PDK1 catalyzes phosphorylation of Thr in the conserved activation loop region of a number of its downstream AGC kinase family members. In addition to the consensus sequence at the site of phosphorylation, a number of PDK1 substrates contain a PIF sequence (PDK1-interacting fragment), which binds and activates the kinase domain of PDK1 (PDK1(ΔPH)). To gain further insight to PIF-dependent catalysis, steady-state kinetic and inhibition studies were performed for His9-PDK1(ΔPH)-catalyzed phosphorylation of PDK1-Tide (Tide), which contains an extended “PIF” sequence C-terminal to the consensus sequence for PDK1 phosphorylation. In two-substrate kinetics, a large degree of negative binding synergy was observed to occur on formation of the active ternary complex (αKdTP = 40 μM and αKdTide = 80 μM) from individual transitory binary complexes (KdATP = 0.6 μM and KdTide = 1 μM). On varying ATP concentrations, the ADP product and the (T/E)-PDK1-Tide product analog (p’Tide) behaved as competitive and noncompetitive inhibitors, respectively; on varying Tide concentrations, ADP and p’Tide behaved as noncompetitive and competitive inhibitors, respectively. Also, negative binding synergy was associated with formation of dead-end inhibited ternary complexes. Time progress curves in pre-steady-state studies under “saturating” or kcat conditions showed (i) no burst or lag phenomena, (ii) no change in reaction velocity when adenosine 5’-O-(thiotriphosphate) was used as a phosphate donor, and (iii) no change in reaction velocity on increasing relative microviscosity (0 ≤ η/η0 ≤ 3). Taken together, PDK1-catalyzed trans-phosphorylation of PDK1-Tide approximates a Rapid Equilibrium Random Bi Bi system, where motions in the central ternary complex are largely rate-determining.

Phosphoinositide-dependent protein kinase-1 (PDK1)2 is a member of the AGC subfamily of serine-threonine protein kinases, which includes different isoforms of cAMP-dependent protein kinase (PKA), protein kinase B (PKB), Ca2+-activated protein kinase (PKC), protein kinase G, 70-kDa 40 S ribosomal protein S6 kinase (S6K), 90-kDa 40 S ribosomal protein S6 kinase (RSK), serum- and glucocorticoid-induced protein kinase (SGK), and mitogen- and stress-activated protein kinase (1). Among these kinases, amino acid sequences are conserved in a segment of the kinase domain known as the activation loop or T-loop, as well as in a segment C-terminal to the kinase domain known as the hydrophobic motif; and phosphorylation sites or acidic residues in these regions play important roles in their catalytic regulation and/or stability. PDK1 has been termed the “master kinase” (2) in that it has been shown to phosphorylate the critical residue in the activation loops of AGC kinase family members (Fig. 1A) including PKBα (3–6), PKBβ(7), PKBγ (7), SGK1 (8), SGK2 (9), SGK3 (9, 10), S6K (10, 11), PKA (12), PKCα (13), PKCβII (13), PKCδ (14), PKCε (14, 15), RSK (16, 17), and protein kinase N (18, 19). Whereas the activation loop residue appears to be constitutively phosphorylated in PKA, PKCα, and PKCβII, PDK1-catalyzed phosphorylation of PKB, SGK, S6K, RSK, PKCδ, and PKCε occurs in response to cellular agonists acting through a common phosphatidylinositol-3-kinase pathway, albeit with differing temporal regulation (20–23).

The common ability of PDK1 to catalyze phosphorylation of the activation loop of its numerous protein targets, but with varying degrees and mechanisms of regulation, has been explained from the intriguing perspective that PDK1 has the ability to “sense the conformation” of many of its substrates (24). PDK1 and other AGC kinases possess a “PIF (PDK1-interacting fragment) pocket” on the catalytic kinase domain (25, 26). In contrast to other AGC kinases, PDK1 does not possess a hydrophobic motif C-terminal to its catalytic domain. Therefore, the PIF pocket of PDK1 is accessible for interaction with the phosphorylated hydrophobic motifs of its target kinases. Such intermolecular interaction promotes activation of PDK1, enabling PDK1-catalyzed phosphorylation of target kinases at the activation loop. Upon phosphorylation of the activation loop, the phosphorylated C-terminal hydrophobic motif of the target kinase is released from the PIF pocket on PDK1, and it forms an intramolecular interaction with its own PIF pocket, which fully stabilizes an active conformation (24). Both in vivo
and in vitro studies indicated the important role of intermolecular PIF interactions in activating PDK1-catalyzed phosphorylation of S6K, RSK, and SGK (27–29).

To further investigate the role of PIF interactions, a model “PIF-Tide” was synthesized to contain the C-terminal hydrophobic motif region of protein kinase C-related kinase-2. The PIF region in protein kinase C-related kinase-2 contains high sequence homology to the PIF regions of PDK1 protein substrates, except that the Ser/Thr phosphorylation site is replaced by a negatively charged Asp residue (Fig. 1B) (25). By conjugating the PIF-Tide to the C terminus of T308-Tide, the PDK1-Tide was generated (Fig. 1B) (25). PKB phosphorylation of the small T308-Tide, which represents the consensus motif near the site of phosphorylation in PKBo (Fig. 1B) (25). By conjugating the PIF-Tide to the C terminus of T308-Tide, the PDK1-Tide was generated (Fig. 1B) (25). PDK1-Tide remains the prevailing model PDK1 substrate, as it undergoes phosphorylation at a rate 2–10-fold than T308-Tide, and its Km values yielded lower than the Km of T308-Tide.

Whereas x-ray structural and mutagenesis studies have clearly defined PIF pocket residues important to catalytic activation of PDK1 (25, 26), a detailed kinetic study of this process awaits description. Here, we report kinetic and chemical/solution perturbation studies aimed to establish the steady-state kinetic mechanism for PDK1-catalyzed trans-phosphorylation of PDK1-Tide. The results of these studies are best approximated a Rapid Equilibrium Random Bi Bi system, where conformational steps in the central ternary complex are largely rate-determining. Most interesting was the apparently large degree of negative binding synergism exhibited between the peptide and nucleotide substrates/products. Such synergism may derive from conformational transitions important to catalytic turnover.

**EXPERIMENTAL PROCEDURES**

**Materials and General Methods—**The catalytic kinase domain of PDK1 (His6-PDK1(ΔPH), residues 51–359) (26), containing an N-terminal His6 tag followed by a PreScission protease recognition sequence prior to residue 51, was expressed using the Bac-to-Bac® Baculovirus Expression System (Invitrogen) and His6 affinity purified as described (31). Protein concentration was estimated using the Bio-Rad Protein Assay Kit with bovine serum albumin as a standard. PDK1-Tide and (T/E)-PDK1-Tide were from 21st Century Biochemicals, Inc. (Marlboro, MA). [γ-32P]ATP and [γ-35S]ATP were from MP Biomedical (Irvine, CA). All other chemicals, salts, and buffers were from Sigma.

**Two-substrate Steady-state Kinetic Assays—**Steady-state kinetic assays were carried out for His6-PDK1(ΔPH)-catalyzed trans-phosphorylation of the PDK1-Tide model peptide substrate (Fig. 1B). The 65-μl peptide phosphorylation reactions were performed at 30 °C in reaction buffer containing final concentrations of 50 mM Tris-HCl buffer, pH 7.5, 0.1% 2-mercaptoethanol, 10 mM MgCl2, and 0.2 mM sodium vanadate. Initial velocities were measured for varying PDK1-Tide concentrations (0.3, 1, 3, 10, 30, 100, and 300 μM) at different fixed concentrations of [γ-32P]ATP (~500 cpmpmol; 0.3, 1, 3, 10, 30, 100, and 300 μM). Data collection in this manner provided all information necessary to construct companion reciprocal plots for the two varied substrates.

After 5 min preincubation of a 60-μl reaction mixture containing both substrates at 30 °C, the assays were initiated by addition of a 5-μl amount of a stock concentration of His6-PDK1(ΔPH) to yield final enzyme concentrations of 5–30 nM. For kinase assays where both the ATP and PDK1-Tide concentrations were ≥3 μM, 20-μl aliquots were removed and quenched at three different times (ranging from 5 to 60 min). For kinase assays where either the ATP or PDK1-Tide concentration was ≤1 μM, it was necessary to extend some reaction times up to 3 h to obtain the minimal detectable amounts of 32P-radiolabeled PDK1-Tide (300–600 cpm) in a 20-μl reaction aliquot (~0.6–1.2 pmol or 30–60 nm). Each 20-μl reaction aliquot was quenched by mixing with 20 μl of 50 mM phosphoric acid, which was then applied to P81 phosphocellulose paper (2 × 2 cm). After 30 s, the papers were washed in 50 mM phosphoric acid for 10 min, then rinsed with acetone, and placed in the hood (≤5 min) to dry. The amount of 32P-labeled peptide was determined by scintillation counting of the paper in 10 ml of scintillation mixture.

Initial rates for reactions containing ≥1 μM of both substrates were measured under conditions where total product formation represented ≤10% of the initial concentration of the limiting substrate. Due to detection limits, initial rates for reactions containing 0.3 μM of either substrate were measured under conditions where total product formation (30–60 nm) represented 10–20% of the initial limiting substrate concentration. Because it was often necessary to use 30 nm enzyme to catalyze 30–60 nm product under these limiting conditions, it is important to point out these initial velocities were obtained under pre-steady-state conditions. However, the initial velocities obtained under these conditions approximate those obtained under true steady-state conditions, as demonstrated by more rigorous pre-steady-state experiments described later in this paper, which provide no evidence of burst or lag phenomena. Initial velocities (v, μmol.min−1) were normalized to enzyme concentration to yield apparent first-order rate constants (k, min−1), which better facilitate kinetic comparisons between steady-state and pre-steady-state kinetic results.

Control assays were carried out in which either the enzyme or PDK1-Tide were omitted; these values were always ≤5% of the activity measured in the presence of both the lower- and upper-bound concentrations of these reagents (0.3 and 300 μM [γ-32P]ATP; 5 and 30 nm enzyme). Control assays containing the enzyme and only the [γ-32P]ATP substrate were further analyzed to measure ATPase activity. The amount of [32P] inorganic phosphate released from [γ-32P]ATP was determined by addition of 50 μl of the reaction mixture to 100 μl of a quench solution containing a 21% suspension of acid-washed (HCl) activated charcoal in 75 mM phosphoric acid. The quenched solution was mixed and placed on ice for 5 min, which provided for effective removal of the [γ-32P]ATP nucleotide from solution by the charcoal. The charcoal with bound [γ-32P]ATP was
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pelleted by centrifugation for 10 min, and a 60-μl aliquot of the supernatant was analyzed for [32P]inorganic phosphate. 32P radioactivity in the supernatant was always ≤5% of the radioactivity measured in the absence of enzyme, indicating that the ATPase activity of His\textsubscript{6}-PDK1(ΔPH) is significantly lower than activity to the PDK1-Tide substrate.

Product and Dead-end Inhibition Steady-state Kinetic Assays—Diagnostic enzyme inhibition studies were carried out for His\textsubscript{6}-PDK1(ΔPH)-catalyzed transphosphorylation of PDK1-Tide exactly as described for two-substrate kinetics. ADP was tested as a product inhibitor and a phospho-PDK1-Tide mimetic in which the number 2 Thr residue that undergoes phosphorylation was replaced by a Glu residue ((T/E)-PDK1-Tide, Fig. 1B) was used as a dead-end product analog inhibitor. In both cases, initial velocities were measured for varying substrate concentrations (0.3, 1, 3, 10, 30, 100, and 300 μM) at different fixed concentrations of the inhibitor (0, 10, 30, 50, 70, and 100 μM) at an unsaturating concentration of the other substrate (30 μM). Data collection in this manner provided information necessary to construct companion plots for “diagnostic” analysis of the effect of either the ADP or (T/E)-Tide inhibitors on the two different varied substrates.

In contrast to the diagnostic inhibition studies, a full array of data sets was collected for product inhibition with ADP. Initial velocities were measured for varying PDK1-Tide concentrations (0.3, 1, 3, 10, 30, 100, and 300 μM) at different fixed concentrations of ADP (0, 1, 3, 10, 30, and 100 μM). In this case, ADP product inhibition was also measured at different fixed concentrations of [γ\textsuperscript{32P}]ATP (0.3, 1, 3, 10, 30, 100, and 300 μM). Data collection in this manner provided all information necessary to construct companion plots for complete kinetic analysis of the effect of ADP on the two different varied substrates.

Pre-steady-state Kinetic Assays—Pre-steady-state kinetic assays were carried out for His\textsubscript{6}-PDK1(ΔPH)-catalyzed transphosphorylation of PDK1-Tide using a KinTek Corporation (Austin, TX) model RQF-3 rapid quench-flow apparatus thermostatted at 30 °C. Reaction buffer refers to 50 mM Tris-HCl buffer, pH 7.5, 0.1% 2-mercaptoethanol, 10 mM MgCl\textsubscript{2}, and 0.2 mM sodium vanadate. The left and right drive syringes in the RQF apparatus were filled with reaction buffer, and the middle (quench) syringe was filled with 75 mM phosphoric acid. One sample loop was loaded with 15 μl of reaction buffer containing 600 μM of both PDK1-Tide and [γ\textsuperscript{32P}]ATP; and the other sample loop contained 15 μl of varying concentrations of His\textsubscript{6}-PDK1(ΔPH) enzyme (2, 6, and 10 μM) in reaction buffer. Thiostabilization effects were determined by equivalent substitution of [γ\textsuperscript{35S}]ATPγS for [γ\textsuperscript{32P}]ATP. The phosphorylation reaction was initiated by mixing the contents of the sample loops that was allowed to react for a time \( t \) (0.05 ≤ \( t \) ≤ 10 s) before the reaction was acid quenched. For each time point, two 20-μl aliquots of each quenched reaction solution were individually applied to P81 phosphocellulose paper (2 × 2 cm), and the amount of PDK1-Tide phosphorylation was quantified as described for the steady-state kinetic assays. The time courses were performed in triplicate and the data points represent averaged values ± S.E.

Control assays were carried out in which the PDK1-Tide substrate was omitted for each enzyme concentration in the presence of either [γ\textsuperscript{32P}]ATP or [γ\textsuperscript{35S}]ATPγS. The amounts of 32P or 35S radioactivity detected on the filter paper were always ≤5% of the measured radioactivity in the presence of PDK1-Tide, indicating that purified active pS241 His\textsubscript{6}-PDK1(ΔPH) catalyzes little or no nonspecific autophosphorylation during reaction times ≤10 s. In addition, the amounts of 32P or 35S radioactivity hydrolyzed from either [γ\textsuperscript{32P}]ATP or [γ\textsuperscript{35S}]ATPγS during this time period were shown to be negligible by the charcoal filtration assay.

Solution Viscometric Studies—Pre-steady-state assays containing either [γ\textsuperscript{32P}]ATP or [γ\textsuperscript{35S}]ATPγS as described above were also carried out in reaction buffer containing varying amounts of either microviscogen (0–30% (w/v) glycerol or sucrose) or macroviscogen (0–6.3% (w/v) polyethylene glycol). The relative viscosities (\( \eta / \eta_0 \)) of reaction buffers containing either a micro- or macroviscogen were calculated at 30 °C, using an Ostwald viscometer to measure transit times and correcting for density. Relative solvent viscosities of 1.0 and 3.0 were obtained for buffers containing 0 and 30% sucrose, respectively. The measurements were made in triplicate and did not deviate by more than 3%.

Data Analysis—Initial rates determined in the two-substrate steady-state kinetic studies were globally fitted to Equation 1,

\[
\nu (\text{m s}^{-1}) = \frac{[PDK1](M)}{K_p} = k(s^{-1})
\]

\[
= \frac{k_{\text{cat}} [\text{ATP}][\text{Tide}]}{\alpha K_{\text{app}}^\text{ATP} K_{\text{app}}^\text{Tide} + [\text{ATP}][\text{Tide}]} \tag{1}
\]

the general velocity equation derived for a Rapid Equilibrium Random Bi Bi system (32). In this equation, \( \nu /[PDK1] = k(s^{-1}) \) yields the observed pseudo-first order rate constant as a function of ATP and PDK1-Tide concentrations ([ATP] and [Tide]) according to the dissociation constants of ATP and PDK1-Tide (\( K_{\text{app}}^\text{ATP} \) and \( K_{\text{app}}^\text{Tide} \)). The symbol \( \alpha \) is a proportionality constant, which quantifies the degree that the binding of one substrate either increases (\( \alpha < 1 \)) or decreases (\( \alpha > 1 \)) the affinity of the enzyme for the other substrate. In addition, initial rates for varying the concentrations of either [ATP] or [Tide] were individually fitted to the standard Michaelis-Menten equation to obtain values of \( k_{\text{cat(app)}} \) and either \( K_{m(app)}^\text{ATP} \) (at different fixed [Tide]) or \( K_{m(app)}^\text{Tide} \) (at different fixed [ATP]), respectively, which were further analyzed by the appropriate secondary expressions derived from Equation 1. Secondary plots of \( k_{\text{cat(app)}} \) versus either [Tide] or [ATP] were fitted to Equations 2 and 3, respectively (32).

\[
k_{\text{cat(app)}} = \frac{k_{\text{cat}} [\text{Tide}]}{\alpha K_{\text{app}}^\text{Tide} + [\text{Tide}]} \tag{2}
\]

\[
k_{\text{cat(app)}} = \frac{k_{\text{cat}} [\text{ATP}]}{\alpha K_{\text{app}}^\text{ATP} + [\text{ATP}]} \tag{3}
\]

Secondary plots of either \( K_{m(app)}^\text{ATP} \) versus [Tide] or \( K_{m(app)}^\text{Tide} \) versus [ATP] were fitted to Equations 4 and 5, respectively (32).
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Initial rates determined in steady-state kinetic dead-end inhibition studies were fitted to the general velocity equations derived for competitive and noncompetitive mechanisms of inhibition, respectively (32). In these equations, values of $k_{\text{cat(app)}}$, $K_m^\text{(app)}$, and $K_i^\text{(app)}$ were determined for varying substrate and inhibitor concentrations at a single fixed concentration of the other substrate.

Initial rates determined in the complete array of ADP inhibition studies were globally fitted to Equation 6, which is the general velocity equation derived for a Rapid Equilibrium Random Bi Bi system with a dead-end PDK1-ADP-Tide ternary complex (32). In this equation, the parameters are the same as described for Equation 1, but further include $K_d^\text{ADP}$, the dissociation constant of the ADP product inhibitor, and $\beta$, the proportionality constant to quantify the degree that the binding of Tide substrate either increases ($\beta < 1$) or decreases ($\beta > 1$) the affinity of the enzyme for ADP. Values of $k_{\text{cat(app)}}$, $K_m^\text{(app)}$, $K_i^\text{(app)}$, and $K_d^\text{ADP}$ that were obtained from fits of individual ADP inhibition data sets were further analyzed by the appropriate secondary expressions derived from Equation 6. In these cases, secondary expressions for $k_{\text{cat(app)}}$ versus either [Tide] or [ATP] are the same as Equations 2 and 3, respectively; and secondary expressions for either $K_m^\text{(app)}$ versus [Tide] or $K_i^\text{(app)}$ versus [ATP] are the same as Equations 4 and 5, respectively (32). Secondary expressions of either $K_i^\text{(app)}$ versus [Tide] or $K_m^\text{(app)}$ versus [ATP] are given by Equations 7 and 8, respectively (32).

$$k(s^{-1}) = \frac{k_{\text{cat}} [\text{ATP}][\text{Tide}]}{1 + [\text{ATP}] + [\text{Tide}] + [\text{ADP}] + [\text{ATP}][\text{Tide}] + [\text{ADP}][\text{Tide}] + \frac{K_m^\text{(app)} K_i^\text{(app)}}{\beta K_d^\text{ADP} K_d^\text{Tide}} K_d^\text{Tide}}$$

$$K_{\text{m(app)}} = \frac{\alpha K_d^\text{ATP} K_d^\text{Tide}}{[\text{Tide}] + \alpha K_d^\text{ATP}} \quad (\text{Eq. 4})$$

$$K_i^\text{(app)} = \frac{K_i^\text{ADP} K_d^\text{Tide}}{\alpha K_d^\text{ATP} [\text{ATP}] + \beta K_d^\text{ADP}} \quad (\text{Eq. 5})$$

$$K_{\text{D(app)}} = \frac{\beta K_i^\text{ADP} [\text{ATP}] + \beta K_d^\text{ADP}}{\alpha K_d^\text{ATP} [\text{ATP}]} \quad (\text{Eq. 7})$$

$$K_{\text{D(app)}} = \frac{\beta K_i^\text{ADP} [\text{ATP}] + \beta K_d^\text{ADP}}{\alpha K_d^\text{ATP} [\text{ATP}]} \quad (\text{Eq. 8})$$

All data were plotted and fitted using the GraFit 4.0 software (Erithacus Software, UK), which utilizes an iterative least squares algorithm (33).

RESULTS

Two-substrate Steady-state Kinetics of His$_\gamma$-PDK1(ΔPH)—

As demonstrated in a previous study, the recombinant catalytic domain construct of PDK1 (His$_\gamma$-PDK1(ΔPH), residues 51–359) was affinity purified (>95%) from Sf9 insect cell lysate in its Ser$^{241}$ phosphorylated and catalytically active form (26). Whereas His$_\gamma$-PDK1(ΔPH) exhibited detectable activity toward trans-phosphorylation of T308-Tide, we confirmed that His$_\gamma$-PDK1(ΔPH), similar to full-length PDK1 (25), exhibits very low affinity for T308-Tide with $K_m \approx 10$ mM. In contrast to T308-Tide, His$_\gamma$-PDK1(ΔPH) exhibited significantly higher activity to PDK1-Tide, which contains the extended PIF residues C-terminal to the sequence of T308-Tide (Fig. 1B). Most important, saturating conditions could be approached with PDK1-Tide enabling steady-state kinetic determinations of values for $k_{\text{cat(app)}}$, $K_i^\text{(app)}$, and $K_m^\text{(app)}$. Therefore, all kinetic studies and mechanistic depictions are given in reference to PDK1-Tide (Tide) as the substrate for phosphorylation by His$_\gamma$-PDK1(ΔPH).

Fig. 2 shows all steady-state kinetic data and secondary plots for titration of His$_\gamma$-PDK1(ΔPH) with varying concentrations of one substrate at different fixed concentrations of the other substrate. During initial attempts to carry out a full titration analysis, it became apparent that titrations using substrate concentrations spanning two log units (3–300 $\mu$M) were yielding seemingly parallel lines in double reciprocal plots. Only by spanning substrate concentrations over three log units (0.3–300 $\mu$M) was it possible to clearly identify points of reciprocal plot intersection, which lie far to the left of the 1/k or y axis and well below the 1/1[S] or x axis (Fig. 2, A and B). Such a phenomenon reflects a large negative binding synergism associated between the ATP and Tide substrates. Due to the results obtained in inhibition studies (see below), analysis of these data were best approximated and described for a Rapid Equilibrium Random Bi Bi system (Fig. 2G) (32). Global fitting of the data to Equation 1 yielded values of $k_{\text{cat}} = 0.356 \pm 0.010$ s$^{-1}$, the dissociation constants of ATP ($K_d^\text{ATP} = 0.57 \pm 0.24$ $\mu$M) and Tide ($K_d^\text{Tide} = 1.1 \pm 0.5$ $\mu$M) in the absence of the other substrate, and the proportionality constant of $\alpha = 77 \pm 32$. In Equation 1, the calculated dissociation constants of ATP ($\alpha K_d^\text{ATP} = 44.3$ $\mu$M) and Tide ($\alpha K_d^\text{Tide} = 84.3$ $\mu$M) in the ternary complex reflect the large value of $\alpha$ and approximate their $K_m^\text{(app)}$ values under saturating conditions.

Because the nature of these data preclude both (i) close examination in double reciprocal plots and (ii) statistical appreciation in global analysis, the data are further displayed in individual plots, where both the global fit to Equation 1 and individual direct fits to the Michaelis-Menten equation are indicated (supplemental Fig. S1). To better highlight the effects of the large negative binding synergism ($\alpha >> 1$), values of $k_{\text{cat(app)}}$ and $K_m^\text{(app)}$ (supplemental Fig. S1A) and $K_i^\text{(app)}$ (supplemental Fig. S1B) obtained from individual fits were analyzed in secondary plots, where both global and direct fits are indicated (Fig. 2C–F). Direct fits of $k_{\text{cat(app)}}$ values obtained for varying [ATP] at different fixed [Tide] (Fig. 2C, Equation 2) and varying [Tide] at different fixed [ATP] (Fig. 2D, Equation 3) yielded well defined values of $k_{\text{cat}} = 0.351 \pm 0.015$ s$^{-1}$ and $\alpha K_d^\text{ATP} = 39.3 \pm 2.2$ $\mu$M (Fig. 2C) and $k_{\text{cat}} = 0.346 \pm 0.006$ s$^{-1}$ and $\alpha K_d^\text{Tide} = 80.7 \pm 9.2$ $\mu$M (Fig. 2D). However, the difficulty toward accurate determinations of the dissociation constants of ATP and Tide in each individual binary complex are clearly illustrated by the secondary plots of $K_m^\text{(app)}$ (Fig. 2E, Equation 4) and $K_i^\text{(app)}$ (Fig. 2F, Equation 5), where the corresponding $K_d^\text{ATP}$ and $K_d^\text{Tide}$ values are given by the y intercepts (insets). Table 1 summarizes the fitted values ± S.E. of the kinetic constants determined by global and secondary plot data.
fitting to the equations derived for the Rapid Equilibrium Random Bi Bi system (Fig. 2G).

Dead-end Inhibition Steady-state Kinetics—The pattern of intersecting lines shown in Fig. 2, A and B, provide little evidence toward the steady-state kinetic mechanism of peptide phosphorylation other than ruling out both (i) Ping-Pong and (ii) Rapid Equilibrium Ordered Bi Bi systems (32). For these mechanisms, respectively, either parallel lines are observed or lines intersect on the y axis for the second binding substrate. Discrimination between other possible mechanisms (e.g. Steady-State Ordered, Steady-State Random, or (Partial) Rapid Equilibrium Random Bi Bi systems) may be achieved by comparing the effects of product (or dead-end) inhibitors in double reciprocal plots constructed for varying each substrate, while the other substrate is fixed at an unsaturated concentration (32).

For His$_n$-PD1K1(APH), ADP was shown to be (i) competitive with the ATP substrate (Fig. 3A; $k_{cat(app)} = 0.0939 \pm 0.0007$ s$^{-1}$, $K_m^{app} = 11.3 \pm 0.4$ $\mu$M, and $K_i^{TP} = 29.6 \pm 1.8$) and (ii) non-competitive with the Tide substrate (Fig. 3B; $k_{cat(app)} = 0.145 \pm 0.001$ s$^{-1}$, $K_m^{app} = 33.1 \pm 0.9$ $\mu$M, and $K_i^{ADP} = 73.3 \pm 1.9$). Because a wide range of concentrations were used for the varied substrate, insets are included in Fig. 3 to magnify views of the line patterns crossing the y axis. In complementary inhibition studies, the phosphopeptide product analog (T/E)-Tide (p'Tide) was shown to be (i) noncompetitive with the ATP substrate (Fig. 3C; $k_{cat(app)} = 0.0954 \pm 0.0009$ s$^{-1}$, $K_m^{app} = 13.6 \pm 0.4$ $\mu$M, and $K_i^{TP} = 74.2 \pm 2.0$) and (ii) competitive with the Tide substrate (Fig. 3D; $k_{cat(app)} = 0.142 \pm 0.002$ s$^{-1}$, $K_m^{Tide} = 31.7 \pm 1.9$ $\mu$M, and $K_i^{TP} = 31.0 \pm 2.6$).

The observation of competitive inhibition exhibited between both (i) the ATP substrate and the ADP product inhibitor and (ii) the Tide substrate and the p'Tide product analog rule out Steady-State Ordered and Steady-State Random Bi Bi systems (32). In a Steady-State Ordered system, competitive inhibition between a given substrate/product pair would be observed only for the substrate that binds first. In a Steady-State Random system, only mixed-type inhibition patterns would be observed. Thus, the line patterns in Fig. 3 remain consistent with either (i) a Rapid Equilibrium Random Bi Bi system, which can form both E-ADP-Tide and E-ATP-p'Tide types of dead-end ternary complexes or (ii) a Theorell-Chance system, which is a special case of an Ordered Bi Bi system (32). In a Theorell-Chance system, only the substrate that binds first can form a stable binary complex, whereas the concentrations of the central substrate and product ternary complexes are essentially zero.

Binding Synergism between ADP and Tide—To distinguish between the Rapid Equilibrium Random and Theorell-Chance Bi Bi mechanisms, the effect of ADP product inhibition was tested for varying one substrate concentration at different fixed concentrations of the other substrate (32). Due to the extensive substrate concentration ranges required in this experiment, the ADP inhibition data are displayed in arrays of individual plots, which permit close inspection of the analyses (supplemental Figs. S2 and S3). Similar to Fig. 3A, ADP was shown to be competitive with ATP as the varied substrate (supplemental Fig. S2), and values of $k_{cat(app)}$ (Fig. 4A), $K_m^{TP}$ (Fig. 4C), and $K_i^{ADP}$ (Fig. 4E) were obtained for different fixed Tide concentrations. Similar to Fig. 3B, ADP was shown to be noncompetitive with Tide as the varied substrate (supplemental Fig. S3), and values
of $k_{\text{cat(app)}}$ (Fig. 4B), $K_{\text{Tide(app)}}^\text{Tide}$ (Fig. 4D), and $K_{\text{ADP(app)}}^\text{ADP}$ (Fig. 4F) were obtained for different fixed ATP concentrations. The critical observation whereby the $K_{\text{ADP(app)}}^\text{ADP}$ increases hyperbolically with increasing fixed [Tide] (Fig. 4E) rules out the special case of a Theorell-Chance system, where $K_{\text{ADP(app)}}^\text{ADP}$ would show no dependence on different fixed [Tide] (32). Thus, the ADP inhibition data can be appropriately analyzed according to equations derived for a Rapid Equilibrium Random Bi Bi system with a $E$-ADP-Tide dead-end ternary complex (Fig. 4G, Equation 6) (32).
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Table 1

| Parameter | Data analyzed | Two-substrate* | ADP inhibition* |
|-----------|---------------|----------------|---------------|
|           | Eq. | Value | Eq. | Value |
| $k_{cat}$ (s$^{-1}$) | Global | 1 | 0.356 ± 0.010 | 6 | 0.334 ± 0.007 |
| $k_{cat, app}$ versus [Tide] | 2 | 0.351 ± 0.015 | 2 | 0.336 ± 0.010 |
| $k_{cat, app}$ versus [ATP] | 3 | 0.346 ± 0.006 | 3 | 0.324 ± 0.014 |
| $K_d^{ATP}$ (μM) | Global | 1 | 0.57 ± 0.24 | 6 | 0.54 ± 0.15 |
| $k_{cat, app}$ versus [ATP] | 4 | 1.6 ± 1.1 | 4 | 0.804 ± 0.231 |
| $K_m^{ADP}$ (μM) | Global | 5 | 1.0 ± 1.4 | 5 | (1.37)$^c$ |
| $k_{cat, app}$ versus [ATP] | 5 | 39.3 ± 2.2 | 3 | 43.4 ± 6.0 |
| $K^{Tide}$ (μM) | Global | 4 | 40.8 ± 2.6 | 4 | 42.7 ± 0.9 |
| $k_{cat, app}$ versus [ATP] | 5 | 25 ± 10 | 5 | 59 ± 32 |
| $K_d^{Tide}$ (μM) | Global | 1 | 1.1 ± 0.5 | 6 | 1.0 ± 0.3 |
| $k_{cat, app}$ versus [ATP] | 4 | 3.2 ± 2.0 | 4 | (1.52)$^c$ |
| $K^{ADP}$ (μM) | Global | 5 | 2.1 ± 2.2 | 5 | 1.9 ± 4.6 |
| $k_{cat, app}$ versus [ATP] | 6 | (84.3)$^c$ | 6 | (78.2)$^c$ |
| $K^{Tide}$ (μM) | Global | 1 | 77.7 ± 32 | 6 | 78 ± 21 |
| $k_{cat, app}$ versus [Tide] | 4 | (25.2)$^c$ | 4 | (53.1)$^c$ |
| $K^{ADP}$ (μM) | Global | 5 | (39.3)$^c$ | 5 | (42.7)$^c$ |
| $k_{cat, app}$ versus [Tide] | 7 | 75.7 ± 7.2 | 7 | 81 ± 13 |
| $K^{ADP}$ (μM) | Global | NA$^d$ | NA | 6 | 1.2 ± 0.5 |
| $k_{cat, app}$ versus [ATP] | 7 | NA | 7 | (84)$^d$ |
| $K^{Tide}$ (μM) | Global | NA | NA | 8 | 19.4 ± 2.8 |
| $k_{cat, app}$ versus [Tide] | NA | NA | 7 | 49 ± 20 |
| $K^{Tide}$ (μM) | Global | NA | NA | 7 | 11 ± 4 |

* The steady-state kinetic values ± S.E. were determined for the rapid equilibrium random Bi Bi mechanism depicted in Fig. 2G.

* The steady-state kinetic values ± S.E. were determined for the rapid equilibrium random Bi Bi mechanism with a dead-end $E$-$Tide$-ADP complex depicted in Fig. 4G.

* This value was calculated from the appropriate relationship given between fitted values of $K_d^{ATP}$, $αK_d^{Tide}$, $αK^{ADP}$, or $α$ determined in the indicated method of analysis.

* This value was calculated from the appropriate relationship given between fitted values of $K^{ADP}$ and $β$ determined in the indicated method of analysis.

Global fitting of the data to the general rate in Equation 6 yielded values of $k_{cat}$, $K_d^{ATP}$, $K_d^{Tide}$, and $α$ in agreement with those obtained from two-substrate steady-state kinetic analysis (Table 1). In addition, a $K^{ADP} = 1.2 ± 0.5$ μM was determined for the dissociation constant of ADP, whereas $β = 49 ± 20$ indicated large negative binding synergism between ADP product and Tide substrate. The global fitted lines to Equation 6 are indicated for comparison to fitted lines obtained for the competitive (supplemental Fig. S2, varying [ATP]) and noncompetitive (supplemental Fig. S3, varying [Tide]) individual ADP inhibition data sets.

Table 1 summarizes the fitted values ± S.E. of the kinetic constants determined by both global and secondary plot data fitting to the equations derived for the Rapid Equilibrium Random Bi Bi system with a $E$-ADP-Tide dead-end ternary.
FIGURE 4. Binding synergism between ADP and Tide. Values of \( A, k_{\text{cat(app)}} \) from varying [ATP]; \( B, k_{\text{cat(app)}} \) from varying [Tide]; \( C, K_{m}^{\text{ATP}} \) from varying [ATP]; \( D, K_{m}^{\text{Tide}} \) from varying [Tide]; \( E, K_{i}^{\text{ATP}} \) from varying [ATP]; and \( F, K_{i}^{\text{Tide}} \) from varying [Tide] were determined from fits of ADP inhibition data sets to the equation for either competitive inhibition (supplemental Fig. S2) or noncompetitive inhibition (supplemental Fig. S3) and plotted against the corresponding fixed substrate concentrations. Dashed lines represent direct fits of the secondary plots to \( A, \) Equation 2; \( B, \) Equation 3; \( C, \) Equation 4; \( D, \) Equation 5; \( E, \) Equation 7; and \( F, \) Equation 8. The solid lines were generated by these equations using the kinetic constants determined from a global fit of the initial rates shown in supplemental Figs. S2 and S3. Insets in panels C–F magnify views of the data and fitted line patterns near the y axis. G, equilibria of the Rapid Equilibrium Random Bi Bi system with an \( E \)-ADP-Tide dead-end ternary complex according to the general rate Equation 6. The proportionality constants \( \alpha \) and \( \beta \) quantify binding synergism exhibited between either ATP and Tide or ADP and Tide, respectively.
complex (Fig. 4G). The primary source of error in the global fitted value of $\beta$ results from deviations of the $K_i^{ADP}$ values obtained at the highest fixed concentrations (300 $\mu$M) of both Tide (Fig. 4E) and ATP (Fig. 4F), where little ADP inhibition was detected (supplemental Figs. S2 and S3, bottom rows). These relatively small upward deviations cause more dramatic effects on the $y$ intercepts, which yield the values of $K_i^{ADP}$ (Fig. 4E, inset) and $bK_i^{ADP}$ (Fig. 4F, inset).

Pre-steady-state Thio-substitution Effects—Because the steady-state kinetic and inhibition results were best approximated by a Rapid Equilibrium Random Bi Bi system, it was of great interest to next investigate the nature of the enzymatic step governing the relatively slow turnover rate of $k_{cat} \approx 0.34$ s$^{-1}$. Therefore, His$_6$-PDK1($\Delta$PH)-catalyzed peptide phosphorylation reactions were carried out under pre-steady-state conditions ([E] = 1–5 $\mu$M) with “saturating” amounts (300 $\mu$M) of both ATP and Tide. By using enzyme concentrations in this range, it was possible to distinguish whether rates of peptide chemical phosphorylation changed on progressing from the initial turnover (i.e. observed velocity includes all steps leading to and including chemical phosphorylation) to subsequent turnovers (i.e. observed velocity further includes product dissociation steps) (34). Fig. 5A shows the time progress curve of phosphopeptide product (pTide) formed during the first three catalytic turnovers using 5 $\mu$M enzyme. No changes in the velocity of pTide formation (slope) could be detected on transition between the initial and subsequent turnovers. If the rate of release of either ADP or pTide product was significantly slower than chemical phosphorylation, then a burst of pTide formation would have been observed during the first turnover, followed by slower linear accumulation of pTide during subsequent turnovers. The constant velocity (i.e. absence of a "burst" of pTide) observed in this pre-steady-state experiment is further supported by the $y$ intercept, which closely approaches zero (Fig. 5A).

To more closely probe the nature of the rate-determining step(s), the pre-steady-state time progress curve was also generated using ATP$_S$ as an alternative substrate. If the chemical transfer step was (partially) rate-determining, then such “thio-substitution” could lead to either an increased or decreased reaction velocity, depending on the degree of either dissociative or associative character, respectively, which may occur in the chemical step (35). Similar to the ATP reaction (Fig. 5A), pTide showed linear accumulation with time when ATP$_S$ was the substrate (Fig. 5B). Pre-steady-state time progress curves were also generated for [enzyme] = 1 and 3 $\mu$M; and the slopes ("$\Delta$"/[$E$]) determined from plots of the reaction velocities with either the ATP or ATP$_S$ substrates yielded similar values of $k_{cat}^{ATP} = 0.310 \pm 0.015$ s$^{-1}$ and $k_{cat}^{ATP}$ = 0.306 $\pm 0.017$ s$^{-1}$ (Fig. 5C). Importantly, these values approximated the $k_{cat}$ values obtained in two-substrate steady-state kinetics (Table 1), indicating that (i) saturating conditions are observed in these pre-steady-state experiments, (ii) reaction velocities are linearly proportional over a 10$^5$-fold concentration range of His$_6$-PDK1($\Delta$PH) (0.005 $\mu$M $\leq$ [E] $\leq$ 5 $\mu$M), and (iii) thio-substitution causes no significant change in the magnitude of $k_{cat}$. Whereas similar values of $k_{cat}^{ATP}$ and $k_{cat}^{ATP_S}$ suggest that the chemical step is not rate-determining, it cannot be completely ruled out, as small thio-substitution effects may occur if the reaction proceeds with very little change in P-S bond order.

Pre-steady-state Solution Viscometric Effects—As described in Fig. 5, A–C, pre-steady-state kinetic values of both $k_{cat}^{ATP}$ and $k_{cat}^{ATP_S}$ were also determined in reaction buffers containing increasing amounts of a microviscogen (e.g. glycerol and sucrose). Similar to reaction buffer alone (Fig. 5, A and B), pTide showed linear accumulations with time, which yielded similar reaction velocities for all viscogen-containing buffers. Fig. 5D highlights the similar glycerol-independent va-
ues of $k_{\text{cat}}^0$ (buffer with no viscogen) and $k_{\text{cat}}^* \text{ATP}$ (buffer with viscogen) for both the ATP and ATP$\gamma$S substrates. The observations that all pre-steady-state kinetic time courses remained linear and independent of viscogen indicate that dissociation of both ADP and p$'$/Tide occur significantly faster than the rate-limiting step(s). These results remain consistent with the good approximation of the steady-state kinetic results to a Rapid Equilibrium Random Bi Bi system, where motions in the central ternary complex are largely rate-determining (Figs. 2G and 4G).

**DISCUSSION**

Protein kinases comprise the largest enzyme family, with ~500 being encoded by the human genome (36). The large number of cellular protein kinases reflects the large number of signal transduction pathways required in regulating proper cellular growth, survival, and proliferation. The complexity in elucidating cellular signaling networks is compounded by analyses indicating that individual protein kinases may target and phosphorylate numerous different protein substrates (37, 38). Thus, the mechanisms by which protein kinases selectively recognize protein substrates are of fundamental interest; and only recently, have detailed kinetic, binding, and mutagenesis studies revealed the importance of protein-protein docking complexes in regulating protein phosphorylation specificities (39, 40).

Central to the framework of such mechanistic understanding is development of kinetic mechanisms, describing time scales and equilibria of chemical steps involved in selective substrate recognition, catalytic phosphorylation, and release of products. On review of the steady-state kinetic mechanisms reported for all protein kinases to date, it became apparent that a leading majority of protein kinase reactions best approximate a Rapid Equilibrium Random Bi Bi system (Fig. 2G). In addition, such protein kinases may be further subcategorized according to whether the ATP and protein substrates exhibit either (i) apparently positive binding synergism ($\alpha < K_{\text{cat}}^0/K_{\text{cat}}^* \text{ATP}$), (ii) apparently little binding synergism ($\alpha > K_{\text{cat}}^0/K_{\text{cat}}^* \text{ATP}$), or (iii) apparently negative binding synergism ($\alpha > K_{\text{cat}}^0/K_{\text{cat}}^* \text{ATP}$). Examples of protein phosphorylation reactions exhibiting random type mechanisms with apparently positive binding synergism include p21-activated kinase-2 (PAK2) with myelin basic protein ($\alpha \approx 0.5$) (41), Csk with Src ($\alpha \approx 0.25$) (42), Sky1p with nuclear protein localization-3 (Npl3) ($\alpha \approx 0.07$) (43), and p38$\alpha$ with ATF2$\Delta$115 (activating transcription factor-2 (residues 1–115)) ($\alpha \approx 0.04$) (44); apparently little binding synergism ($\alpha \approx 1$) has been reported for EGFR-TK(+EGF) with GAT-Tide (45), Csk with poly(Glu,Tyr) (46), and cyclin-dependent kinase-activating kinase of budding yeast (Cak1p) with cyclin-dependent kinase 2 (47); whereas apparently negative binding synergism has been reported for ERK2 with Ets$\Delta$138 ($\alpha \approx 2$) (48), Sky1p with peptide ($\alpha \approx 3$) (49), ROCKII with N6 peptide ($\alpha \approx 4$) (50), and EGFR-TK with GAT-Tide (45). Thus, the $\alpha$ value of ~70 for the reaction of PDK1 with PDK1-Tide represents the largest $K_{\text{cat}}^0/K_{\text{cat}}^* \text{ATP}$ ratio yet determined for a protein kinase reaction.

Strict interpretation of the $\alpha$ value according the Rapid Equilibrium Random Bi Bi system (Fig. 2G) requires that $\alpha$ quantify the degree that the binding of one substrate either increases ($\alpha < 1$) or decreases ($\alpha > 1$) the affinity of the enzyme for...
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the other substrate (i.e. $K_d$ and $aK_d$ are true dissociation constants). However, recently reported results of transient kinetic studies have revealed that apparent $aK_d$ values obtained in steady-state kinetic analyses may contain terms related to steps occurring in the central ternary complex. For example, pre-steady-state kinetic studies of both Csk with Src ($\alpha \leq 0.25$) (42) and Sky1p with nuclear protein localization-3 ($\alpha \sim 0.07$) (43) showed that a rapid burst of chemical phosphorylation precedes steady-state rate-limiting steps involving release of products. For these cases, Adams and co-workers (39, 42, 43) demonstrated how fast and favorable phosphoryl transfer can function as a reversible “clamp” that grasps onto the substrate, pulling it toward product and overcoming weak interactions between the enzyme and substrate (Fig. 6A). Under such conditions ($k_{-3} < k_3 > k_4$), the complex expression relating to the apparent $K_m$ (apparent $aK_m$) is reduced to the approximation given by Equation 9 (39, 42, 43).

$$K_{m(app)} \approx K_d \frac{k_{-3}}{k_3} \quad \text{(Eq. 9)}$$

In contrast to the thermodynamically coupled systems described above ($K_{m(app)} < K_m$), Adams and co-workers (39) further explained how uncoupled fast phosphoryl transfer can either lower or raise $K_m$ relative to $K_d$ in cases where substrates bind with high intrinsic affinity. However, this explanation cannot account for the observed $K_m > K_d$ relationship exhibited by PDK1, because pre-steady-state kinetics showed no evidence of a chemical burst preceding rate-limiting product release (Fig. 5). Furthermore, the absence of both thio-substitution and solution microviscosity effects suggested that a rate-limiting conformational change likely precedes chemical phosphorylation ($k_{3a} < k_{3b} < k_{-3}$), as depicted in Fig. 6B. Under conditions where the conformational equilibrium is unfavorable ($k_{3a} < k_{3b} < k_{-3}$), the complex expression relating to the apparent $K_m$ (apparent $aK_m$) is reduced to the approximation given by Equation 10.

$$K_{m(app)} \approx K_d \frac{k_{-3a}}{k_{3a}} \quad \text{(Eq. 10)}$$

Thus, the high affinity that PDK1 exhibits for both ATP and PDK1-Tide in individual binary complexes ($K_{ATP}^d$ and $K_{Tide}^d$, Table 1) would appear to decrease ~70-fold ($aK_{ATP}^d$ and $aK_{Tide}^d$, Table 1), because formation of the ternary complex may be coupled to both a rate-limiting and a thermodynamically unfavorable conformational transition preceding chemistry. Such a conformational step has been included for the reaction of ERK2 with ETS138, whereby mutagenesis studies revealed the important role of distal contacts in mediating “proximity-induced” catalysis (40, 51, 52). In addition, the apparently negative binding synergism observed between ADP and PDK1-Tide ($K_{ADP}^d < \beta K_{ADP}^d$) may also reflect a thermodynamically unfavorable conformational transition in the dead-end ternary complex.

Similar to the important role of docking interactions exhibited for the catalytic reactions of Csk, Sky1p, p38α, and ERK2 with their protein substrates, His$_8$-PDK1(ΔPH) reactivity was greatly enhanced by PIF docking interactions to the model PDK1-Tide substrate (e.g. where the $K_m \sim 70 \mu$m observed for PDK1-Tide compared with $K_m \approx 10 \mu$m for T308-Tide). However, the significant effect of this specific interaction has yet to lower the apparent substrate $K_m(aK_d)$ values to $\approx K_d$ values for the individual binary complexes. Thus, the kinetic constants determined for the PDK1 reaction with PDK1-Tide now serve as a benchmark for future comparisons to kinetic constants determined for reactions of PDK1 with protein substrates (Fig. 1A). Of particular interest will be to identify whether other possible “docking” interactions will serve to overcome the rate-limiting unfavorable conformational transition (Fig. 6B), which currently hinders PDK1-Tide phosphorylation.

In conclusion, the results of steady-state kinetic and inhibition studies for PDK1-catalyzed trans-phosphorylation of PDK1-Tide were best approximated by a Rapid Equilibrium Random Bi Bi system. ATP, PDK1-Tide, ADP, and (Thr/Glu)-Tide were shown to form high affinity binary complexes, which significantly decreased upon formation of either catalytically competent or dead-end inhibited ternary complexes. In addition, time progress curves in pre-steady-state kinetic studies under saturating or $k_{cat}$ conditions showed (i) no burst or lag phenomena, (ii) no change in reaction velocity when ATPγS was used as a phosphate donor, and (iii) no change in reaction velocity on increasing relative microviscosity. Taken together, a reaction mechanism is proposed whereby a rate-limiting and unfavorable conformation transition coupled to substrate recognition could account for the large degree of apparently negative binding synergism. To further address this phenomenon, extensive transient kinetic and equilibrium binding studies of the coupled interactions will be forthcoming.

REFERENCES

1. Peterson, R. T., and Schreiber, S. L. (1999) Curr. Biol. 9, R521–R524
2. Mora, A., Komander, D., van Aalten, D. M., and Alessi, D. R. (2004) Semin. Cell Dev. Biol. 15, 161–170
3. Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R. J., Reese, C. B., and Cohen, P. (1997) Curr. Biol. 7, 261–269
4. Alessi, D. R., Deak, M., Casamayor, A., Caudwell, F. B., Morrice, N., Norman, D. G., Gaffney, P., Reese, C. B., MacDougall, C. N., Harbison, D., Ashworth, A., and Bownes, M. (1997) Curr. Biol. 7, 776–789
5. Stooke, D., Stephens, L. R., Copeland, T., Gaffney, P. R., Reese, C. B., Painter, G. F., Holmes, A. B., McCormick, F., and Hawkins, P. T. (1997) Science 277, 567–570
6. Stephens, L., Anderson, K., Stooke, D., Erdjument-Bromage, H., Painter, G. F., Holmes, A. B., Gaffney, P. R. J., Reese, C. B., McCormick, F., Tempest, P., Caudwell, J., and Hawkins, P. T. (1998) Science 279, 710–714
7. Walker, K. S., Deak, M., Paterson, A., Hudson, K., Cohen, P., and Alessi, D. R. (1998) Biochem. J. 331, 299–308
8. Kobayashi, T., and Cohen, P. (1999) Biochem. J. 339, 319–328
9. Kobayashi, T., Deak, M., Morrice, N., and Cohen, P. (1999) Biochem. J. 344, 189–197
10. Alessi, D. R., Kozlowski, M. T., Weng, Q. P., Morrice, N., and Avruch, J. (1998) Curr. Biol. 8, 69–81
11. Pullen, N., Dennis, P. B., Andjelkovic, M., Dufner, A., Kozma, S. C., Hemmings, B. A., and Thomas, G. (1998) Science 279, 707–710
12. Cheng, X., Ma, Y., Moore, M., Hemmings, B. A., and Taylor, S. S. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 9849–9854
13. Dutil, E. M., Toker, A., and Newton, A. C. (1998) Curr. Biol. 8, 1366–1375
14. Le Good, J. A., Ziegler, W. H., Parekh, D. B., Alessi, D. R., Cohen, P., and Avruch, J. (1999) Curr. Biol. 8, 1069–1077
15. Chou, M. M., Hou, W., Johnson, J., Graham, L. H., Lee, M. H., Chen, C. S., Newton, A. C., Schaffhausen, B. S., and Toker, A. (1998) Curr. Biol. 8, 1069–1077
16. Jensen, C. J., Buch, M.-B., Krag, T. O., Hemmings, B. A., Gammeltoft, S., and Frödin, M. (1999) *J. Biol. Chem.* **274**, 27168–27176
17. Richards, S. A., Fu, J., Romanelli, A., Shimamura, A., and Blenis, J. (1999) *Curr. Biol.* **9**, 810–820
18. Dong, L. Q., Landa, L. R., Wick, M. I., Zhu, L., Mukai, Y., Ono, Y., and Liu, F. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 5089–5094
19. Torbett, N. E., Casamassima, A., and Parker, P. J. (2003) *J. Biol. Chem.* **278**, 32344–32351
20. Belham, C., Wu, S., and Avruch, J. (1999) *Curr. Biol.* **9**, R93–R96
21. Vanhaesebroeck, B., and Alessi, D. R. (2000) *Biochem. J.* **346**, 561–576
22. Newton, A. C. (2003) *Biochem. J.* **370**, 361–371
23. Biondi, R. M., and Nebreda, A. R. (2003) *Biochem. J.* **372**, 1–13
24. Manning, G., Whyte, D. B., Martinez, R., Hunter, T., and Sudarsanam, S. (2002) *Science* **298**, 1912–1934
25. Manning, B. D., and Cantley, L. C. (2002) *Sci. STKE* **162**, PE49
26. Obenauer, J. C., and Yaffe, M. B. (2004) *Methods Mol. Biol.* **261**, 445–468
27. Manning, B. D., and Cantley, L. C. (2002) *Sci. STKE* **162**, PE49
28. Obenauer, J. C., and Yaffe, M. B. (2004) *Methods Mol. Biol.* **261**, 445–468
29. Manning, B. D., and Cantley, L. C. (2002) *Sci. STKE* **162**, PE49
30. Balendran, A., Casamayor, A., Deak, M., Paterson, A., Gaffney, P., Currie, R., Downes, C. P., and Alessi, D. R. (1999) *Biochem. J.* **346**, 561–576
31. Gao, X., Yo, P., and Harris, T. K. (2005) *Protein Expression Purif.* **43**, 44–56
32. Segel, I. H. (1975) *Enzyme Kinetics*, John Wiley & Sons, Inc., New York
33. Leatherbarrow, R. J. (1998) *GraFit*, version 4.0, Erithacus Software Ltd., Staines, United Kingdom
34. Adams, J. A. (2001) *Chem. Rev.* **101**, 2271–2290
35. Herschlag, D., Piccirilli, J. A., and Cech, T. R. (1991) *Biochemistry* **30**, 4844–4854
36. Manning, G., Whyte, D. B., Martinez, R., Hunter, T., and Sudarsanam, S. (2002) *Science* **298**, 1912–1934
37. Manning, B. D., and Cantley, L. C. (2002) *Sci. STKE* **162**, PE49
38. Obenauer, J. C., and Yaffe, M. B. (2004) *Methods Mol. Biol.* **261**, 445–468
39. Leiser, S. A., Aubol, B. E., Wong, L., Jennings, P. A., and Adams, J. A. (2005) *Biochim. Biophys. Acta* **1754**, 191–199
40. Rainey, M. A., Callaway, K., Barnes, R., Wilson, B., and Dalby, K. N. (2005) *J. Am. Chem. Soc.* **127**, 10494–10495
41. Wu, H., Zheng, Y., and Wang, Z.-X. (2003) *Biochemistry* **42**, 1129–1139
42. Leiser, S. A., Shindler, C., Aubol, B. E., Lee, S., Sun, G., and Adams, J. A. (2005) *J. Biol. Chem.* **280**, 7769–7776
43. Aubol, B. E., Unger, L., Lukasiewicz, R., Ghosh, G., and Adams, J. A. (2004) *J. Biol. Chem.* **279**, 30182–30188
44. Szafranska, A. E., and Dalby, K. N. (2005) *FEBS J.* **272**, 4631–4645
45. Posner, I., Engel, M., and Levitzki, A. (1992) *J. Biol. Chem.* **267**, 20638–20647
46. Cole, P. A., Burn, P., Takacs, B., and Walsh, C. T. (1994) *J. Biol. Chem.* **269**, 30880–30887
47. Enke, D. A., Kaldis, P., and Solomon, M. J. (2000) *J. Biol. Chem.* **275**, 33267–33271
48. Waas, W. F., and Dalby, K. N. (2002) *J. Biol. Chem.* **277**, 12532–12540
49. Aubol, B. E., Nolen, B., Vu, D., Ghosh, G., and Adams, J. A. (2002) *Biochemistry* **41**, 10002–10009
50. Trauger, J. W., Lin, F.-F., Turner, M. S., Stephens, J., and LoGrasso, P. V. (2002) *Biochemistry* **41**, 8948–8953
51. Waas, W. F., Rainey, M. A., Szafranska, A. E., and Dalby, K. N. (2003) *Biochemistry* **42**, 12273–12286
52. Waas, W. F., Rainey, M. A., Szafranska, A. E., Cox, K., and Dalby, K. N. (2004) *Biochim. Biophys. Acta* **1697**, 81–87.