AGC protein kinase phosphorylation and protein kinase C
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Abstract
Protein kinase cascades feature in many signal transduction pathways. For those discussed here, a single upstream protein kinase appears to be responsible for the control of multiple downstream targets. So how is specificity introduced into these events? For the downstream kinases (substrates) described here, it would appear that specificity is determined by substrate-directed events that are permissive for phosphorylation. There are also distinctions relating to the turnover of these phosphorylations providing a further element of specificity.

Introduction
The AGC [cAMP-dependent protein kinase (PKA)/protein kinase G/protein kinase C (PKC)] protein kinase extended family embraces a collection of protein kinase families that display a high degree of primary sequence conservation within their respective kinase domains (see The Protein Kinase Resource; http://www.sdsc.edu/Kinases). Outside these catalytic domains, the AGC protein kinases generally show little similarity. This lack of regulatory domain conservation reflects the very distinct controls that operate on these proteins. These range from the classical cAMP-induced dissociation of regulatory catalytic subunits for PKA (recently reviewed in [1]), to the acute phosphorylation of protein kinase B (PKB) within its activation loop, at Thr308, by phosphatidylinositol 3,4,5-triphosphate (PtdIns(3,4,5)P3)-dependent protein kinase 1 (PDK1) [2].

Phosphorylation of activation loops is a characteristic activation step for many different protein kinases. The basis for this is manifested in the determined X-ray structures. For example, the dual phosphorylation of extracellular responsive kinase (ERK) 2 on its Thr-Glu-Tyr motif stabilizes a conformation of the kinase with an appropriately aligned substrate-binding site and phosphotransfer catalytic centre [3]. For the AGC kinases, the structure of PKA is compatible with a similar role for Thr197 phosphorylation in its activation loop [4]; mutagenesis supports this contention [5]. Similarly, for other AGC kinases, structure prediction and mutational analysis indicate a common underlying need for activation loop phosphorylation to achieve optimal activity. Among the AGC kinases, different strategies are employed to effect these phosphorylations, reflecting the underlying distinctions in control. Here, we exemplify the types of mechanism that are employed for these phosphorylations in AGC protein kinases. To illustrate one other dimension to these mechanisms, a discussion of the AGC protein kinase C subfamily follows.

AGC protein kinase activation-loop phosphorylation
Despite a high degree of sequence conservation within this protein kinase extended family, there appear to be two classes of phosphorylation events associated with activation-loop phosphorylations. The first involves autophosphorylation, while the second appears to be dependent on PDK1 (see below).

The autophosphorylation of an activation-loop site is exemplified by the cAMP-dependent protein kinase (PKA) family. The catalytic subunit of PKAα, when expressed in bacteria, becomes phosphorylated at its Thr286 activation-loop site (as well as at other sites), indicative of the capacity for autophosphorylation [6]. Furthermore, despite an ability of PDK1 to phosphorylate this site in vitro [7], embryonic stem cells that have lost both PDK1 alleles retain a phosphorylated, active PKA [8]. It would appear, therefore, that under normal growth conditions, autophosphorylation is sufficient for PKA activation-loop phosphorylation. Once phosphorylated, the heterotetramer of the holoenzyme [RαCγ (R, regulatory subunit; C, catalytic subunit)] is primed for

Key words: autophosphorylation, input/signal, permissive, phosphoinositide, signal transduction.
Abbreviations used: AGC, cAMP-dependent protein kinase/protein kinase G/protein kinase C extended family; DAG, diacylglycerol; ERK, extracellular responsive kinase; PH, pleckstrin homology; PI-3K, phosphoinositide 3-kinase; PKA, protein kinase A; PKB, protein kinase B; PKC, protein kinase C; PKG, protein kinase G; PDK1, phosphatidylinositol 3,4,5-triphosphate-dependent protein kinase 1; PRK, PKC-related kinase; PtdIns(3,4,5)P3, phosphatidylinositol 3,4,5-triphosphate.
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its acute response to the elevation in cAMP levels that causes dissociation of the complex and release of fully active C subunits.

In contrast with PKA, the PKB family (α, β, γ; also referred to as Akt 1–3) does not autophosphorylate on its activation-loop site, Thr308. In elucidating the control of this AGC protein kinase family, two groups independently identified PDK1 [9, 10]. This enzyme phosphorylates PKB on its activation loop site. Both PKB (at the N-terminus) and PDK1 (C-terminus) have pleckstrin homology (PH) domains. These have been shown to bind PtdIns(3,4,5)P₃, although the PDK1 PH domain has a greater affinity than PKB domain [10]. The process of PDK1 phosphorylation of PKB is most efficiently achieved in vitro through their co-recruitment to membranes/liposomes containing PtdIns(3,4,5)P₃. The activation of the phosphoinositide 3-kinase (PI-3K) pathway, which is responsible for PtdIns(3,4,5)P₃ production, serves to trigger PKB phosphorylation in intact cells and, consistent with this, inhibitors of PI-3K block PKB phosphorylation [9, 10]. It is surmised that the co-recruitment of PDK1 and PKB underlies the latter’s phosphorylation.

A related pattern of behaviour has been reported for PKC, in which bacterially-expressed PKCδ can be phosphorylated on its activation-loop site (Thr505) by PDK1 [11]. It is notable that PKCδ does not autophosphorylate at this site when expressed in bacteria [11, 12]. In contrast with PKB, PKCδ phosphorylation by PDK1 is optimally achieved through co-recruitment to membranes/liposomes via its own allosteric activator [diacylglycerol (DAG)], in combination with PtdIns(3,4,5)P₃ [11]. Thus, despite a common requirement for PtdIns(3,4,5)P₃ and PDK1, PKB and PKC display specificity at the level of their own selective effectors.

**Figure 1**

Conditional inputs to substrates of PDK1

The protein kinases PKB, p70S6k, p90RSK and PKC are substrates for PDK1. All are phosphorylated within their protein kinase domains. The specificity of action of PDK1 in effecting these phosphorylations is governed by conditional inputs that determine selectivity for the different downstream targets. These inputs include second messengers [PtdIns(3,4,5)P₃, Ca²⁺ and DAG] and protein kinases (ERK) as illustrated (see text).
This theme is developed further in the case of two other AGC protein kinases, p70^S6kinase and p90^rsk. For p70^S6kinase, the nature of the permissive inputs remain to be elucidated completely, however there is evidence that its phosphorylation in its extended C-terminal domain may, in part, serve such a permissive role [13]. This is more clearly the case for p90^rsk. This protein contains two tandem kinase domains. The activation, via the mitogen-activated protein kinase cascade, of the C-terminal kinase domain leads to phosphorylation of the linker region, the subsequent binding of PDK1 and then phosphorylation of the N-terminal kinase domain at its activation-loop site [14].

In general, the phosphorylation of AGC protein kinases by PDK1 is a common, but non-exclusive, event, the specificity of its action being dictated by the idiosyncrasies of the control of the target protein kinase (Figure 1).

Before considering the PKC family in more detail, it should be made clear that, for all of these PDK-1 substrates, there is a second hydrophobic phosphorylation site closer to the C-terminus [a Phe-Xaa-Xaa-Phe-(Ser/Thr)-(Phe/Tyr) motif], which also contributes to activation and/or stabilization of the active conformer [15]. The mechanism(s) of phosphorylation of these sites are unclear with both trans- and auto-phosphorylation events being proposed. Interestingly however, this region can act as a binding site for PDK1 [16], contributing to the ordered mechanism of p90^rsk phosphorylation (see above). In the case of the PKC-related kinase (PRK) family, the equivalent region has a Phe-Xaa-Xaa-Phe-Asp-Tyr motif and this appears to be sufficient for PDK1 binding [16]. For PRK1 and PRK2, allosteric activation through a GTP-bound Rho family member enables assembly of a PRK-PDK1 complex [17]. It is evident that it will be important to establish the controls acting upon these hydrophobic sites, in order to provide a fuller insight into AGC protein kinase control.

**PKC and the fourth dimension**

In stark contrast with some of the kinases discussed above (PKB, p70^S6kinase and p90^rsk), which all display acute agonist-induced phosphorylation, classical and novel isotypes of PKC (cPKC, nPKC) usually display a high degree of phosphorylation within their activation loops (i.e. PDK1 sites) under normal cell-culture conditions. It is also the case that, despite inhibition of the upstream pathways leading to c/nPKC phosphorylation, their dephosphorylation is usually slow and, indeed, it is most readily detected under conditions of chronic activation [18]. This contrasts with PKB where treatment with a PI-3K inhibitor rapidly (in periods of seconds or minutes) reverses an agonist-induced phosphorylation [19]. These distinctive properties of PKC have some interesting implications. Under unfavourable conditions for net PDK-1 phosphorylation of PKC (e.g. a fibroblast in a quiescent state, or the acute presence of a PI-3K inhibitor), PKC can still be phosphorylated by this same kinase. Presumably, the concentration of PtdIns(3,4,5)P3 is adequate for PDK1 (transient) recruitment to a DAG-containing compartment, but insufficient to recruit the lower affinity PH domain of PKB. It can be surmised also that, even without an acute stimulus, one or other PI-3K pathway displays a sufficient constitutive function to support this basal level of PtdIns(3,4,5)P3. This, presumably, operates in synergy with the apparently distinct protein phosphatase sensitivities of PKB and PKC.

The most intriguing feature of this behaviour of c/nPKC, is that its response to agonists not only integrates the signalling information flow from more than one pathway, but that it does so over time. Thus, while PI-3K may have exerted appropriate activity many hours before the arrival of an agonist that acts solely via DAG on PKC, a full response may ensue, since PKC remains highly phosphorylated and is, therefore, primed for action.

Do cells exploit this 'stored' information? In principle, this is a simple form of signalling memory and could be used in this way, for example to modify synaptic transmission. However, the behaviour of the system perhaps lends itself more readily to act as a signalling buffer, where, for example, environmental changes influencing (basal) PI-3K activity would not lead to an acute loss of PKC responsiveness, but rather a slow, gradual decline. Such behaviour may protect cells from short-term privations.

**References**


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Abstract
The Tec kinases have been implicated as important components of signalling pathways downstream of lymphocyte antigen receptors. Activation of these kinases requires two steps: (i) phosphorylation by Src family kinases and (ii) plasma membrane localization, which is mediated by interaction between the pleckstrin homology (PH) domains of Tec kinases and the products of phosphoinositide-3 kinase (PI-3K). Itk and Rlk/Txk are Tec kinases expressed in T-lymphocytes. Despite similarity to other Tec kinases, Rlk/Txk lacks a PH domain and instead possesses a palmitoylated cysteine-string motif. We have found that both Rlk/Txk and Itk are phosphorylated in response to T-cell receptor stimulation and can be activated by phosphorylation by Src family kinases. However, consistent with its lack of PH domain, Rlk/Txk is phosphorylated independent of PI-3K activity. Furthermore, we demonstrated that like Itk, Rlk/Txk is associated with lipid RAFTs (detergent-insoluble, cholesterol-rich regions of the membrane), but unlike Itk, Rlk/Txk's RAFT association is independent of PI-3K activity. Despite these differences, Rlk/Txk partially compensates for loss of Itk in gene-targeted animals, suggesting overlapping functions for these kinases.

Introduction
The Tec family of non-receptor tyrosine kinases first emerged as important components of lymphocyte signalling in 1993, when several groups found that mutations affecting Btk, a member of this family, were the cause of the human primary immunodeficiency X-linked agammaglobulinemia, and a similar disorder in mice, X-linked immunodeficiency [1,2]. These disorders are characterized by variable defects in B-cell development associated with reduced serum immunoglobulin levels and impaired B-cell signalling. These findings provided the first example of mutations affecting a non-receptor tyrosine kinase causing a human immunodeficiency and paved the way for defining the Tec kinases as important players in antigen receptor signalling.

Biochemical and genetic analyses of the Tec kinases Itk and Rlk/Txk
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Key words: phosphatidylinositol 3-kinase, RAFTs, T lymphocytes, tyrosine kinase.
Abbreviations used: PH, pleckstrin homology; PI-3K, phosphoinositide-3 kinase; SH, Src homology; TCR, T-cell receptor.
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Received 13 July 2001