. It is further shown that the interaction of PDK1 with MRCK requires the integrity of the PIF-pocket (MRCK does not interact with the PDK1 L155E mutant); it was therefore suggested that the HM could mediate the interaction of MRCK with the PIF-pocket of PDK1 [156]. If the HM of MRCK interacted with the PIF-pocket of PDK1, then the dimeric structure of MRCK would likely be compromised, which could modulate kinase activity. It thus appears that MRCKs, DMPKs, and ROCKs are constitutive dimers.

6.1. The G-protein coupled receptor kinases, GRKs

GRKs have evolved to phosphorylate G-protein coupled receptors (GPCRs) specifically, and thereby participate broadly in the desensitization of GPCR-mediated cell signalling [157]. Due to their ability to modulate GPCR activity, GRKs are emerging as potent onco-modulators [158]. Since there are only six GRKS and more than 800 GPCRs, each GRK could have multiple targets. GRKs have mechanisms for interaction with cell membranes (Table 1) [6]; also the specific phosphorylation of GPCRs is supported by its interaction with Gβγ [159].

GRKs possess an N-terminal helix (αNT), an RH dimerization domain that is inserted in a loop within the kinase domain, and a C-terminal extension to the catalytic core (termed AST by authors working on GRKs) that contains an atypical shorter HM, with the motif Leu/Phe-Phe/Tyr-Xaa-Xaa-Phe. GRKs are constitutive dimers that show an overall active conformation. Compared to other AGC kinases, the HM appears to occupy the hydrophobic PIF-pocket more superficially. The GRK6 structure shows that the helix αNT sits in close proximity with the AST segment with which it interacts (PDB, 3NYN) [160]. Studies on GRK1, GRK2 and GRK6 indicate that physiological substrates form specific regulated docking interactions with the αNT and residues from AST, which in turn mediate the stabilization of the active form of the catalytic domain [160–164], indicating that the selective phosphorylation of GPCRs by GRKs is mediated by a coordinated docking interaction of GPCRs coupled to the stabilization of the active structure of the GRK kinase domain, thereby activating the kinase to phosphorylate its substrate. Mutations within the αNT and AST segments inhibit the kinase activity, inhibit the docking interaction with substrate, inhibit the activation by substrate docking and can even enhance the specific phosphorylation of GPCRs. Thus, the regulation of the physiological activity of GRKs is mediated by the modification of the substrate GPCR that enables its docking interaction with GRKs, which resembles the mechanism by which PDK1 phosphorylates its substrates (see above).

6.2. Cyclic nucleotide-dependent protein kinases: PKA and PKG

The cAMP-dependent protein kinase (PKA) has pleiotropic effects on the cell physiology and is an important player in cancer biology, where it is involved in hyperproliferation, tumourigenesis, cell migration, and resistance to chemotherapy [165]. On the other hand, cGMP-dependent protein kinase (PKG) is a major downstream effector of the cGMP signalling cascade and is a central regulator of cardiovascular and neurological functions [166]. PKG is also considered a potential target for colon and breast cancer [167,168].

PKAs in mammals are tetramers, formed by two catalytic subunits and two regulatory subunits. The structure of the isolated subunits, catalytic subunit alpha (Cα) and regulatory subunit (R), as well as of the holoenzyme ($RI\beta_2$-$C\alpha_2$; PBD, 4DIN) have been solved by X-ray crystallography, and the extensive work on the structure and regulation of PKAs has been reviewed [13,169]. In brief, the catalytic subunits fold into an overall active conformation, where the PIF-pocket is always occupied by the truncated HM. The activity of PKA is inhibited by the regulatory subunits, which block the active site but do not affect the overall active conformation of the kinase. The binding of cAMP to the regulatory subunits releases the inhibition. In PKA, the allosteric mechanisms that activate the kinase are mediated by the regulatory subunit [170]. Molecular dynamics simulations of the PKA catalytic subunit indicate that the hinge motion correlates with the actual turnover of the kinase [171].

In contraposition to PKA, PKG regulatory and catalytic subunits are part of the same polypeptide chain. PKG is a homodimer, and its classical activation mechanism involves the binding of cGMP to the regulatory sites, which rearranges the structure, removing the inhibition on the catalytic core and yielding a fully active enzyme [172]. As an alternate mechanism, PKGIa was shown to be activated in the absence of cGMP by H_2O_2 through the generation of an intermolecular disulfide between Cys42 of each member of the homodimer [173]. The oxidation of Cys42 seems to lead to the same conformational changes as the binding of cGMP. Knock-in mice with the redox dead mutant Cys42Ser PKGIa are hypertensive [174] and do not respond to nitro-glycerine treatment [175] giving physiological relevance to the redox sensing of Cys42. It is expected that compounds that mimic cGMP or catalyse the generation of this disulfide bridge would activate PKGIa independently of the cGMP concentration.

6.3. Opportunities and challenges for the discovery and development of allosteric mode of action compounds

Schematic representations summarizing important aspects of the mechanisms of regulation of selected families of AGC kinases are presented in Fig. 7. As depicted in the figure, the mechanisms of regulation within AGC kinases are vast but often follow simple lines. 1-Post-translational modifications modulate the conformation of the kinase domain. Since phosphorylation stabilizes the active conformation of AGC kinases, the allosteric inhibitors will most efficiently stabilize the kinase domains when the kinases are not phosphorylated. Therefore, it should be expected that the compounds will be more potent inhibitors of the kinase activity in cell systems where the kinases can be dynamically phosphorylated-dephosphorylated than in *in vitro* activity assays, which are usually performed with phosphorylated-active kinases. 2- Additional proteins or domains allosterically activate or inhibit the catalytic activity of a given kinase. To exploit such situations, allosteric inhibitors or activators could either mimic the effect of the interacting domain or stabilize such interactions. 3- Second messengers bind to allosteric domains, releasing inhibition. Therefore, compounds acting on the second messenger binding domain would mimic the effect of this signal. On the other hand, compounds acting on the kinase domain could, directly or allosterically, affect the inhibitory interaction and stabilize kinases in inactive conformations. An important consequence of the above mechanisms of regulation, which can ultimately affect the ATP-binding site, is the existence of the "reverse" allosteric modulation, where compounds binding at the ATP-binding site can modulate the conformation of the regulatory site and have the potential to stabilize inactive conformations, affect the localization of the kinase, enable the phosphorylation or dephosphorylation of the kinase domain in *in vivo* situations, and so on.

In addition to the finding of regulatory binding pockets, the development of new medicines will require the development of compounds with sufficient potency. In this regard, targeting allosteric-regulatory mechanisms have the advantage that they will not have to directly compete with the binding of ATP, which is at mM concentrations in cells. Furthermore, allosteric sites provide a much higher potential for the development of selective drugs. The case study of Akti1/2 and its derivatives experimentally shows that it is possible to develop allosteric compounds that are selective within members of the same family. Allosteric drugs would certainly enable enough selectivity to target the orthologous kinase in an infective organism. On the other hand, the challenge is whether the new binding sites can be exploited for the development of sufficiently potent compounds. The good news is that compounds with excellent selectivity and pharmacological properties have been developed to target the stabilization of pH domain interaction with the catalytic domain of PKB/Akt, stabilizing the OFF-switch of the mechanism of regulation of the kinase.

It is worth stressing that Akti1/2 was discovered without the structural information on the conformation of the inhibited form of PKB/Akt that is available today. Most interesting is that the discovery and publication of Akti1/2 led to the understanding of the molecular details of how the pH domain intramolecularly stabilizes the inhibited form of PKB/Akt. However, despite lacking access at the time to the molecular details of full-length PKB/Akt, a rich set of biochemical information was already available. For example, in early 2000s, biochemical characterizations and the published structure of the active and inactive structures of the catalytic domain of PKB/Akt already indicated that PIF and the phospho-HM polypeptide of PKB/Akt, when added "in trans" *in vitro*, could activate the kinase by stabilizing the active form of PKB. With this information, one could already deduce that the PIF-pocket regulatory site existed in at least two conformations, an active conformation bound to PIF/P-HM polypeptides and an inactive

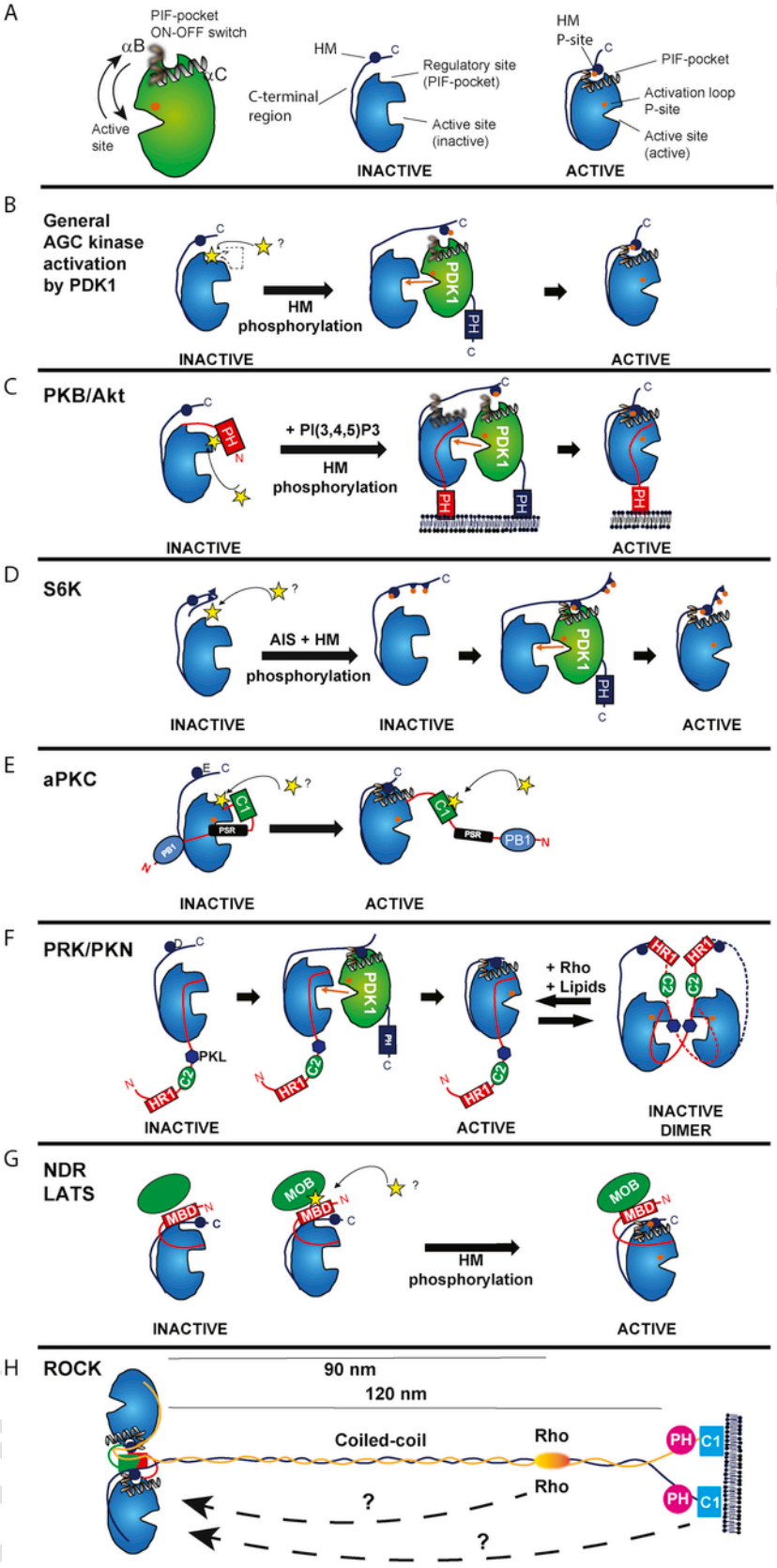


Fig. 7. Schematic representation of the mechanisms of regulation of AGC kinases. A, General representation of the AGC kinase catalytic domain, focusing on the flexibility of the PIF-pocket, the allosteric communication between the PIF-pocket regulatory site and the ATP-binding site and the different common regions found in AGC kinases. The different shapes of the αB represents its flexibility. B, Representation of a putative substrate of PDK1 that requires docking of the HM with the PIF-pocket for phosphorylation at the activation loop. The inactive conformation can be stabilized by additional domains. Small compounds (represented by a yellow star) binding to the inactive conformation of the regulatory site would be al-

losteric inhibitors. C, Structure-based representation of the inhibited form of PKB/Akt in complex with allosteric inhibitor MK2206 and the mechanism of activation by PDK1. D, Representation of the mechanism of activation of S6K. S6K has a C-terminal autoinhibitory domain that needs to be phosphorylated to enable the HM phosphorylation and the docking interaction with PDK1. E, Represented is the prephosphorylated aPKCs kept in the inhibited form of with the C1 domain clamped onto the small lobe of the kinase. Shown is also a putative allosteric inhibitor that stabilizes this intramolecular interaction, as MK2206 on PKB/Akt. Described activators of PKCs bind to the C1 domain; however, compounds binding to the PIF-pocket could also displace the N-terminal inhibition. F, Representation of the mechanism of activation of PRK/PKN. The suggested mechanism has two stages; in a first stage, upon synthesis, PRK/PKN requires docking interaction with PDK1 to become phosphorylated. Phosphorylation of the turn-motif/zipper site helps to release the interaction with PDK1. In a second stage, phosphorylated PRK/PKN remains inactive by dimerization (binding of a pseudosubstrate region to a neighbour molecule). Binding to Rho or to lipid activators releases the inter-molecular inhibition, stabilizing active monomers. G, Representation of the inactive form of NDR/LATS based on the crystal structure of their yeast homologue Cbk1. In the inhibited form, the HM is positioned away from the PIF-pocket in proximity of the α-Mob and MOB activator binding site. Since LATS-PIF is constitutively active, it is suggested that in the active form the phosphorylated HM stabilizes the PIF-pocket (αB and αC helices) as in other active AGC kinases. H, Representation of the structure of ROCK. ROCK is a rather rigid elongated protein with the kinase domain and the C1-pH membrane binding domains 120 nm apart. Modulation of ROCK kinase activity *via* Rho binding or lipid binding to C1-pH domain has been described. However, there is no model to explain such long distance regulation. Alternatively, it is suggested that ROCK is constitutively active sitting at approximately 120 nm from the membrane, and that the specific phosphorylation of substrates would be regulated by the access of substrates to the specific location of ROCK catalytic domain.

structure that does not bind PIF/P-HM polypeptide. Compounds displacing the interaction of PIF/P-HM polypeptides with an AGC kinase could prove to be modulators, activators or inhibitors of AGC kinases [89]. In the case of PKB/Akt, such an assay could have identified compounds stabilizing the inactive form, such as Akti1/2. Here, we have reviewed some key aspects of the mechanism of regulation of representative members of different AGC kinase families. There is abundant biochemical information supporting the molecular mechanism of allosteric regulation of different families. The available knowledge can guide specific screenings strategies designed for individual cases. For example, the availability of homogeneous assays that probe the interaction between the N-terminal regulatory region of aPKC with the catalytic domain, or the dimer interaction in PRK2, could be employed to identify compounds that enhance the interaction and hence the inhibited state of the kinases. Similarly, it may be possible to design interaction-displacement assays to stabilize the interaction of the C-terminal autoinhibitory domain of S6K, or the inactive dimer of NDR/LATS.

Allosteric modulators present a challenge for drug discovery. For example, compounds binding to a given extracellular site on GPCRs can allosterically affect the intracellular domain, stimulating or inhibiting downstream signals; variations of a starting agonist compound may prove to be antagonists, or have partial effects. The PIF-pocket regulatory site can also produce activators, inhibitors, partial activators and partial inhibitors. This situation presents a challenge for rational drug design. In a case-by-case situation, particular features in the compound-target interaction can be experimentally identified to lead to a given allosteric effect. It can be envisioned that MD simulations, as described for the PDK1 allosteric system, may enable future MD-based strategies for the rational design of allosteric drugs with a given allosteric effect. A particular kind of allosteric compounds that can be identified for AGC kinases are those that stabilize an intramolecular interaction that allosterically affects the active site, inhibiting the intrinsic activity of the kinase, such as Akti1/2. A consequence of the allosteric mechanism of regulation is that all improvements in the potency of such allosteric compounds will be in allosteric inhibitors.

Conflict of interest

None.

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