Kinase phosphorylation: **Keeping it all in the family**

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The identification of PDK1 as a kinase that phosphorylates the AGC family of kinases led to a hunt for 'PDK2', a hypothetical regulated kinase(s) that would be required for full activation of the AGC kinases. Recent findings suggest that the elusive PDK2 may actually be a familiar kinase with an atypical associate.

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Current Biology 1999, 9:R521–R524
http://biomednet.com/elecref/09609822009R0521

Encounters with kinases are virtually unavoidable in many areas of biological study. Not only are kinases abundant (an estimated 1000 human genes encode kinases), but they participate in critical processes ranging from cell growth and division to memory. Frequently, kinases are themselves activated or inactivated through phosphorylation by other kinases, and intracellular signals often pass through several kinases in a linear phosphorylation cascade before reaching their destination. Recent studies [1–4] may have finally identified the kinases that control the phosphorylation and activation of the important 'AGC' family of kinases (so named because the family includes protein kinases A, G, and C). The kinases identified are surprisingly familiar, yet they appear to combine in unusual ways to control not only the phosphorylation of the AGC kinases, but their localization, substrate specificities, and responsiveness to incoming signals as well.

The structurally related AGC kinases phosphorylate their substrates at serine and threonine residues and participate in a variety of well-known signaling processes. For example, members of this family mediate cyclic AMP signaling, the response to insulin, apoptosis protection, diacylglycerol signaling, and control of protein translation (see Table 1). As well as having a high degree of sequence similarity in their catalytic domains, the AGC kinases share a number of features in the ways they are regulated. Many family members are stimulated by the production of the lipid second messenger phosphatidylinositol 3,4,5-trisphosphate (PIP_3), and a means of translocation to the cell membrane has been identified for most AGC kinases [5,6] (Table 1). In each case, membrane localization is an important step in kinase activation.

Another trait shared by the AGC kinases is that full kinase activity depends upon phosphorylation of their catalytic domain activation loops. Each family member has a highly conserved activation loop phosphorylation site within the consensus sequence T(F/L)CGT (in single-letter amino acid code). Several family members also possess a second conserved motif outside the catalytic domain that is approximately 160 amino acids carboxy-terminal to the activation loop phosphorylation site (Figure 1). In protein kinase B (PKB), the conventional and novel PKCs, and the 70 kDa S6 kinase ($p70^{S6K}$), this carboxy-terminal site

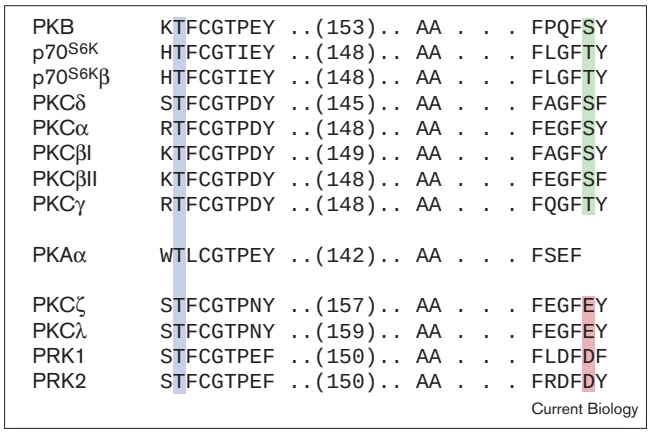
Table 1

Properties of several AGC kinases.

Kinase	Classification	Mechanism of membrane localization	Cellular processes mediated
PKA	cAMP-dependent protein kinase	Myristoylation?	cAMP signaling
PKB (c-Akt)	Protooncogene	Amino-terminal PH domain	Insulin signaling and apoptosis protection
PKC α,β,γ	Conventional PKCs	DAG	DAG and calcium signaling
PKC δ,ε	Novel PKCs	DAG	DAG signaling
PKC ζ,λ,ι	Atypical PKCs	PIP_3	PI 3-kinase signaling
PRK1,2	PKC-related kinases 1 and 2	PDK1 association?	Cytoskeletal organization
$p70^{S6K}$	Ribosomal subunit S6 kinase	Rac1/Cdc42 association	Translation of 5′ TOP mRNAs
PDK1	3-Phosphoinositide-dependent kinase	Carboxy-terminal PH domain	Phosphorylation of AGC kinase activation loops

DAG refers to diacylglycerol and TOP to terminal oligopyrimidine tract.

Figure 1



Alignment of the conserved sequences surrounding the kinase domain activation loop and the carboxy-terminal phosphorylation sites of several AGC kinases. The number of amino acid residues between the activation loop and the carboxy-terminal phosphorylation site are indicated for each kinase. The activation loop phosphorylation sites are phosphorylated by PDK1 and are highlighted in blue. The carboxy-terminal phosphorylation sites, termed PDK2 sites, are highlighted in green. The pseudosubstrate sites present in the atypical PKCs and the PKC-related kinases are highlighted in red. PKAα does not contain a conserved carboxy-terminal PDK2 site or pseudosubstrate site.

has the consensus sequence FXXF(S/T)(Y/F) — where X indicates a non-conserved residue — and phosphorylation at this site is required for protein stability and/or kinase activity [5]. The atypical PKCs (PKCζ and PKCλ/ι) and the PKC-related kinases PRK1 and PRK2 possess the same sequence except that an aspartate or glutamate residue replaces the serine or threonine residue; presumably the negatively charged aspartate or glutamate mimics the presence of phosphate at this site. With few exceptions, phosphorylation of both the activation loop site and the carboxy-terminal site (when present) are critical for full activation of the AGC kinases [5].

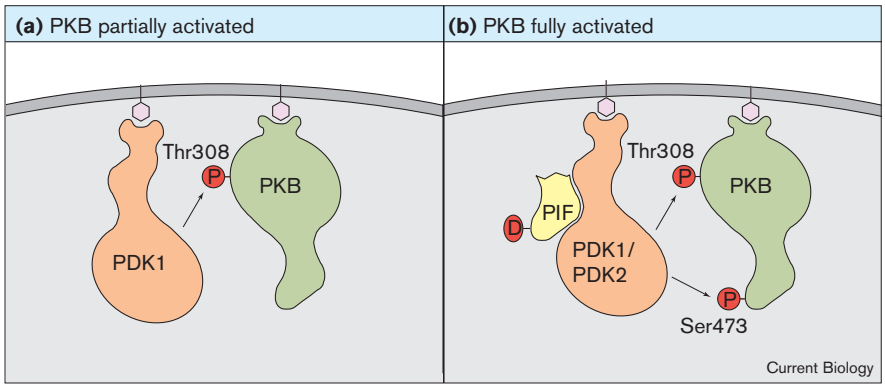
A kinase capable of phosphorylating the activation loop site of PKB in a PIP_3-dependent manner was identified less than two years ago [7,8]. Cloning and sequencing of the kinase revealed a novel protein with a familiar profile. This activation loop kinase, called 3-phosphoinositide-dependent kinase 1 (PDK1) is itself related to the AGC kinases. It has the characteristic activation loop consensus sequence and a carboxy-terminal pleckstrin homology (PH) domain that mediates PIP_3-dependent membrane localization. PDK1 has since been shown to have broad substrate specificity for the AGC kinase activation loops, phosphorylating PKA, PKB, PKCβII, PKCδ, PKCζ, and $p70^{S6K}$ *in vitro* and promoting the activation loop phosphorylation of several of these kinases upon coexpression *in vivo* [5].

Strikingly, PDK1 kinase activity appears to be constitutive. Mitogenic stimuli do not affect PDK1 kinase activity [7], nor does rapamycin, a small molecule that induces dephosphorylation of the $p70^{S6K}$ activation loop [9]. Even 3-phosphoinositides do not alter intrinsic PDK1 activity; the observed dependence of PDK1 on PIP_3 appears merely to reflect an ability of PIP_3 to increase the access of PDK1 to its substrates — both by colocalizing kinase and substrate at the membrane and, in the case of PKB, by displacing the inhibitory PKB PH domain [7]. Because PDK1 kinase activity is constitutive, activation loop phosphorylation of the AGC kinases is likely to depend upon the control of kinase localization, regulated dephosphorylation, or regulated phosphorylation of the carboxy-terminal phosphorylation sites. Several attempts have been made to identify the kinase(s) responsible for phosphorylation of the AGC kinase carboxy-terminal sites, and in anticipation of its identification, the predicted kinase was provisionally named 'PDK2'. For simplicity, we will therefore refer to the carboxy-terminal sites in the AGC kinases as PDK2 sites.

A number of candidate 'PDK2s' have been reported recently. Purified integrin-linked kinase (ILK), an ankyrin-repeat-containing serine/threonine kinase, has been shown to catalyze phosphorylation of the PDK2 site (Ser473) of PKB *in vitro*, whereas overexpression of a kinase-inactive ILK diminished phosphorylation of the Ser473 residue *in vivo* [10]. The analogous PDK2 site in $p70^{S6K}$, Thr389, becomes dephosphorylated upon rapamycin treatment [11] and is regulated by the rapamycin target FRAP (also called RAFT or mTOR), a member of the phosphatidylinositol kinase (PIK)-related kinase family [12]. FRAP has been shown to phosphorylate a bacterially expressed fragment of $p70^{S6K}$ on Thr389 *in vitro* [13]; this fragment is not responsive to FRAP regulation *in vivo*, however, and direct phosphorylation of full-length $p70^{S6K}$ has not been demonstrated. The extent to which ILK and FRAP phosphorylate the PDK2 sites of PKB and $p70^{S6K}$ *in vivo* remains unclear.

Four recent reports suggest that phosphorylation of AGC kinase PDK2 sites, like phosphorylation of their activation loop sites, is under the control of other AGC kinase family members [1–4]. Phosphorylation of the kinases containing PDK2 motifs relies upon those family members that are 'activated' at that site constitutively because a glutamate mimics the phosphorylated serine or threonine. For example, $p70^{S6K}$ and PKCδ appear to become phosphorylated at their FXXF(**S/T**)Y motif only in the presence of the atypical PKCs PKCλ or PKCζ, which possess the pseudosubstrate PDK2 motif FXXF**E**Y [1–3]. Overexpression of kinase-inactive PKCλ or PKCζ blocks activation of $p70^{S6K}$ induced by serum or epidermal growth factor [2,3]. Similarly, overexpression of kinase-inactive PKCζ suppresses serum-induced phosphorylation of the PDK2 site of PKCδ, Ser662 [1]. Involvement of the atypical PKCs in phosphorylation of PKCδ is further supported by the observation that expression of an activated PKCζ

Figure 2



Conversion of PDK1 to 'PDK2'. **(a)** PIP_3 production causes PDK1 and PKB to colocalize at the cell membrane, where PDK1 phosphorylates PKB at the activation loop site, Thr308 (P). Phosphorylation at this site results in partial activation of PKB. **(b)** Full activation of PKB also requires phosphorylation of its carboxy-terminal Ser473. PDK1 can phosphorylate this carboxy-terminal site as well, but only when complexed to the fragment of PRK2 called PIF that contains a phosphate-mimicking aspartate (D). The pink hexagon represents PIP_3.

causes hyperphosphorylation of the PKCδ Ser662 residue [1]. Activated alleles of PKCζ and PKCλ activate $p70^{S6K}$ modestly [2] or not at all [3] by themselves, but an activated PKCζ is able to activate $p70^{S6K}$ when coexpressed with PDK1 [2]. Thus, it appears that atypical PKCs are necessary but not sufficient for activation of $p70^{S6K}$ and may cooperate with PDK1 to activate $p70^{S6K}$ fully.

How do the atypical PKCs affect phosphorylation at the PDK2 sites of PKCδ and $p70^{S6K}$? One simple explanation is that the atypical PKCs may phosphorylate PKCδ and $p70^{S6K}$ directly, and indeed activated PKCζ immune complexes promote phosphorylation of PKCδ Ser662 *in vitro* [1]. This phosphorylation has not been shown to be direct, however, and others have been unsuccessful at detecting direct phosphorylation of $p70^{S6K}$ by the atypical PKCs [3]. Another possibility is that atypical PKCs allow autophosphorylation of AGC kinase PDK2 sites. Precedent for PDK2 site autophosphorylation exists: PKCβII can catalyze phosphorylation of its own PDK2 site *in vitro*, and kinase-inactive PKCβII mutants exhibit defective PDK2 site phosphorylation *in vivo* [14]. Further experimentation is required to determine whether direct phosphorylation by atypical PKCs or autophosphorylation contribute significantly to PDK2 site phosphorylation during activation of PKCδ and $p70^{S6K}$.

It is also possible that the atypical PKCs regulate PKCδ and $p70^{S6K}$ indirectly by regulating a separate AGC kinase kinase. A similar mechanism was recently proposed by Balendran *et al.* [4] who examined the role of PRK2 in phosphorylation of the PDK2 site on PKB. Like the atypical PKCs, PRK2 has a PDK2 pseudosubstrate motif (Figure 1) and promotes phosphorylation at the PDK2 site of PKB. However, PRK2 does not directly phosphorylate PKB. Instead, it appears to interact with PDK1 via its PDK2 pseudosubstrate motif, termed the PDK1-interacting fragment (PIF). When this fragment of PRK2 interacts with PDK1, it converts PDK1 from a kinase with activation loop specificity to a kinase capable of phosphorylating both the activation loop and the PDK2 site of PKB (Figure 2). PIF binding also converts PDK1 from an enzyme that is not directly activated by PIP_3 to one that is activated by PIP_3. PRK2 kinase activity is not required for binding to PDK1 or for *in vitro* phosphorylation of PKB — even synthetic peptides from PIF can confer PDK2 activity upon PDK1, whereas mutation of any of the conserved residues in the PIF pseudosubstrate greatly abrogates binding to PDK1 and phosphorylation of the PKB Ser473 residue. By analogy, it seems possible that the pseudosubstrate sites of PKCζ and PKCλ influence phosphorylation of PKCδ and $p70^{S6K}$ by interacting with PDK1 (or a related kinase) and conferring upon it specificity for the PKCδ and $p70^{S6K}$ PDK2 sites. Such a mechanism is consistent with the observations that PDK1 and PKCζ interact and that coexpression of PDK1 and constitutively active PKCζ can activate $p70^{S6K}$ more potently than expression of either PDK1 or constitutively active PKCζ alone [2].

The role of the atypical PKCs and the PRKs in regulation of AGC kinases may extend beyond the conversion of PDK1 to PDK2 — they may also affect AGC kinase localization. A striking number of associations between family members have been observed by coimmunoprecipitation. PDK1 has been reported to associate with PRK2, PKCα, PKCβI, PKCδ, PKCε, PKCι, PKCζ, and $p70^{S6K}$ [2,4,15]. In addition, $p70^{S6K}$ associates with PKCζ and PKCλ [2,3], and an association between PKB and PKCζ has also been observed [16]. In general, the interactions appear to be stable, independent of kinase activity, and mediated by regions both within and outside the kinase catalytic domains. It appears likely that several family members congregate at the membrane in large, multimeric complexes and perhaps even exhibit cooperativity in responding to PIP_3 or other membrane-associated signals.

Several questions remain unanswered. How does binding of PIF, a PDK2 motif pseudosubstrate, confer PDK2

activity upon PDK1, and how does this interaction render PDK2 activity sensitive to the presence of PIP_3? Does a similar interaction govern phosphorylation of the PDK2 site of other AGC kinases such as $p70^{S6K}$ and PKCδ? What effects do the many other intra-family associations have on AGC kinase substrate specificity, activity, and localization? What role do the kinase activities of PRK2 and the atypical PKCs play in phosphorylation of the PDK2 site? The answers to these questions are likely to provide insight into the function of several, if not all, members of this diverse family of kinases. Indeed, these recent findings about the nature of PDK2 motif phosphorylation serve as an excellent illustration of how protein phosphorylation depends upon much more than just the inherent substrate specificity of a kinase as measured *in vitro*. Furthermore, the findings demonstrate that a linear phosphorylation cascade cannot adequately describe the function of many kinase signaling pathways. In the end, characterization of PDK2 may do more than provide a missing link in a chain of phosphorylations — it may tell us something about the fundamental nature of kinases.

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