Signal transduction pathways use protein kinases for the modification of protein function by phosphorylation. A major question in the field is how protein kinases achieve the specificity required to regulate multiple cellular functions. Here we review recent studies that illuminate the mechanisms used by three families of Ser/Thr protein kinases to achieve substrate specificity. These kinases rely on direct docking interactions with substrates, using sites distinct from the phospho-acceptor sequences. Docking interactions also contribute to the specificity and regulation of protein kinase activities. Mitogen-activated protein kinase (MAPK) family members can associate with and phosphorylate specific substrates by virtue of minor variations in their docking sequences. Interestingly, the same MAPK docking pocket that binds substrates also binds docking sequences of positive and negative MAPK regulators. In the case of glycogen synthase kinase 3 (GSK3), the presence of a phosphate-binding site allows docking of previously phosphorylated (primed) substrates; this docking site is also required for the mechanism of GSK3 inhibition by phosphorylation. In contrast, non-primed substrates interact with a different region of GSK3. Phosphoinositide-dependent protein kinase-1 (PDK1) contains a hydrophobic pocket that interacts with a hydrophobic motif present in all known substrates, enabling their efficient phosphorylation. Binding of the substrate hydrophobic motifs to the pocket in the kinase domain activates PDK1 and other members of the AGC family of protein kinases. Finally, the analysis of protein kinase structures indicates that the sites used for docking substrates can also bind N- and C-terminal extensions to the kinase catalytic core and participate in the regulation of its activity.

Key words: docking, glycogen synthase kinase 3 (GSK3), mitogen-activated protein kinase (MAPK), phosphoinositide-dependent protein kinase-1 (PDK1), phosphorylation, protein kinase.

INTRODUCTION

Protein phosphorylation is ubiquitous and is the most common mechanism of protein function regulation known to date. In eukaryotes, phosphorylations are carried out by protein kinases, a family of evolutionarily related enzymes that transfer the terminal phosphate from ATP to a specific Ser, Thr or Tyr residue on protein substrates. Protein kinases represent about 2% of the proteins encoded by eukaryotic genomes [1–4], and this makes them the third most common protein domain in the human genome [4].

As key signalling enzymes, protein kinases participate in the regulation of multiple cellular responses and have evolved two properties that are essential for their function: sensitive means of regulation and high specificity for substrates. Recently, protein kinases have emerged as major targets in drug development, totalling almost one-third of newly validated targets in the pharmaceutical industry [5]. It is expected that elucidation of the molecular mechanisms underlying regulation and specificity may prompt novel approaches for the pharmacological modulation of protein kinase activities. Examples of mechanisms that generate specificity for protein kinases have been described [6], and include subcellular co-localization, interaction with substrates via a scaffolding protein, modular docking interactions and specific protein kinase-substrate docking mechanisms.

This review will focus on recent findings regarding the function and regulation of direct docking interactions with substrates in protein kinases from the mitogen-activated protein kinase (MAPK), glycogen synthase kinase 3 (GSK3) and phosphoinositide-dependent protein kinase-1 (PDK1) families (Figure 1). We also speculate on the importance of docking interactions in other protein kinases and the regulatory properties of docking sites.

SPECIFICITY OF SER/THR PROTEIN KINASES: HISTORICAL PERSPECTIVE

Early studies on cAMP-dependent protein kinase (PKA) and phosphorylase kinase revealed that the principal substrate specificity determinants for these kinases were "recognition

Abbreviations used: CD domain, common docking domain; CDK, cyclin-dependent kinase; D-domain, docking domain; DEF domain, domain characterized by sequence Phe-Xaa-Phe-Pro; ERK, extracellular-signal-regulated kinase; FRAT, frequently rearranged in advanced T-cell lymphomas; FRATide, FRAT residues 188–226; GSK3, glycogen synthase kinase 3; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MAPKAPK, MAPK-activated protein kinase; MAPKK, MAPK kinase; MKP, MAPK phosphatase; pCIP, p21-activated kinase; MEK, MAPK/ERK kinase; MEF, myocyte enhancer factor; PAK, p21-activated kinase; MEK, MAPK/ERK kinase; MKK, MAPK-interacting kinase; MSK, mitogen- and stress-activated protein kinase; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PKR2, PKC-related protein kinase 2; PTP, protein tyrosine phosphatase; RSK, p90 ribosomal S6 kinase; S6K, p70 S6 kinase; STEP, striatum-enriched phosphatase.

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motifs’, located in short segments of the primary sequence around the phosphorylation sites [7,8]. These were key discoveries that shaped most of the future studies on the substrate specificity of protein kinases, and the recognition motif concept has since been successfully applied to many protein kinases [9]. As a consequence, protein kinase phosphorylation sites are tabulated and synthetic peptides are commonly used as specific substrates for diverse protein kinases. Furthermore, the sole identification of the phosphorylated residue within a particular sequence has been, in many cases, an essential hint in the discovery of the physiological kinase that phosphorylates that site. Nevertheless, it is unlikely that ‘recognition motifs’ provide the specificity required in vivo for several reasons: (i) many protein kinases share in vitro ‘recognition motifs’, for example Ser/Thr-Pro sites for different MAPKs and cyclin-dependent kinases (CDKs), or Arg residues N-terminal to the phosho-acceptor for related kinases, including p90 ribosomal S6 kinase (RSK), PKA and protein kinase B (PKB); (ii) recognition motifs within proteins may not be physiologically phosphorylated; (iii) many protein kinases do not seem to possess a clear consensus ‘recognition motif’ within substrate peptides; and (iv) peptides do not always mimic the phosphorylation kinetics of the whole protein [10].

Direct docking interactions that facilitate efficient phosphorylation of some substrates by Ser/Thr protein kinases were first postulated in the transcription factor c-Jun, a substrate for the MAPK JNK (c-Jun N-terminal kinase). It was proposed that binding of the δ-domain of c-Jun to JNK2 increased the local concentration of the substrate and contributed to its efficient phosphorylation [11,12]. Phosphorylase kinase was also postulated to possess secondary interaction sites with its substrate glycogen phosphorylase [13]. In recent years, sequences that facilitate kinase–substrate interactions have been reported in substrates of several Ser/Thr protein kinases, suggesting that this may be a wide-spread specificity mechanism. In addition to the focus of this review, MAPK, GSK3 and PDK1, docking sites have also been identified in the Smad substrates of the transforming growth factor-β receptor [14,15] and on some CDK substrates (p107, p130, E2F1) and inhibitors (p27Kip1, p21Cip1) [16,17]. In particular, the docking interactions of CDK2, which are mediated by cyclin A, have been the subject of elegant structural studies [18,19].

MAPK

MAPK pathways convert different extracellular stimuli into specific cellular responses via phosphorylation of particular groups of substrates. There are three major mammalian MAPK subfamilies: ERK (extracellular-signal-regulated kinase), JNK and p38. Each subfamily has different biological functions, but all MAPKs can phosphorylate substrates containing the minimal consensus sequence Ser/Thr-Pro. In recent years, several MAPK-recognition modules that are involved in the specific binding to MAPKs have been reported. Equivalent motifs are found in substrates (both nuclear and cytoplasmic), as well as in positive and negative regulators of MAPK activity [20,21]. Thus the docking interactions with MAPKs must be somehow sequentially organized (Figure 2). It is now becoming clear that these interaction motifs are crucial for efficient and specific signal transduction by MAPKs.

Docking of transcription factors to MAPK

The docking site of c-Jun, known as the δ-domain, is required for specific binding to JNK, and includes the sequence Lys-Xaa-Xaa-Arg/Lys-Xaa-Leu-Xaa-Leu, where Xaa is any amino acid [12]. Sequences almost identical with the δ-domain of Jun were later identified in many transcription factors, including members of the bZIP, ETS and MADS families that are regulated by ERK, JNK and/or p38 MAPKs (reviewed in [20]). In all of these cases, the kinase-docking domains (or D-domains) enhance substrate phosphorylation by MAPKs. D-domains are also believed to contribute to specificity determination, although some of them can be recognized by more than one class of MAPKs [22]. The importance of the D-domains was also emphasized by mutation of specific acidic residues in the MAPK that were predicted to interact with the Lys or Arg residues of the substrate docking sequences [23].
Interestingly, the Leu-Xaa-Leu motif, preceded by 3–5 basic residues, has been found in transcription factors phosphorylated by ERK and JNK MAPKs, whereas some p38 substrates, such as myocyte enhancer factor (MEF) 2A and MEF2C, contain three hydrophobic residues instead of a Leu-Xaa-Leu motif [20]. Thus small changes in the consensus docking sequence may contribute to substrate specificity by different MAPKs [24].

D-domains are generally located N-terminal to the transcription-activation domain, less than 100 amino acids upstream from the MAPK phosphorylation site, which is important for the activation of the transcription factor. Recently, the Pointed domain of a subset of ETS transcription factors has been shown to contain an ERK2 docking sequence that is located C-terminal to the MAPK phosphorylation site [25].

Analysis of gain-of-function mutations of the ETS transcription factor LIN-1, which is negatively regulated by MAPKs, has identified a new motif named the DEF domain. This domain, characterized by the sequence Phe-Xaa-Phe-Pro (where one of the Phe residues may be a Tyr), is also required for efficient phosphorylation by ERK [26]. DEF domain sequences, together with potential MAPK phosphorylation sites, have also been identified by database searches in GATA family transcription factors, MAPK phosphatases and other ERK regulators [26]. In the transcription factor Elk-1, the DEF and D-domains have been shown to direct phosphorylation of different Ser/Thr-Pro residues by ERK [27]. DEF domains have also been proposed to allow immediate early gene products, such as c-Fos, to translate differences in the kinetics of ERK activation into specific cellular responses [28].

It therefore appears that different types of MAPK recognition modules may provide unique functions by working either synergistically or competitively. When combined, DEF and D-domains may stabilize kinase–substrate binding, enhance kinase selectivity and improve efficiency of phosphorylation.

Docking of cytoplasmic MAPK substrates

D-domains specific for ERK have been found in substrates other than transcription factors, including protein kinases, protein phosphatases and cyclic AMP phosphodiesterases (PDEs). The docking sites of PDE4D [29] and protein tyrosine phosphatases (PTPs) [30,31] resemble those found in transcription factors [26]. In particular, DEF domains have been shown to be functionally relevant for the phosphorylation of PDE4D3 and the KSR-1 (kinase suppressor of Ras-1) scaffold by ERK [27].

In contrast, docking sequences of MAPK-activated protein kinases (MAPKAPKs) contain the sequence Leu-Xaa-Xaa-Arg-Arg followed by several basic residues. This docking sequence is required for the efficient phosphorylation and activation of MAPKAPKs of the RSK family in vitro and in cells. When fused to another protein, it is sufficient for the stable and specific association with ERK [32,33]. Mutagenesis analysis indicates that the dissociation of the ERK–RSK complex is actually due to the phosphorylation of RSK. Interestingly, an N-terminally truncated RSK mutant can constitutively interact with ERK [34].

The sequence Leu-Xaa-Xaa-Arg-Arg may be also a relevant docking site in other ERK MAPKAPKs, such as MNK (MAPK-interacting kinase) and mitogen- and stress-activated protein kinase (MSK) [35–39]. Moreover, the same motif is present and required for ERK binding in the PTPs PTP-SL and STEP (striatum-enriched phosphatase) [30]. It is therefore possible that this class of MAPK docking site may allow the association of inactive ERK MAPKs with cytoplasmic targets. This would ensure the preferential phosphorylation of the associated substrate, especially for those cases in which partial or transient levels of active MAPK are produced. Furthermore, it may be of particular importance when the targets themselves are protein kinases or phosphatases with the potential to modulate the intensity of the signal.

Although RSK family members are specifically activated by ERK, other MAPKAPKs are activated either by p38 (MAPKAPK-2 and -3) or by both ERK and p38 (MNK1 and MSK1). Where does the specificity p38/ERK come from in the D-domains of MAPKAPKs? The generation of chimaeric proteins has indicated that MAPK docking sites are located in the C-terminal tails of the MAPKAPKs. These sequences show similar specificity for ERK or p38 binding as the full-length proteins [40]. As mentioned above for transcription factors, the variability in the number and position of hydrophobic and basic residues within the docking sequences may determine specificity. The number of basic residues, in particular, makes an important contribution but does not appear to be the only specificity determinant [40,41].

What about MAPK substrates that lack D-domains? It is conceivable that new kinds of docking sequences remain to be identified. However, there may also be substrates that contain no MAPK-interaction modules other than the phospho-acceptor motif. MAPKs could promiscuously phosphorylate these proteins if their local concentration is high enough, for example by association with mutual scaffolding molecules.

D-domains in MAPK regulatory proteins

MAPK D-domains have been found in many MAPK regulatory proteins, including activating kinases, down-regulating phosphatases and scaffold proteins [21,42]. All of these proteins are likely to interact with the same residues of MAPK as the substrates discussed above. The binding site on the p38δ MAPK structure is shown in Figure 3(A) (box), and is represented as a striped oval at the back of the kinase domain in Figure 2.

The N-terminal region of MAPK kinases (MAPKs) contain a docking site that is very similar in sequence to the D-domain of MAPK substrates [43]. In many cases, this docking site is known to be required for binding and activation of the MAPKs; both basic and hydrophobic residues are involved in MAPK recognition. However, the presence of the N-terminal docking site does not appear to be essential for MAPK activation and there is evidence that other docking mechanisms, in addition to the D-domain, might regulate MAPK–MAPKK interactions [44,45].

Cytoplasmic PTPs, such as PTP-SL and STEP [30,31], also contain functional MAPK docking sites that are similar to the D-domain. There are mechanistic differences, however, as PTPs associate with inactive MAPKs in the cytoplasm and the complex dissociates upon ERK activation and PTP phosphorylation. On the other hand, ERK interacts more efficiently with the phosphorylated form of transcription factors, such as Elk-1. Interestingly, the docking interaction between ERK and PTP- SL can be blocked by PKA-catalysed phosphorylation of Ser 311, within the kinase interaction motif of PTP-SL [46]. Phosphorylation of PTP-SL may interfere with down-regulation of ERK, according to the model presented in Figure 2.

Dual-specificity phosphatases that dephosphorylate both phospho-Thr and phospho-Tyr residues in MAPKs (termed MKPs) also contain a D-domain-like sequence in their non-catalytic N-terminus. In some cases, like MKP3, the D-domain has been shown to be involved in selectivity [47]. As mentioned for other MAPK interactors, the number and spacing of positively charged residues might account for specificity in MKP docking. Mutation of an N-terminal cluster of basic residues in MKP1
has also suggested the existence of distinct binding determinants for different MAPKs [48]. In general, there is good correlation between binding of a MKP to a MAPK and its activity towards the MAPK, but there are also examples where MAPK–MKP interactions do not appear to ensure specificity [49,50].

In some cases, D-domains are not involved in the efficient and accurate phosphorylation of substrates, but serve to recruit MAPKs into signalling complexes. For example, the JNK scaffold JIP-1 (Janus kinase interacting protein-1) contains a D-domain [51]. Another example is JunB, which through its D-domain recruits JNK to phosphorylate its heterodimerization partner JunD [52]. Docking interactions have also been described between the MAPKs MEK (MAPK/ERK kinase) 1/2 and MKK (MAP kinase kinase) 4 and their upstream activators Raf1 and MEKK1 (MEK kinase 1) respectively [53,54]. These interactions should prevent cross-talk and contribute to efficient and specific MAPK signalling.

MAPK specificity determinants that recognize docking motifs

Based on the generation of JNK1 and JNK2 chimaeras, it was proposed that the δ domain of Jun binds to a short surface-exposed segment in the large lobe of the JNK2 kinase domain [12]. One motif containing acidic residues, termed the common docking (CD) domain, has been identified in all MAPKs. It was suggested that this domain establishes electrostatic interactions with the basic residues of the D-domain of MAPK activators, substrates and regulators [23,55,56]. In addition, two Tyr residues close to the CD domain of ERK2 are also required for MEK1 docking [56,57]. In some cases, like the ERK1b spliced variant, mutation of the CD domain does not affect activation by MEK1, but the mutant protein shows reduced sensitivity to phosphatases [58]. The Drosophila ERK ‘sevenmaker’ allele is also mutated in the CD domain and has decreased sensitivity to dephosphorylation [59].

Other regions of MAPKs that have been implicated in specificity regulation include the Glu-Asp domain of p38α, which is required for docking of MAPKAPKs and MKPs [41], the activation loop of p38β/2 [44] and N-terminal domains of ERK2 [60]. Mutations at distinct regions of the C-terminus of ERK2 and p38α have also been shown to differentially affect their association with PTPs [61]. Recently, ERK2 point mutants have been identified that are selectively impaired in binding to the MAPKK MEK1 but not to MNK1, RSK or MKP3 [62]. The position of these mutations in the ERK structure suggests that a 30-amino-acid sequence, known as the MAPK insert [63] (Figure 3A), which is homologous to

Figure 3 MAPK docking interactions with substrates, activators and regulators

The docking groove for MEF2A (1LEW) and MKK3b (1LEZ) on p38α MAPK is shown to be a site which in other kinases is occupied by extensions to the catalytic domain. (A) p38 MAPK structure is shown as ribbons and the co-crystallized peptide (docking site) backbone as sticks. The docking groove is shown bound to MEF2A and MKK3b (right panels). The involvement of the different secondary structure regions of the kinase is indicated in the p38α MAPK–MEF2A peptide magnification. The red arrow indicates the region termed ‘MAPK insert’. (B) A site equivalent to the p38α MAPK docking groove is occupied by sequences C-terminal to the catalytic domain in the protein kinases PAK1 (1F3M) and CDK2 (1HCL). The numbers/letters in parentheses are Protein Data Bank codes for the structures used in the Figure.

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GSK3

GSK3 has been known for more than 20 years as part of the insulin signalling pathway, and is still receiving a great deal of attention, due to its role in this and other disease-related signalling pathways. Recent reviews on GSK3 have been published both on general issues [64–66] and on its involvement in neurodegenerative disease, cell fate and tumorigenesis [67–69]. The importance of GSK3 is highlighted by its possible use as a drug target for the treatment of neurodegenerative diseases and diabetes [70,71].

GSK3 interaction with primed substrates of the insulin signalling pathway is linked to the mechanism of GSK3 inhibition by phosphorylation

Glycogen synthase is the terminal enzyme in the insulin signalling pathway that regulates glycogen synthesis. In response to insulin, GSK3 becomes inhibited, facilitating the dephosphorylation and activation of glycogen synthase [72] (Figure 1). It was subsequently found that GSK3 becomes inhibited upon insulin stimulation by PKB-mediated phosphorylation of an N-terminal Ser (Ser²¹ in GSK3α and Ser¹⁹ in GSK3β) [73], but the actual molecular mechanism of inhibition was not deciphered until recently.

In glycogen synthase, as in other substrates downstream of insulin and growth factor signalling, GSK3 requires the previous phosphorylation of a Ser located four amino acids C-terminal to the actual phosphorylation site (n + 4); this was termed ‘primed phosphorylation’ [74]. Recent biochemical and structural work has revealed that docking of primed substrates is achieved by direct interaction with a specific phosphate-binding site within the catalytic core of GSK3 [75–77]. Interestingly, the molecular mechanism of GSK3 inhibition is also based on the occupancy of the same phosphate-binding site by a phosphorylated N-terminal Ser that may act as a pseudosubstrate [75,76]. As indicated in Figure 4(A), glycogen synthase is normally maintained in an inactive state by GSK3-mediated phosphorylation. Upon insulin stimulation, PKB phosphorylates the N-terminus of GSK3. This blocks the GSK3 phosphate-binding site, which in turn interferes with the docking (and phosphorylation) of glycogen synthase, facilitating its dephosphorylation and activation. Thus the GSK3 phosphate-binding site has a fundamental role both in binding primed (active) glycogen synthase and in the inhibition of GSK3 by insulin. As noted above, docking interactions can produce highly efficient concentration of substrates. It is therefore possible that phosphorylation of the N-terminal Ser may not inhibit the phosphorylation of substrates that possess high affinity for GSK3, for example by having additional docking sites.

GSK3 interactions with substrates of the Wnt signalling pathway

Apart from its role in the insulin signalling pathway, a pool of GSK3 forms part of multiprotein complexes that participate in the Wnt signalling pathway, phosphorylating at least two of its components, axin and β-catenin (Figure 1). Wnts constitute a family of secreted glycoproteins that are involved in embryonic development throughout the animal kingdom and have been also implicated in some forms of human cancers [78,79]. Multiprotein complex interactions of proteins involved in Wnt signalling have been extensively studied (reviewed in [80]). As shown in Figure 4(B), the key effector of this pathway is β-catenin, which upon phosphorylation by GSK3 is targeted for proteasome degration. In response to Wnt signalling, GSK3 is inhibited and the non-phosphorylated (stable) β-catenin binds to transcription factors of the TCF (T-cell factor) family, which in turn activate the expression of Wnt target genes.

The Wnt signalling multiprotein complex includes GSK3β, β-catenin, the tumour suppressor adenomatous polyposis coli (APC) and the scaffolding protein axin. Axin binds tightly to β-catenin phosphorylation (Figure 4B). The axin/FRAT-docking complex dissociates, resulting in β-catenin dephosphorylation and stabilization, which stimulates gene expression. The phosphorylation of FRAT upon Wnt signalling is speculative.
Figure 5  GSK3 docking interactions with substrates

The GSK3β structure depicts the phosphate binding site and FRATide/axin binding site.

(A) Ribbon representation of GSK3β with a phosphate bound to the phosphate binding site and FRATide (backbone represented as sticks) bound to the FRAT/axin binding site (1GNG). The right panel shows a magnification of the phosphate-binding site that interacts with ‘primed substrates’ of GSK3. The phosphate interactions are equivalent to those of the activation loop phosphorylation site in other protein kinases. GSK3 does not possess an analogous activation loop phosphorylation site, and phospho-Tyr216 in the activation loop points outward, similarly to phospho-Tyr185 in MAPK (1CM8). The bottom panel shows a magnification of the GSK3β-FRATide binding site. (B) The equivalent region in protein kinase CK2 (1DAW) interacts with a helix (red arrow) formed within a large insertion at the bottom of the large lobe of the catalytic domain. The numbers/letters in parentheses are Protein Data Bank codes for the structures used in the Figure.

GSK3. Thus axin and β-catenin phosphorylation is abolished by displacement of axin from GSK3 by FRAT. What regulates FRAT binding to GSK3 is not known. One possibility is that FRAT contains a second docking site [in addition to the one depicted in the GSK3/FRATide (FRAT residues 188–226) crystal structure], which may be regulated by phosphorylation and docks into the GSK3 phosphate docking site (Figure 4B). If this was the case, the physiological FRAT that binds GSK3 in response to Wnt signalling should be phosphorylated and able to inhibit GSK3 activity on primed substrates. This could explain the inhibition of GSK3 activity on primed peptide substrates observed upon Wnt stimulation.

The primed phosphorylations that have been most studied are from the insulin/growth factor signalling pathway (e.g. glycogen synthase; Figure 4A). More recently, it has also been suggested that β-catenin, a substrate of GSK3 in the Wnt signalling pathway, is phosphorylated in a site dependent on an (n+4) priming phosphate [85]. This is consistent with the effect of a GSK3 mutant disrupted in the phosphate docking site (Argx6 → Ala) on Wnt signalling [86]. However, the kinase responsible for the priming phosphorylation is casein kinase-1, which itself usually requires an (n−4) priming phosphate. Thus phosphorylation of this site could be dependent on the activity of either of both kinases or perhaps, more interestingly, on the exposure of the sites by other mechanisms. Thus β-catenin may be an example of a GSK3 substrate that relies on the combined effect of interaction with the scaffold protein axin and priming phosphate docking for efficient phosphorylation.

In summary, GSK3 activity is regulated by the displacement of docking interactions with its substrates. In one case, axin is displaced by FRAT, whereas most primed substrates are displaced by the phosphorylated N-terminus of GSK3.

Figure 6  Schematic representation of PDK1 and other AGC protein kinases

PDK1 and its substrates, members of the AGC kinase family, contain a phosphorylation site at the activation loop of the catalytic domain (black arrows, Thr308 in PKBα) which is necessary for activity. Most PDK1 substrates also have a hydrophobic motif phosphorylation site (red arrows, Ser473 in PKBα) within the motif Phe-Xaa-Xaa-Phe-Ser/Thr(Tyr)-Tyr, but some have a negatively charged residue in that position (Asp in PRK2). The hydrophobic motif phosphorylation is essential for some substrates to dock to PDK1. PDK1 has a C-terminal PH domain but does not possess the hydrophobic motif. PKA has a truncated hydrophobic motif that lacks the phosphorylation site. SGK regulates its interaction with PDK1 via a C-terminal autoinhibitory domain, whereas PKR2 interaction with PDK1 is regulated by Rho binding. RSK requires prior docking to ERK in order to phosphorylate and activate its C-terminal domain, which in turn phosphorylates the hydrophobic motif. This phosphorylation allows docking to PDK1, which phosphorylates the activation loop of the N-terminal domain and enables full RSK activity.

PDK1

PDK1 is a key enzyme of the insulin/growth factor signalling pathway that lies downstream of phosphatidylinositol 3-kinase (PI3K) (P3K) activation (Figure 1) [87,88]. PDK1 consists of a protein kinase catalytic core with a 75-amino-acid extension N-terminal to the catalytic core and a pleckstrin homology (PH) domain at the C-terminal end (Figure 6). PDK1 was originally isolated for its ability to phosphorylate the activation loop of PKB (Thr308 in PKBα) in the presence of phosphatidylinositol 3,4,5-trisphosphate (PIP3), the product of P13K [89,90]. PIP3 binds the PH domain of both PDK1 and PKB. Thr308 phosphorylation is crucial for PKB activation and, together with Ser473, which is located at the C-terminus within a hydrophobic motif, these
residues are the two phosphorylation sites known to regulate PKB activity (Figure 6). The phosphorylation of Ser^{473} is not sufficient for the activation of PKB but stimulates the kinase activity of Thr^{308}-phosphorylated PKB. Sites equivalent to the active site (Thr^{308}) and the hydrophobic motif (Ser^{473}) in PKBα are strikingly conserved, and are also important for activity of evolutionarily related protein kinases that are grouped within the AGC kinase family, to which PDK1 also belongs (Figure 6). PDK1 can also phosphorylate the activation loop of several other AGC kinases, including RSK, PKA, p70 S6 kinase (S6K), serum- and glucocorticoid-induced kinase (SGK), protein kinase C (PKC) isoforms, PKC-related protein kinase 2 (PRK2) and MSK1 [91,92]. Studies using PDK1-knockout cells have shown that this enzyme phosphorylates most of these protein kinases, except for PKA and MSK1 [93,94].

PDK1 is thought to be a constitutively active kinase that may use distinct mechanisms for the phosphorylation of different substrates inside cells. Accordingly, many studies have found no increase in the activity of PKD1 immunoprecipitated from cells in which the phosphorylations of endogenous PDK1 substrates were stimulated. It should be noted, however, that there is in vitro evidence suggesting the activation of PDK1 upon binding to its substrates [95,96] (see below). A small change in the in vitro PDK1 activity towards PKB has also been reported in some studies [97,98]. Nevertheless, the work of several groups strongly suggests that docking interactions are very important specificity determinants for PDK1.

**PDK1 specificity**

The analysis of the sequences surrounding PDK1 phosphorylated residues indicates that the possible ‘recognition motif’ should be located C-terminal to the phosphorylation site. This region bears significant similarity among different substrates (Figure 6). Peptides based on the activation loop of protein kinase substrates of PDK1 are useful to measure intrinsic PDK1 activity [95,98,99]. However, these peptides are very poor substrates [95,100], indicating that they lack determinants found in the native proteins [100].

Early studies found that PDK1 can form stable complexes with some of its substrates, including PKCs and S6K [101–103]. Moreover, the C-terminal 76 amino acids of PRK2 were found to bind PDK1 in a yeast two-hybrid screen [104]. This peptide, termed PDK1-interacting fragment (PIF), has homology with residues present at the C-terminus of most AGC kinases (Figure 6). Initially, it was suggested that binding of PIF might allow PDK1 to phosphorylate Ser^{473} of PKB [104]. An alternative possibility is that the interaction of PIF with PKB [105,106] accounts for the observed phosphorylation of Ser^{473} in vitro. Consistent with the latter possibility, the mutant PDK1-Leu^{159} → Glu (which does not bind PIF) can only phosphorylate Ser^{473} of PKB upon addition of PIF (R. Biondi and D. Alessi, unpublished work). PRK2 and PDK1 can also interact inside cells (see below).

The hydrophobic and the Asp residues within the motif Phe-Xaa-Xaa-Phe-Asp-Tyr in PIF were found to be critical for the binding to PDK1. The hydrophobic motif of AGC kinases is likely to interact with their own catalytic core, in a similar way to that of PKA (Figure 7A). This has recently been supported by the determination of the structure of active PKBα [107]. Thus binding to PDK1 through the hydrophobic motif sequence may be subjected to competition between inter- and intra-molecular interactions [108,109] (Figure 7B).

**PDK1 docking with substrates is a requirement for efficient phosphorylation**

Fusion of the hydrophobic motif of PRK2 (PIF) to a particular peptide makes a much better substrate for PDK1 than the same peptide alone [95], suggesting that the interaction between PDK1 and its substrates is of functional significance. Similarly, RSK2 phosphorylation by PDK1 requires the prior phosphorylation of RSK2 at the hydrophobic motif site that promotes PDK1 docking [96]. Interestingly, RSK2 mutants that do not contain the hydrophobic motif docking sequences are not phosphorylated in vivo on the activation loop unless they are co-expressed with PDK1. These results suggest that increasing the concentration of the two proteins may be sufficient to trigger their interaction in vivo [96].

Studies with other AGC family kinases further support the theory that activation loop phosphorylation of different PDK1 substrates is ultimately modulated by distinct regulatory docking interactions [96,109–112]. Additional variations come from the participation of other domains in the PDK1 substrates. For example, phosphorylation of C-terminal residues in S6K may expose the hydrophobic motif to make it available for interaction with PKD1 [111,113,114]. A similar mechanism may operate in the case of PRK2, which requires prior binding of the small GTP-binding protein Rho for interaction with PKD1 [112].

Figure 7(B) shows a simplified model of how the PIF-like hydrophobic motif of PDK1 substrates can regulate their interaction with, and phosphorylation by, PDK1. In contrast, PKB activation does not seem to rely on this type of docking mechanism to gain proximity to PDK1 [111].
Figure 8  PDK1–substrate docking interaction

The docking pocket in PDK1 is equivalent to a site that, in other AGC kinases, binds a hydrophobic motif located in the C-terminal extension to the catalytic core. The hydrophobic motif Phe-Xaa-Xaa-Phe of PDK1 substrates binds to the PDK1 docking pocket in a manner equivalent to the binding of the PKA hydrophobic motif to its own pocket. (A) The ribbon structure of PKA (1ATF), indicating N-terminal (N-T) or C-terminal (C-T) extensions to the catalytic domain, is shown. The Phe of the hydrophobic motif and the activation loop phosphorylation site (pThr197) interacting with His87 from αC-helix are shown as sticks. The right panel shows a magnification of the PKA pocket where Phe residues bind. (B) The PDK1 crystal structure (1H1M), depicting a hydrophobic pocket equivalent to that in PKA, and a phosphate-docking site that interacts with the hydrophobic motif of PDK1 substrates are shown. Sticks represent residues from αB- and αC-helices, as well as from β4 and β5 that have been implicated in binding and activation of PDK1. The numbers/letters in parentheses are Protein Data Bank codes for the structures used in the Figure.

PKB: a peculiar PDK1 substrate

PKB is different from the rest of the PDK1 substrates in that it possesses a PH domain (Figure 6) that, similar to PDK1, can bind PIP3 lipids at the plasma membrane. Both PKB and PDK1 translocate to specific regions in the plasma membrane where PIP3 is generated as a consequence of PI3K activity [115,116]. There is also evidence that the binding of PIP3 to PKB is sufficient to produce a conformational change that is required for PDK1 phosphorylation. In fact, PKB is the only substrate of PDK1 that requires the presence of PIP3 to be efficiently phosphorylated by PDK1. Moreover, PKB phosphorylation in vivo takes place seconds after PI3K stimulation and production of PIP3, while the phosphorylation of other PI3K-dependent substrates, such as S6K, is maximal 15–40 min later [111]. Furthermore, PIP overexpression does not affect PKB activation, but inhibits the phosphorylation and activation of other PDK1 substrates [110,111,117]. Mutagenesis analysis also supports that, in contrast with other PDK1 substrates, PKB does not appear to rely on the hydrophobic motif docking interaction for phosphorylation. However, the situation appears to be different for PKB mutants lacking the PH domain, which may require docking with the PDK1 PIF binding pocket, similar to other substrates of PDK1 [111,118].

In conclusion, the mechanism of PKB phosphorylation appears to rely less on the hydrophobic motif–PIF pocket interaction, perhaps to facilitate phosphorylation only when PKB and PDK1 co-localize with PIP3 at the plasma membrane.

Structural analysis of the PDK1 PIF pocket that docks the hydrophobic motif of substrates

In contrast with other PDK1 substrates, PKA only possesses a truncated hydrophobic motif (Phe-Xaa-Xaa-Phe), where the second Phe corresponds to the C-terminus of the protein. In the PKA crystal structure, the Phe residues occupy a deep hydrophobic pocket in the small lobe of the catalytic domain [119] (Figure 8A). Biochemical studies demonstrated that these two Phe residues are important for PKA activity [120,121]. Molecular modelling of PDK1, based on PKA, allowed the biochemical characterization of the PDK1 pocket where PIF binds [95]. Recently, the crystal structure of PDK1 was solved and the details of the ‘PIF pocket’ revealed (see below).

Participation of the PIF pocket in PDK1 activation

In order to study the effect of docking sequences on the intrinsic kinase activity, it is necessary to use a substrate that does not require a docking mechanism for interaction. For PKD1, kinase assays were developed with either a peptide substrate based on the activation loop of PKB [95,98], or a truncated RSK2 [96]. These assays revealed that peptides encompassing the hydrophobic motifs increase PDK1 intrinsic activity 4–6-fold. Interestingly, mutation of Leu155 or Lys115 in the PIF-binding pocket increases the activity of PDK1 4-fold. Other mutants, such as Ile119 → Ala and Gln150 → Ala, having decreased affinity for PIF, are fully activated only at higher concentrations of PIF [95].
The mechanism of activation of PDK1 has recently been shown to be similar to that of other AGC kinases [106,122,123]. Structural studies suggest that the mechanism of PKB activation involves the stabilization of the α-C helix by binding of the phosphorylated hydrophobic motif to the ‘PIF pocket’ site on PKB [106,107]. In addition, the interaction of an Asp from the PKB C-terminal extension with the Arg in substrates [107] could suggest a secondary mechanism of activation.

In summary, the docking of PDK1 substrates is coupled to the activation of PDK1 via a mechanism similar to the intramolecular activation of AGC kinases. PDK1 does not possess the hydrophobic motif itself, but can complement its protein kinase catalytic core using the hydrophobic motif of AGC kinases (Figure 7).

WHERE DO DOCKING SEQUENCES DOCK?

Protein kinase structures

PKA was the first protein kinase to be crystallized and its structure has been a hallmark for the whole protein kinase family [119]. Protein kinases consist of an N-terminal small lobe and a C-terminal large lobe that give the catalytic core a characteristic ‘bean-like’ structure. ATP binds between the two lobes, directing the γ-phosphate outwards while the adenine ring lies deep in the cleft between the two lobes. The substrate binding site for peptides was structurally depicted by co-crystallization of PKA with its pseudo-substrate inhibitor PKI [124]. It was later confirmed that other crystallized peptide substrates bind to protein kinases in an extended conformation [125].

Protein kinases usually possess both N-terminal and C-terminal extensions from the kinase catalytic core. In many cases, these regions fold back into the catalytic core, forming important specific interactions. In PKA, N- and C-terminal extensions from the catalytic core promote stability or activity [126–128]. Alternatively, these extensions may promote inhibitory interactions by acting as pseudosubstrates [129].

Structural analysis of protein kinase sites involved in docking interactions

MAPK

Recently the structural basis for docking interactions in MAPKs has started to be elucidated by the co-crystallization of p38α MAPK with the D-domains of the substrate MEF2A and the activating kinase MKK3b [130]. This work confirmed that both domains bind to the same docking groove in p38α, with Ile116 and Gln120 playing a key role in the interaction with the hydrophobic motif of the D-domain (Figure 3A). Similar docking grooves exist in other MAPK family members, but the p38α residues in contact with the docking peptides are not always conserved, suggesting that small differences in the structure of the groove may contribute to MAPK specificity. Unexpectedly (based on previous mutagenesis studies), no interactions between the acidic residues of the MAPK CD domain and basic residues of the substrate/activator D-domain were observed in the p38α crystal structures. This might be due to the use of non-phosphorylated (inactive) p38α and docking site peptides (instead of the whole proteins) for the crystallographic study. The MAPK Glu-Asp site also does not participate in these interactions although it is located close to the docking groove. Interestingly, the binding of two D-domain peptides induces conformational changes in the p38α MAPK, which are different in the case of MEF2A and MKK3b, suggesting that the docking interactions may also regulate MAPK activity [130].

The interaction of p38α with both peptide substrates is mainly hydrophobic, with an important participation of the residues Leu-Arg-Val and Leu-Arg-Ile. As other docking sequences are known to be hydrophobic, the question of whether the same docking pocket is used for other docking sequences arises. Mutations within the hydrophobic pocket of MAPK family members may reveal whether this is the case.

GSK3

Structural insight into the phosphorylation-mediated docking interaction between GSK3 and primed substrates has recently been obtained [76,77]. In the GSK3 structures, the phosphate-binding site is depicted by a sulphonate moiety from the Hepes buffer, or by a sulphate group present in the crystallization conditions. The phosphate binding site of GSK3 is defined by residues Arg95, Arg108 and Lys105 (Figure 5). These residues are structurally equivalent to the residues defining the activation loop phosphate-binding site in a number of other protein kinases, such as PKA, PDK1 and ERK2 (reviewed in [131,132]). This finding raises the possibility that docking of primed substrates could activate GSK3. In this case, a short phosphopeptide that specifically interacts with the docking pocket (phosphate-binding site) would be expected to promote GSK3 activity on a non-primed substrate. However, experimental evidence to support this point is lacking [75]. Recently, crystals of active GSK3 have also been formed in the absence of sulphonate/phosphate moieties, arguing that these are not indispensable for the active conformation to be adopted [133]. The elucidation of the possible role of the priming phosphate in GSK3 activation may require the use of shorter phosphopeptides, or chemical compounds that specifically interact with the phosphate-binding site without interfering with the catalytic binding site.

Recent biochemical work has also characterized the axin and FRAT binding sites on GSK3 [134,135]. Furthermore, the crystal structure of GSK3β bound to a polypeptide derived from FRAT residues 188–226 (FRATide) has been solved [133]. The FRATide binding site is located at the front of the large kinase lobe, in a position that does not block the substrate-binding site (Figure 5). This may explain why FRATide does not block the phosphorylation of primed peptide substrates. FRATide bound to GSK3 has a helix-loop-helix structure, where the helices are amphipathic and the main interactions are of a hydrophobic nature. The C-terminal helix of FRATide induces a shift in a GSK3 large loop and exposes the hydrophobic groove where the C-terminal helix binds. This loop is absent in most protein kinases, but has structural similarity to an insert found in CDKs and MAPKs (MAPK insert). It appears that the axin binding site on GSK3 probably overlaps with that for FRATide. Its proximity to the active site may actually explain how axin acts as a scaffold protein promoting β-catenin phosphorylation.

PDK1

The details of PDK1 substrate docking have been revealed by solving the crystal structure of ATP-bound PDK1 (Figure 8). The binding of the hydrophobic motif Phe-XXa-Xaa-Phe to the so-called PIF pocket was previously modelled, based on the structure of PKA, which has itself the homologous pocket occupied by the two C-terminal Phe residues from its own polypeptide (see above). The hydrophobic PIF pocket is thus located between β4–β5 and α-C helix A, with a side formed by the short α-B helix (which
in non-AGC kinases forms a loop instead of a helix) (Figure 8). The phosphate binding site that interacts with the phosphorylated hydrophobic motif of substrates is clearly depicted by an ordered sulphate ion situated next to the PIF pocket of PDK1 and has potential specific interactions with Lys76, Arg131, Thr148 and Gln150. Interestingly, modelling had also revealed that Arg131 and possibly Lys76 could be responsible of this interaction [122]. The structure of PDK1 reveals that the PIF pocket is strikingly similar to the pockets in the active structures of PKA (Figure 8) and PKB [107], which bind their own C-terminal hydrophobic motifs. Thus the PDK1 study provides information on the structure of the ‘active’ conformation that AGC kinases may adopt upon binding of their phosphorylated hydrophobic motif to the pocket. The inactive conformation may be similar to that of a PKB mutant lacking the C-terminal hydrophobic motif [106].

Based on the different biochemical responses obtained with some PDK1 mutations, it is expected that the mode of interaction between PDK1 and each hydrophobic motif sequence of substrates will have specific characteristics. Moreover, it seems likely that each protein kinase substrate may have particular interactions with PDK1, besides its PIF pocket interaction site and the activation loop phosphorylation site (as described above). The specific and common interaction sites will only be fully revealed by resolving the structure of PDK1 bound to the different protein kinase substrates.

**Substrate docking sequences complement regions of the protein kinase catalytic core**

A revision of available structures of Ser/Thr protein kinases shows that regions similar to those used for docking substrates in MAPK, GSK3 and PDK1 are also complemented by regions outside of the catalytic core in other protein kinases.

The docking sequences from MKK3b and MEF2a bind p38α MAPK in a manner strikingly similar to the interactions found between the C-terminal regions of CDK2 and PAK (p21-activated kinase) 1 with its own catalytic cores (Figure 3). Interestingly, PAK1 and CDKs are phylogenetically close to the MAPK family. Moreover, there is also a partial overlap between part of the MAPK interaction site with docking sequences and the C-terminal extensions to the catalytic core of PKA, PDK1 and GSK3.

In GSK3, the phosphate docking site used by primed substrates is homologous with the activation loop phosphate-binding site in many other kinases (Figure 5) [131,132]. One of the α-helices within FRATide binds to GSK3 in a manner that is similar to the binding of a non-conserved α-helix originating from an insertion (equivalent to the MAPK insert) on the large lobe of the protein kinase CK2 catalytic domain (Figure 5).

Several protein kinases complement themselves in the equivalent site to the PIF-binding pocket in PDK1. For example, the N-terminal residue Trp10 complements an equivalent groove in calmodulin kinase-2. In MAPK family members, the site is complemented with C-terminal extensions to the catalytic core. Interestingly, the groove between α-C-helix and β-4–β-5, equivalent to the site used by PIF interaction with PDK1, was recently suggested as the site of interaction for a non-ATP competitive drug against the MAPKK MEK1 [136]. The CDK2–cyclin interaction region occupies a large area, which also includes the same groove between α-C-helix and β-4–β-5 (Figure 8). Binding of cyclins is known to be the first event in the activation of CDKs [137]. Interestingly, viral cyclins show specific interactions that are very different from the bona fide cyclins. However, one core region which binds between α-C-helix and β-4–β-5 is conserved [138].

In summary, structural analysis of protein kinases suggests that interactions of the catalytic core with N-terminal or C-terminal extensions may participate in the regulation of kinase activity. Alternatively, equivalent sites seem to be used for docking of substrates to protein kinases.

**CONCLUDING REMARKS**

Amino acid sequences surrounding the phospho-acceptor motifs have been extensively studied, and have shown to be important determinants for the substrate specificity of many protein kinases. In the last few years, however, it has become increasingly obvious that docking interactions, without any significant requirement for specific phosphorylation motifs, are a major mechanism for substrate recognition in Ser/Thr protein kinases, such as CDK, MAPK, GSK3 and PDK1. It is therefore to be expected that the importance of direct docking interactions may be recognized throughout the whole family of Ser/Thr protein kinases in the future.

In the docking interactions reviewed here, a linear sequence of amino acids are binding to the catalytic domain of a protein kinase. This may just reflect that ‘docking sequences’ are more easily identified than interactions where a tertiary structure site might be involved. Interestingly, docking interactions in PDK1 and GSK3 are regulated by phosphorylation. Docking interactions with MAPKs may be also regulated by phosphorylation, although the precise mechanisms involved remain to be elucidated. Thus the regulation of docking interactions by phosphorylation allows an important additional level of control to these signalling pathways.

The analysis of sites in kinase structures where docking sequences bind supports the idea that protein kinases may require ‘complementation’ of their catalytic domains for stability and/or activity. In some cases, interacting partners, such as substrates, may provide the complementary regions. Further work will be required to verify whether docking interactions could be used as targets for non-ATP competitive drugs against protein kinases. Compounds interfering with docking sites could potentially be useful as substrate selective protein kinase drugs.

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