Disruption of 3-Phosphoinositide-dependent Kinase 1 (PDK1) Signaling by the Anti-tumorigenic and Anti-proliferative Agent N-α-tosyl-L-phenylalanyl Chloromethyl Ketone*

The anti-tumorigenic and anti-proliferative effects of N-α-tosyl-L-phenylalanyl chloromethyl ketone (TPCK) have been known for more than three decades. Yet little is known about the discrete cellular targets of TPCK controlling these effects. Previous work from our laboratory showed TPCK, like the immunosuppressant rapamycin, to be a potent inhibitor of the 70-kilodalton ribosomal S6 kinase 1 (S6K1), which mediates events involved in cell growth and proliferation. We show here that rapamycin and TPCK display distinct inhibitory mechanisms on S6K1 as a rapamycin-resistant form of S6K1 was TPCK-sensitive. Additionally, we show that TPCK inhibited the activation of the related kinase and proto-oncogene Akt. Upstream regulators of S6K1 and Akt include phosphoinositide 3-kinase (PI 3-K) and 3-phosphoinositide-dependent kinase 1 (PDK1). Whereas TPCK had no effect on either mitogen-regulated PI 3-K activity or total cellular PDK1 activity, TPCK prevented phosphorylation of the PDK1 regulatory sites in S6K1 and Akt. Furthermore, whereas both PDK1 and the mitogen-activated protein kinase (MAPK) are required for full activation of the 90-kilodalton ribosomal S6 kinase (RSK), TPCK inhibited RSK activation without inhibiting MAPK activation. Consistent with the capacity of RSK and Akt to mediate a cell survival signal, in part through phosphorylation of the pro-apoptotic protein BAD, TPCK reduced BAD phosphorylation and led to cell death in interleukin-3-dependent 32D cells. Finally, in agreement with results seen in embryonic stem cells lacking PDK1, protein kinase A activation was not inhibited by TPCK showing TPCK specificity for mitogen-regulated PDK1 signaling. TPCK inhibition of PDK1 signaling thus disables central kinase cascades governing diverse cellular processes including proliferation and survival and provides an explanation for its striking biological effects.

Cells respond to a vast array of extracellular cues that direct their intracellular activities and ultimately govern their fates. Such guided regulation fashions and maintains complex and highly specialized tissues in multicellular organisms. However, the misregulation or misinterpretation of such signals can spell disaster for an organism and is the molecular basis for a number of disease states such as cancer, autoimmunity, tissue degenerative disorders, and developmental abnormalities.

These extracellular cues include numerous soluble and cell-tethered ligands for which responsive cells express receptors. For ligands regulating such diverse processes as cell growth, proliferation, motility, survival, and differentiation, the “AGC” family of serine/threonine kinases provides critical links between the extracellular signals and their intracellular effector molecules (1–4). AGC kinases are so-named as they include protein kinase A (PKA), protein kinase G (PKG), protein kinase C (PKC), and their nearest kinase relatives. Although AGC kinases share high structural conservation in their kinase domains, they exhibit rich disparity in the surrounding regulatory regions. This permits similar kinase activities to respond to a spectrum of molecular messengers mobilized by extracellular signals.

Despite such diversity, one critical step in the activation of AGC kinases is highly conserved: phosphorylation of their autoinhibitory activation loops. The activation loop is a structural feature found throughout the greater kinase superfamilly (5). In their inactive state, the activation loops of many kinases block the access of substrate to the catalytic core of the enzyme. However, once phosphorylated, the activation loop is displaced and substrate is no longer sterically or electrochemically excluded (6). 3-Phosphoinositide-dependent kinase 1 (PDK1) has recently been shown to phosphorylate the activation loops of a growing number of its own AGC kinase family members. These include Akt or protein kinase B (PKB) (7), the 70-kilodalton ribosomal S6 kinase 1 (S6K1) (8, 9), PKC isoforms (10–13), serum and glucocorticoid-regulated kinase (14–16), PKA (17), the 90-kilodalton ribosomal S6 kinase (RSK) (18, 19), the protein kinase C-related kinase (20), and the p21-activated kinase 1 (21). Additionally it autophosphorylates its own activation loop (22). Recent work using PDK1−/− embryonic stem cells demonstrated the necessity for PDK1 in insulin-like growth factor-1 activation of Akt and S6K1 and in phospholipid 12-myristate 13-acetate (PMA) activation of RSK. However, lysates from these cells showed no loss in PKA activation implying that PDK1 was required for full activation of Akt, S6K1, and RSK, but PKA could be regulated via a PDK1-independent mechanism (23).

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The anti-tumorigenic and anti-proliferative agents N-α-tosyl-L-phenylalanyl chloromethyl ketone; TLCK, N-α-tosyl-L-lysyl chloromethyl ketone; HEK, human embryonic kidney; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; IL, interleukin; PP2A, protein phosphatase 2A; S3T3, Swiss3T3 cells; NFκB, nuclear factor κB; IκB, NFκB inhibitor; IKK, IκB phosphorylating kinases; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin.

1 The abbreviations used are: PKA, protein kinase A; PKB, protein kinase B; PKC, protein kinase C; PKG, protein kinase G; PDK1, 3-phosphoinositide-dependent kinase 1; PDK2, 3-phosphoinositide-dependent kinase 2; S6K1, ribosomal S6 kinase 1; RSK, ribosomal S6 kinase; PMA, phorbol 12-myristate 13-acetate; Ptcln5, phosphoinositide; PI 3-K, phosphoinositide 3-kinase; TPCK, N-α-tosyl-L-phenylalanyl chloromethyl ketone; TCK, N-α-tosyl-L-lysyl chloromethyl ketone; HEK, human embryonic kidney; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; IL, interleukin; PP2A, protein phosphatase 2A; S3T3, Swiss3T3 cells; NFκB, nuclear factor κB; IκB, NFκB inhibitor; IKK, IκB phosphorylating kinases; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin.
As total PDK1 kinase activity in cells appears largely unaffected by growth factor stimulation, the growth factor steps in PDK1 phosphorylation of AGC kinases involve regulation of their steric and spatial accessibility to PDK1 (1, 3, 4). Our current understanding of the molecular mechanisms of this accessibility shows rich complexity when considering the four best-studied PDK1-activated enzymes: Akt, S6K1, RSK, and PKC isoforms. Akt is activated following the activation of phosphoinositide 3-kinase (PI 3-K). PI 3-K activity generates P3-phosphoinositides. Two of these products, PtdIns3-4,5-P3 and PtdIns-3,4,5-P3, bind to the Akt amino-terminal pleckstrin homology domain and exert a dual activating effect. First, this binding of PI 3-K lipid products relieves structural auto-inhibition and exposes the Akt activation loop to PDK1 (24). Second, as PDK1 harbors a carboxy-terminal pleckstrin homology domain, which also binds PI 3-K lipid products, PI 3-K activity co-localizes Akt and PDK1 at the plasma membrane leading to rapid Akt activation following stimulation (1, 3, 25, 26). S6K1 and RSK likewise appear to require stimulus-regulated exposure of their activation loops. Unlike Akt, however, S6K1 and RSK do not contain pleckstrin homology domains. However, their interaction with PDK1 appears more stable than the interaction between PDK1 and Akt as S6K1 and RSK have been shown to co-immunoprecipitate with PDK1 (11, 18, 27–29). PKC isoforms also interact directly with PDK1 (11, 27), but activation loops of conventional PKC isoforms appear to be freely accessible to PDK1 in unstimulated cells. Thus PDK1 is able to phosphorylate them co-translationally (30). Full activation of conventional PKCs is obtained after subsequent agonist-dependent steps (30).

The development of specific inhibitors of AGC kinase members or of their proximal regulators has been critical in defining their individual roles in signal transduction. More broadly, inhibitors of AGC kinases are being pursued as clinical therapeutics. The fungal macrolide rapamycin is a potent immunosuppressant and inhibits the activation of S6K1. Rapamycin is now being evaluated in phase III clinical trials for its ability to synergize with another mechanistically different immunosuppressant, cyclosporin A, in reducing acute rejection of transplanted tissue (31). As S6K1 mediates important events for cell growth and proliferation (32–34), the immunosuppressant activity of rapamycin may be due, at least in part, to its inhibition of S6K1. To more fully understand the biological role of S6K1, we previously evaluated the effect of the enzyme inhibitors N-o-tosyl-l-phenylalanyl chloromethyl ketone (TPCK) and N-o-tosyl-l-lysyl chloromethyl ketone (TLCK) on S6K1 activation given their known anti-proliferative and anti-tumorigenic capacity (35–40). Interestingly, like rapamycin, TPCK (and less potently, TLCK) inhibited the activation of S6K1 by multiple agonists. However, TPCK, but not rapamycin, inhibited S6K1 activation in rapamycin-resistant T-cell lines (41). This suggested that TPCK and rapamycin inhibited S6K1 via distinct molecular mechanisms. To elucidate potentially novel regulatory mechanisms acting on S6K1 or its upstream activators and to define more precisely the molecular mechanisms whereby TPCK exerts its potent biological effects, we sought to identify the cellular target(s) of TPCK that control the mitogenic activation of S6K1.

**EXPERIMENTAL PROCEDURES**

**Mammalian Cell Culture and Transfection—**E1A-transformed human embryonic kidney 293 (HEK 293E) cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS). Swiss3T3 (S3T3) cells were maintained in DMEM supplemented with 5% FBS and 5% calf serum. NIH3T3 cells harboring Myr-Akt-HA-MER (a gift of R. Roth) were maintained in DMEM supplemented with 10% calf serum and 0.4 μg/ml G418. 32D cells (a gift of M. Meyers) were maintained in RPMI supplemented with 10% FBS and 5% WEHI-conditioned media as a source of IL-3. C2C12 myoblasts harboring each of the empty vector pMV7 or pMV7-HA-S6K1 were generated by D. Fingar and J. Blenis and were maintained in 20% FBS and 0.4 mg/ml G418. All cells were maintained in the presence of penicillin (200 units/ml) and streptomycin (20 μg/ml) as antibiotics. Transfection of NIH3T3 cells was by calcium phosphate precipitation and the total amount of DNA per transfected 60- and 100-mm dish between 3 and 5 μg and 6 and 10 μg, respectively. After 4–6 h exposure to the precipitate, cells were washed once with DMEM and then treated as indicated in the figure legends.

**Immunoprecipitation, in Vitro Kinase Assays, Immunoblotting, and Antibodies—**For HEK 293E cells, S3T3 cells, NIH3T3 cells harboring HEK 293E, S3T3 cells, NIH3T3 cells harboring Myr-Akt-HA-MER, and C2C12 myoblasts harboring Myr-Akt-HA-S6K1, cells were washed once with serum-free DMEM and then returned to the same for 24 h prior to drug treatment and stimulation as indicated in the figure legends. For 32D cells, the cells were washed three times in 10–15 ml volumes of RPMI and then incubated in the same for 3–4 h prior to drug treatment and stimulation as indicated in the figure legends. Cell lysis, immune complex kinase assays (RSK, Akt, S6K1), and immunoblotting were performed essentially as described previously (42). PKD1 kinase assays were performed as for RSK, Akt, and S6K1 except that protein G-Sepharose was used to immunoprecipitate Myc-tagged PKD1 proteins, a GST-PKCα fragment was used as substrate, and kinase reactions were allowed to proceed for 15–20 min. Assays in Fig. 6E evaluating the direct effect of TPCK on PDK1 in vitro were performed as follows: using baculovirus-purified His6-PDK1 (a gift from A. Tavigation, no. 6549) and His6-PDK1 (25–50 μg/ml) with either ethanol or 250 μM TPCK at room temperature for 30 min in kinase buffer without ATP. The final concentration of ethanol did not exceed 3%. Subsequently, 20 μl of each master mix were aliquoted into tubes containing 10 μl of a substrate mix consisting of kinase buffer, ATP, and various concentrations of GST-PKCa as indicated in the figure legends. Kinase assays were allowed to proceed for 15 min before the addition of 10 μl of 4× sample buffer. Samples were boiled and analyzed as indicated in the figure legends. For PKA assays, 5 μl of cleared lysates was used in a 30–40-μl kinase reaction using 8-Bromo-cAMP (Sigma) and protein kinase inhibitor (Sigma catalog #8140) as indicated in the figure legends. GST-BAD S112A/S136A and GST-BAD S136A were used as substrates as indicated. PI 3-K assays were performed essentially as described previously (43). Briefly, cells were lysed in 25 mM Tris, pH 7.2, 137 mM NaCl, 10 mM glycerol, 1% I-GEPAL (Nonidet P-40), 10 mM sodium phosphate buffer with either ethanol or 250 μM TPCK for 15 min at room temperature. 20 μl of each lysate were used in a 30–40-μl kinase reaction using 8-Bromo-cAMP (Sigma) and protein kinase inhibitor (Sigma catalog #8140) as indicated in the figure legends. GST-BAD S112A/S136A and GST-BAD S136A were used as substrates as indicated. PI 3-K assays were performed essentially as described previously (43). Briefly, cells were lysed in 25 mM Tris, pH 7.2, 137 mM NaCl, 10 mM glycerol, 1% I-GEPAL (Nonidet P-40), 10 mM sodium phosphate buffer with either ethanol or 250 μM TPCK for 15 min at room temperature. 20 μl of each lysate were used in a 30–40-μl kinase reaction using 8-Bromo-cAMP (Sigma) and protein kinase inhibitor (Sigma catalog #8140) as indicated in the figure legends.
of [32P]orthophosphate (ICN no. 64013). Cells were radiolabeled for 100 min prior to drug treatments as indicated in the figure legends.

**Apoptosis Assays**—For IL-3 withdrawal-induced apoptosis, 32D cells were washed three times in 10–25 ml of RPMI and resuspended in RPMI supplemented with 10% FBS without WEHI-conditioned media for 13–18 h. Cells were then washed once with ice-cold phosphate-buffered saline and resuspended in phosphate-buffered saline with 40 mg/ml of propidium iodide and analyzed by fluorescence-activated cell sorting. Dead cells stained positive for propidium iodide. For TPCK-induced apoptosis cells were washed as described above and then starved of IL-3 for 2–3 h prior to adding the concentrations of drug indicated in the figure legends. After incubation with a drug for 20 min, cells were given recombinant IL-3 (0.5 ng/ml) and then assayed by fluorescence-activated cell sorting.

**Fluorescence-activated cell sorting as described above.**

**Cells** were given recombinant IL-3 (0.5 ng/ml) and then assayed by fluorescence-activated cell sorting. Dead cells stained positive for propidium iodide. For TPCK-induced apoptosis cells were washed as described above and then starved of IL-3 for 2–3 h prior to adding the concentrations of drug indicated in the figure legends. After incubation with a drug for 20 min, cells were given recombinant IL-3 (0.5 ng/ml) and then assayed by fluorescence-activated cell sorting as described above.

**Plasmids and Constructs**—pKH3-HA-S6K1 (rat) and pGEX-4T-1-GST-BAD (murine) wild type and mutant plasmids (42) were described previously. The pGEX-3X-GST-PKCζ fragment was generated by cutting full-length PKCζ (rat) using a 5′-internal BglII site and a 3′-vector EcoRI site and ligated to pGEX-3X digested with BamHI and EcoRI, pCDNA3-Myc-PDK1 S241E (human) was generated using the QuikChange approach (Stragene) and introduced an internal EcoRI site. pCMV6-HA-Akt1 (murine) and pCMV6-Myr-Akt1-HA were gifts of P. Tsichlis. pCDNA3-HA-Akt1 T308A was a gift of A. Toker. pCDNA3-Myc-PDK1 and pCDNA3-Myc-PDK1 K1111 were gifts of P. Hawkins.

**RESULTS**

A rapamycin-resistant form of S6K1 was described previously (46) and was generated by deletion of its amino and carboxyl termini (S6K1 ΔNT/CT). The basis for the rapamycin resistance of this allele is proposed to be due to the deletion of a protein phosphatase 2A (PP2A)-binding region in S6K1 thus preventing PP2A negative regulation of S6K1. PP2A is itself negatively regulated by the mammalian target of rapamycin (47). Thus, in the presence of rapamycin the mammalian target of rapamycin is unable to repress PP2A allowing PP2A to bind to and inactivate wild type S6K1 but not S6K1 ΔNT/CT. To determine whether TPCK and rapamycin employed different methods in their inhibition of S6K1, we asked if the rapamycin-resistant S6K1 was sensitive to TPCK. This mutant was transiently expressed in E1A-transformed HEK 293E cells, and its activation was found to be rapamycin-resistant but TPCK-sensitive (Fig. 1) confirming the distinct inhibitory mechanisms of rapamycin and TPCK on S6K1.

Akt has been shown to positively regulate S6K1 when constitutively activated by the addition of a membrane-targeting myristoylation sequence (48). This alteration of Akt approximates the Gag-Akt fusion found in its transforming viral homologue v-Akt (49). A biological connection between Akt and S6K1 was further suggested as targeted disruption of the Dro sophila alleles of Akt and S6K1 give strikingly similar small fly phenotypes (50, 51). We therefore asked whether Akt activation is also prevented by TPCK. Pretreatment of HEK 293E cells with TPCK inhibited insulin activation of Akt to an extent comparable with the inhibition seen with the PI 3-K inhibitor wortmannin (Fig. 2A). Similar results were obtained using Swiss3T3 (S3T3) cells (data not shown). The concentration of TPCK required to fully inhibit Akt and S6K1 in S3T3 cells and HEK 293E cells was between 25 and 50 μM. The IC₅₀ for Akt in S3T3 cells was half the IC₅₀ for Akt in HEK 293E cells when compared directly (data not shown).

The biochemical properties of halomethyl ketones such as TPCK make them effective, irreversible alkylating agents. Alkylation occurs when the structure of the target protein can simultaneously accommodate the drug and bring it into the proximity of the R-group of either a histidine or cysteine residue. TPCK alkylation of histidine occurs at position 1 of the imidazole ring (52), whereas for cysteine TPCK alkylation occurs at the sulfhydryl group (53). To determine whether TPCK is an irreversible inhibitor of Akt activation, S3T3 cells were pretreated with increasing amounts of TPCK or the reversible PI 3-K inhibitor, LY294002 (54). Following treatment, the cells were either washed or maintained in the presence of the inhib-
Akt exhibiting constitutive activity. As shown in Fig. 5A, wild-type Akt was fully inhibited by treating the cells with TPCK for 20 min as indicated. The cells were then stimulated with 50 μM TPCK for 20 min. As shown in Fig. 5D, pretreatment of cells with TPCK prevented the phosphorylation of threonine 229 in activated Akt, implying that TPCK was alkylating either Akt directly or one of its upstream activators.

Inhibition of S6K1 and Akt by similar concentrations of TPCK suggested that TPCK was inhibiting an upstream regulator common to both S6K1 and Akt. S6K1 and Akt share at least two upstream activators, PI 3-K and PDK1 (2). The effect of TPCK on PI 3-K was first analyzed. PI 3-K (Class IA) signaling is initiated by the association of the 110-kilodalton catalytic subunit (p110)/85-kilodalton regulatory subunit (p85) heterodimer with phosphotyrosine residues on activated receptor complexes. This brings the heterodimer into proximity of substrate at the plasma membrane. This association occurs rapidly following mitogenic stimuli (3). As a relatively small proportion of active PI 3-K heterodimers finds access to these receptor complexes, a good reflection of mitogen-induced PI 3-K activity can be assessed in lipid kinase reactions following immunoprecipitations using anti-phosphotyrosine antibodies. As shown in Fig. 3, pretreatment of HEK 293 cells with TPCK did not inhibit the recruitment of p85 to the receptor complex and did not inhibit phosphotyrosine-associated PI 3-K activity.

Full activation of Akt requires two PI 3-K-regulated phosphorylation events: PDK1 phosphorylation of the activation loop at threonine 308 as well as phosphorylation of serine 473 in the carboxyl terminus (1–4). The relevant in vivo kinase responsible for phosphorylation of serine 473 has been referred to as “PDK2” (55). PDK2 activity has been reported to be a result of autophosphorylation (56), modified PDK1 activity, (57) as well as heterologous kinase activity (58). To determine whether one or both of these phosphorylation events was sensitive to TPCK, we made use of phosphospecific antibodies generated to specifically recognize either serine 473-phosphorylated Akt or threonine 308-phosphorylated Akt. As shown in Fig. 4A, pretreatment of HEK 293E cells with TPCK prevented the phosphorylation of Akt at serine 473. Similar results were seen in 7S3T3 cells (Fig. 7 and data not shown). The phosphothreonine 308-specific antibody required a higher concentration of epitope than the one that recognizes phosphoserine 473, so it was necessary to first immunoprecipitate overexpressed HA-tagged Akt prior to immunoblotting. Fig. 4B shows the resultant blot indicating that phosphorylation of threonine 308 is also prevented by treating cells with TPCK prior to stimulation.

Once stimulated, TPCK treatment of cells diminished Akt activity only gradually, reducing it to roughly half-maximal after 30 min (Fig. 5A). Thus, activated Akt is much less sensitive to TPCK. This implies that the gradual decline in Akt kinase activity observed after TPCK delivery to stimulated cells represented only the normal kinetics of inactivation via phosphatases or protein instability. A similar experiment was performed assessing Akt activity following TPCK treatment of cells expressing either wild type Akt or a myristoylated allele of Akt exhibiting constitutive activity. As shown in Fig. 5B, wild type Akt was fully inhibited by treating the cells with TPCK prior to stimulation. However, the activated allele of Akt was resistant to 30 min of TPCK treatment. Strikingly, however, this same allele, when under the inducible control of a mutant estrogen receptor (MER) fused to its carboxyl terminus (48), was fully inhibited by TPCK regardless of whether TPCK was washed away prior to addition of the inducing agent, 4-hydroxy-tamoxifen (Fig. 5C). Taken together, these data suggested that TPCK inactivation of Akt was primarily through the inhibition of an upstream activator of Akt. As a result of these observations we asked whether TPCK inhibition of Akt activation represented a unique disruption of PDK1 signaling. To further examine this possibility, we analyzed the effect of TPCK on the phosphorylation state of the PDK1 site, threonine 229, in S6K1. C2C12 myoblasts harboring either empty vector or HA-tagged S6K1 were starved of serum for 24 h prior to pretreatment with either vehicle or with 50 μM TPCK for 20 min as indicated. The cells were then stimulated with 100 nM insulin for 5 min prior to lysis. Lysates were subjected to immunoprecipitation with anti-phosphotyrosine antibodies. Immunoprecipitates were then divided equally for use in A and B as described above, were subjected to immunoblot analysis using antibodies directed against the p85 subunit of PI 3-K as insensitive to TPCK. Anti-phosphotyrosine immune-complexes, partitioned equally for use in A and B as described above, were subjected to phosphorimaging quantitation using anti-phosphotyrosine antibodies. Immune complexes were then divided equally for use in A and B. A, phosphotyrosine-associated PI 3-K immune complexes were subjected to lipid kinase assays using phosphatidylinositol (PtdIns) and phosphatidylinositol-4-phosphate (PtdIns 4-P) as substrates. Products were separated by thin layer chromatography. Thin layer chromatography plates were then analyzed by autoradiography (A) and phosphorimaging quantitation (C). B, phosphotyrosine recruitment of the p85 subunit of PI 3-K is insensitive to TPCK. Anti-phosphotyrosine immune-complexes, partitioned equally for use in A and B as described above, were subjected to phosphorimaging quantitation is shown of PI 3-K-generated lipid products from assays described in A.
inhibition of PI 3-K had no effect on PDK1 kinase activity (Fig. 6A), consistent with PI 3-K lipid products having more bearing on PDK1 localization than on kinase activity per se (59). Although immunoprecipitated PDK1 kinase activity was not significantly affected by TPCK, TPCK did strikingly alter its electrophoretic mobility in SDS-polyacrylamide gels (Fig. 6A). This implied that TPCK was inducing a posttranslational modification that could be responsible for its inability to signal to substrates. PDK1 overexpressed in HEK 293 cells exhibits constitutive, unregulated phosphorylation at five serine residues (22). When mutated individually to alanine, only one of these mutations, serine 241 in the activation loop, affected PDK1 kinase activity toward heterologous substrates in vitro (22). Mutation of serine 241 to glutamic acid (S241E) generated a pseudo-phosphorylated, active kinase with reduced specificity (22). To determine whether the increased electrophoretic mobility of PDK1 was due to loss of phosphorylation at serine 241, we generated the S241E mutant of PDK1 and expressed it in HEK 293E cells alongside wild type PDK1. We then examined the effect of TPCK on the respective electrophoretic mobilities and kinase activities. As seen in Fig. 6B, TPCK increased the electrophoretic mobility of both of these forms of PDK1. Additionally, TPCK had no effect on PDK1 autophosphorylation (Fig. 6B). As expected, the PDK1 S241E mutant has greatly reduced autophosphorylating activity, but its ability to phosphorylate a GST-PKCζ substrate, like wild type PDK1, was unaffected by TPCK (data not shown). This showed that loss of phosphorylation at serine 241 was not responsible for the TPCK-induced mobility profile of PDK1. In a similar experiment a kinase-inactive lysine 111 to an isoleucine mutant of PDK1 exhibited the same electrophoretic mobility as both the wild type and S241E (Fig. 6C) showing that the increase in mobility was independent of catalytic activity and not due to a loss of cis-autophosphorylation. Given these results, we analyzed the effect of TPCK on the steady state levels of PDK1 phosphorylation by [32P]orthophosphate metabolic labeling of cells expressing either wild type PDK1 or S241E PDK1. As shown in Fig. 6D, steady state levels of phosphate incorporation into PDK1 were unaffected by TPCK treatment despite the noticeable increase in PDK1 electrophoretic mobility. This suggested the possibility of novel regulatory mechanisms of PDK1 signaling in vitro. Notwithstanding this dramatic effect in vitro, Fig. 6E shows that TPCK did not appear to react significantly with PDK1 in vitro even when subjected to 5× the concentrations of TPCK used on cells. Additionally, Fig. 6E shows that under these conditions TPCK did not alter PDK1 kinase activity, did not compete directly for substrate, and did not alter the substrate-induced increase in autophosphorylation previously observed using a phospho-RSK peptide (29). This peptide contained the phospho-PDK2 site, the hydrophobic sequence that was shown to serve as a PDK1 docking site in RSK (29). As PKCζ has a glutamic acid at this residue it is able to likewise induce increased PDK1 autophosphorylation.

Because TPCK did not significantly affect PDK1 phosphotransferase activity yet was able to block activation of Akt and S6K1 by preventing phosphorylation of their activation loops, we asked if TPCK disrupted PDK1 signaling to RSK. This served as an important control as RSK activation, unlike the activation of Akt and S6K1, is largely independent of PI 3-K signaling. Recent work has shown that in addition to MAPK, PDK1 activity is critical to RSK activation as PDK1 phosphorylates serine 221 (human) in the activation loop of the RSK amino-terminal kinase domain (18, 19, 23). Additionally, myristoylation of RSK, as with Akt, renders the kinase active even in the absence of growth factors (42). Such targeting to membrane is presumed to both locate RSK more proximal to PDK1 as well as remove autoinhibitory structural constraints on the access of PDK1 to serine 221. Fig. 7 shows TPCK inhibition of RSK in three different cell types. Interestingly, whereas the concentrations of TPCK used to inhibit Akt, S6K1, and RSK were inconsequential to MAPK activation in 32D and S3T3 cells (data not shown, (41)), TPCK enhanced MAPK activation in HEK 293E cells (Fig. 7B). Notwithstanding this enhancement, RSK activity was only marginally increased over baseline in HEK 293E cells treated with TPCK (Fig. 7B) underscoring the role of PDK1 in the activation of RSK.

Recently, in the IL-3-dependent murine hematopoietic cell lines 32D and FL5.12, we showed the ability of RSK to transduce a cell survival signal in part through phosphorylation of the proapoptotic Bad family member, BAD (42). We thus analyzed the effect of TPCK on BAD phosphorylation as well as its effect on cell survival in 32D cells. 32D cells were starved of IL-3 for 2 h and then treated with either vehicle or TPCK prior to the re-addition of IL-3 for 10 min. Following lysis and immunoprecipitation, immune complexes of endogenous BAD were analyzed by immunoblotting. TPCK prevented the IL-3-induced mobility shift of BAD that corresponded with the loss of RSK phosphotransferase activity (Fig. 7C). In agreement with TPCK inhibition of the IL-3-activated prosurvival kinases RSK and Akt, increasing concentrations of TPCK antagonized IL-3 survival signaling with the amount of cell death after 16 h in the presence of 25 μM TPCK plus IL-3 roughly corresponding to the amount of cell death 16 h after IL-3 withdrawal (Fig. 7D).

PDK1 is able to phosphorylate the activation loop of PKA in vitro and lead to an increase in PKA activity when overexpressed in cells (17). PKA, however, can still be activated in embryonic stem cells lacking PDK1 (23). This provided evidence that PDK1 is not required for PKA activation despite the similarities between the primary amino acid sequence surrounding the phosphorylation site in PKA’s activation loop and that of other known PDK1 substrates (Fig. 8A). This also proposes an important control for the specificity of TPCK in PDK1-mediated signaling. If TPCK were to inhibit PKA it would...
suggest that TPCK broadly affects AGC-kinases outside of those that require PDK1 for full activation. Fig. 8, B and C show that PKA from both untreated and TPCK-treated cells was similarly activated by 8-Bromo-cAMP as measured by the ability of PKA to phosphorylate in vitro either GST-BAD S136A or S112A/S136A, which is consistent with reports showing PKA phosphorylation activity toward serine 155 of BAD (60, 61).

DISCUSSION

More than 30 years ago (35) and again 20 years ago (38), topically applied TPCK was shown to potently inhibit tumorigenesis initiated in mouse skin by 7,12-dimethylbenz[a]anthracene and promoted by PMA. This anti-tumorigenic effect was also accompanied by a reduction in erythema and leukocyte infiltration at the site of PMA application (35). It was also reported that the survival of mice prone to spontaneous breast cancer was significantly increased when treated with 1 mg of TPCK/week without signs of toxicity after 45 weeks of treatment (35). Additionally, many cell types treated with TPCK have reduced proliferative rates (36, 37, 39). Despite these earlier observations, little was known about the cellular target(s) of TPCK that govern these effects. Given the role of S6K1 in cell growth and proliferation, and as it is a target of the immunosuppressant rapamycin, we evaluated the effect of coexpression of Akt with gene products encoding the serine protease inhibitors plasminogen activator inhibitor-2 (63), the biochemical properties of TPCK do not directly implicate it as a serine protease inhibitor a priori. Whereas we cannot formally exclude the possibility of chymotrypsin-like serine protease activity regulating PDK1 signaling, we have evaluated the effect of coexpression of Akt with gene products encoding the serine protease inhibitors plasminogen activator inhibitor-2 (64) and the related cowpox virus-encoded cytokine response modifier A (65, 66) and saw no effect on insulin-stimulated Akt activation (data not shown).

However, the inhibitory mechanisms of halomethyl ketones toward serine proteases may permit important inferences about the mechanism of the inhibition of TPCK toward PDK1 signaling. TPCK was shown to irreversibly alkylate chymotrypsin...
sin at a one to one molar ratio at histidine 57 (62). The specificity of TPCK for chymotrypsin was accomplished as chymotrypsin proteolyses proteins containing the large hydrophobic and aromatic residues tyrosine or phenylalanine at the P1 position of the target sequence. Additionally, denatured chymotrypsin was not alkylated by TPCK indicating the requirement for chymotrypsin to be in a folded configuration presumably generating a hydrophobic pocket that can accommodate either an aromatic P1 amino acid or the hydrophobic, aromatic rings of TPCK. Along these lines, the lysyl-derivative TLCK has no effect on chymotrypsin but specifically inhibits trypsin. This follows given that the lysyl derivative mimics the trypsin preference for basic amino acids in the P1 position of target proteins (52). If TPCK is acting directly on PDK1 in vivo, it
implies that PDK1 contains a hydrophobic pocket that could both accommodate an aromatic compound as well as be structurally important for its activation of or interaction with substrates. Indeed one such pocket was recently identified in PDK1 and was shown to accommodate two phenylalanine residues from a hydrophobic motif found in PDK1 substrates and thereby facilitated their activation (28). This hydrophobic motif has been shown recently to be essential for PDK1 docking to and subsequent activation of RSK (29). Interestingly, TPCK, TLCK, and the valyl derivitive, TVCK, exert different effects on the activation of S6K1. In cells TPCK fully inhibits S6K1 between 25 and 50 μM, whereas TLCK becomes fully inhibitory at 350 μM (41), and the valyl derivative, TVCK, becomes inhibitory at 500 μM (data not shown). This implies that the structure of the halomethyl ketone is critical to its ability to prevent PDK1 signaling. However, it is formally possible that the primary reason for the observed differences in inhibition of S6K1...
by these halomethyl ketones is a function of their solubilities or abilities to penetrate the cell membrane.

A number of molecules have shown inhibition by TPCK in vitro. However, the concentrations used in vitro far exceeded the concentrations effective to inhibit PDK1 signaling and induce cell death in vivo. For example, TPCK inhibition of PKC in vitro occurs at an IC_{50} of 8 μM (67). TPCK inhibition of PKA in vitro occurred at concentrations between 340 μM and 1 mM (53) yet we show here that TPCK had no effect on PKA activity from cells treated with concentrations of TPCK that inhibit PDK1 signaling (Fig. 8, B and C). Likewise PKC activation was not inhibited when cells were treated with antiproliferative doses of TPCK (39). We have assayed the in vitro effect of TPCK on PDK1, Akt, and S6K1 purified either from baculoviral expression systems or via immune complexes from mammalian cell lysates. No inhibition of any of these kinases was detected at TPCK concentrations of <500 μM (Fig. 6E, and data not shown), and <5% inhibition was seen after treating the kinases with 1–2 mM TPCK (ref. 41 and data not shown). These data suggest that either the chemical makeup of the cell greatly facilitates TPCK conjugation to PDK1 or that TPCK exerts its effects differentially in vivo and in vitro.

Alternatively, TPCK may not be acting via direct inhibition of the kinase activity of PDK1 or its kinase substrates but may be preventing a productive PDK1-substrate interaction inside the cell. This is consistent with the data presented in Fig. 5 that suggest TPCK is operating at the level of upstream activators of Akt in vivo. This is also consistent with the observation that the activation of conventional PKC isoforms is insensitive to TPCK (39) as they are co-translationally phosphorylated by PDK1 (30), and thus TPCK treatment for 20–30 min prior to stimulation would have little effect on conventional PKC activity. We are aware of only one protein that has been directly conjugated to TPCK in vivo, the transforming protein E7 from the human papillomavirus-18 (68). Concentrations of ~200 μM TPCK or 250 μM TLCK were required to fully modify cysteine 27 of the E7 protein stably expressed in a human foreskin keratinocyte cell line (68). Interestingly, such a modification increased the E7 electrophoretic mobility. This increase in mobility was reversed by mutating cysteine 27 to glycine (68).

TPCK has been shown to induce cell death in a number of cell types at concentrations that should inhibit PDK1 signaling (40, 69, 70). It is interesting that protection from TPCK-induced death has been observed in cells overexpressing Bcl-XL (69) and c-Rel (69) or when flooded with a high concentration of α-acevulin-cysteine (40), perhaps generating competition for TPCK binding. This argues against TPCK-induced cytotoxicity and for its inhibition of signaling pathways that mediate cell survival including kinase cascades that modulate mitochondrial integrity via phosphorylation of Bcl-2 family members.

In recent years TPCK has been shown to be a potent inhibitor of nuclear factor κB (NFκB) signaling (69, 71). Although the exact mechanism behind this inhibition is unknown, TPCK has been shown to inhibit the phosphorylation of the inhibitor of NFκB (IκB), implying TPCK is acting at either the level of the kinases that phosphorylate IκB (IKK) or their upstream activators. If the effect of TPCK on NFκB signaling were via its inhibition of PDK1 signaling then the simplest explanation is explained by the inhibition of the PDK1-activated kinases Akt and RSK, both of which have been shown to positively regulate NFκB signaling (72, 73). However, we find it an intriguing possibility that PDK1 plays a more direct role in NFκB signaling perhaps by directly phosphorylating the activation loops of IKK-1 and IKK-2. The activation loops of IKK-1 and -2 contain two regulated serine residues, serines 176 and 180 for IKK-1 and serines 177 and 181 for IKK-2 (both human). The phosphorylation of these residues is required for full kinase activity (71). Comparison of the amino acid context surrounding the second phosphorylated serine in the activation loops of IKK-1 and -2 with the sequence surrounding known PDK1 phosphorylation sites shows remarkable similarity (Fig. 8A). This raises the possibility that TPCK exerts such a potent inhibitory effect on NFκB signaling by disabling multiple PDK1-dependent inputs.

Considerable research has been conducted using the halomethyl ketone TPCK, and the observed effects of TPCK have extended into multiple aspects of normal and aberrant cellular processes with little knowledge of its specific cellular target molecules. The results presented here show that TPCK disrupts PDK1 signaling to Akt, S6K1, and RSK and thereby disrupts central pathways involved in transducing extracellular stimuli into meaningful cellular events such as growth, proliferation, and survival. This understanding serves not only as a starting point for further elucidation of its precise inhibitory mechanism on PDK1 signaling but invites re-examination of the potential use of TPCK as a therapeutic agent as a means of controlling aberrations resulting from inappropriate PDK1 signal propagation.

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