Study of the PDK1/AKT signaling pathway using selective PDK1 inhibitors, HCS, and enhanced biochemical assays

Alexandra Hofler a, Tim Nichols b, Stephan Grant a, Laura Lingardo a, Edward A. Esposito c, Scott Gridley c, Sean T. Murphy d, John C. Kath d, Ciarán N. Cronin d, Michelle Kraus d, Gordon Alton a, Zhi Xie a, Scott Sutton d, Mike Gehring e, Jacques Ermolieff a,⇑

a Department of Oncology, Pfizer, La Jolla, San Diego, CA 92121, USA
b Department of Drug Safety Research and Development, Pfizer, La Jolla, San Diego, CA 92121, USA
c Blue Sky Biotech, Worcester, MA 01605, USA
d Department of Chemistry, Pfizer, La Jolla, San Diego, CA 92121, USA
*e Department of External Research Solution CoE at Pfizer, La Jolla, San Diego, CA 92121, USA

A R T I C L E   I N F O
Article history:
Received 21 November 2010
Received in revised form 21 February 2011
Accepted 8 March 2011
Available online 12 March 2011

Keywords:
PDK1
AKT
Kinase inhibitor
Signal transduction
Template-directed assembly (TDA)

A B S T R A C T
The PI3K/AKT signaling pathway has an important regulatory role in cancer cell growth and tumorigenesis. Signal transduction through this pathway requires the assembly and activation of PDK1 and AKT at the plasma membrane. On activation of the pathway, PDK1 and AKT1/2 translocate to the membrane and bind to phosphatidylinositol-(3,4,5)-trisphosphate (PIP3) through interaction with their pleckstrin-homology domains. A biochemical method was developed to measure the kinase activity of PDK1 and AKT1/2, utilizing nickel-chelating coated lipid vesicles as a way to mimic the membrane environment. The presence of these vesicles in the reaction buffer enhanced the specific activity of the His-tagged PDK1 (full-length, and the truncated kinase domain) and the activity of the full-length His-tagged AKT1 and AKT2 when assayed in a cascade-type reaction. This enhanced biochemical assay is also suitable for measuring the inhibition of PDK1 by several selective compounds from the carbonyl-4-amino-pyrrolopyrimidine (CAP) series. One of these inhibitors, PF-5168899, was further evaluated using a high content cell-based assay in the presence of CHO cells engineered with GFP-PDK1.

© 2011 Elsevier Inc. All rights reserved.
Not surprisingly, the constitutive activation of the PI3K/AKT pathway plays a major role in the development and the survival of various types of cancers due either to the loss of PTEN activity or to the increase of PI3K and/or AKT activity [5–7]. For instance, AKT1 gene amplification and mutation occur in gastric and colorectal cancer, while AKT2 gene amplification has been observed in breast, ovarian, and pancreatic cancers [8]. In addition, mutations in PI3Kα or PTEN genes lead to aberrant proliferative signals and cellular transformation [9–11]. Currently, several AKT1, mTOR, and PI3Kα inhibitors have been reported in the literature, and a few are now either in preclinical or in advanced clinical stages [12,13]. While no late stage and selective inhibitor has been reported for PDK1, it nevertheless represents an attractive target for drug development. PDK1 belongs to the AGC kinase family and was first identified by Phil Cohen’s group in 1997 [14]. This enzyme has been characterized as a master kinase, due to its propensity to activate other important downstream AGC kinases such as AKT, P70 ribosomal S6 kinase (S6K), serum- and glucocorticoid-stimulated protein kinase (SGK), atypical and typical PKC, and p90 ribosomal S6 kinase (RSK). RNA antisense targeted against PDK1 in PTEN null cells significantly reduced their proliferation and survival [15], while overexpression of PDK1 in epithelial cells results in their transformation [16,17]. In addition, hypomorphic mutation of PDK1 protected PTEN +/- mice from developing a wide range of tumors [18]. Several nonselective inhibitors for PDK1 have already been reported in the literature [19] and have been shown to block survival of cancer cells.

In the present study, we first used a cell free model system composed of lipid vesicles with nickel-chelating head groups, TDA 2.0, that mimics the cellular microenvironment. Controlling the exact composition of the vesicles allowed us to study the mechanism of activation of AKT1 and AKT2 in the presence of PDK1 and mTOR. Under these conditions, we have been able to study the role that a few key residues play on the activity and the stability of the AKT enzymes and to observe the extent of PDK1 inhibition on AKT activation. Also, the potency of several novel inhibitors from the carbonyl-4-amino-pyrolopyrimidine (CAP) series was evaluated against PDK1. Comparative studies were conducted with two different assay formats and our data suggest that the presence of lipid particles does not affect the potency of these compounds. Overall, the addition of TDA 2.0 provides an enhanced biochemical assay method for measuring the activity of membrane anchored protein kinases and may be useful for kinase drug discovery and high-throughput screening platforms. Lastly, we used a GFP-PDK1 engineered CHO cell to highlight the effect of PDK1 selective inhibitors on the recruitment of PDK1 at the membrane, the phosphorylation state of AKT1, and the translocation of FoxO3a from the cytoplasm to the nucleus.

Materials and methods

Reagents and general enzymatic assay conditions

EDTA, Tris and Heps buffer, dimethyl sulfoxide (DMSO), ATP, DTT, magnesium chloride, and Brij35 were all purchased from Sigma-Aldrich (St. Louis, MO). Fluorescent-labeled AKT substrate (5FAM-GRPRSSSAEGCONH2) and PDK1 substrate (5FAM-ARKERTYSFGGHA-COOH) were purchased from Caliper LifeSciences (Hopkinton, MA). The PDK1 Omnia peptide (Ac-Sox-PKFTCGPTEYL APEVREPRLSEEEQEMFRDFDYIAD-NH2) was purchased from Invitrogen Life Technologies (Carlsbad, CA). The full-length human recombinant inactive N-terminal His-tagged AKT1 (aa 1–480) was purchased from Cell Sciences (Canton, MA). The full-length human recombinant His-tagged PDK1, the full-length human recombinant inactive His-tagged AKT2, and active mTOR (aa 1360–2549) were purchased from Life Technologies. TDA 2.0 protein assembly reagent was purchased from Blue Sky Biotech, Inc. (Worcester, MA). Recombinant human His-tagged PDK1 catalytic domain (aa 51–359) was made in-house at Pfizer La Jolla. CHOIR cells (PDK-CHO) stably expressing human PDK1 (GenBank Accession No. NM_0002613) coupled to the C-terminus of enhanced Green Fluorescent Protein (EGFP) were purchased from Thermo Fisher Scientific. Cells were maintained in Ham’s F12 media with 1% penicillin-streptomycin, 0.5 mg/ml Geneticin, and 10% heat-inactivated FBS (Gibco). For the cell assay, the rabbit polyclonal antibodies that specifically bind to phospho-AKT Thr, and FoxO3a were all purchased from Cell Signaling Technology. Hoechst and goat anti-rabbit IgG conjugated to AlexaFluor 532 were purchased from Invitrogen–Life Technologies. For the Western blot assay, anti-GST and anti-phospho-AKT Thr308 and Ser473 antibodies were purchased from Cell Signaling (Beverly, MA). The anti-phospho-AKT Thr450 antibody is from Abcam (Cambridge, MA). The goat anti-rabbit IgG–AP pAb is from Vector Labs (Burlingame, CA), the goat anti-mouse IgG–HRP pAb and the goat anti-rabbit IgG–HRP antibody were from Jackson ImmunoResearch (West Grove, PA). The anti-His mouse mAb was from Clontech (Mountain View, CA).

All the kinetic experiments were conducted at room temperature (~22 °C) and the concentrations of reagents reported in the following sections are reported as final in the media buffer. All the experimental data were generated in duplicate and were fitted using a nonlinear regression analysis software, GraphPad Prism 5 [20].

Compound synthesis and selectivity

The synthesis and selectivity of CAP compounds (Fig. 1) have been briefly described by Murphy et al. [21] and will be described in further details in a subsequent paper. Briefly, PF-5168899 was submitted to a broad kinase selectivity panel (see Supplementary Table 2) provided by Invitrogen and the University of Dundee (UK) as a fee-for-service and data were generated in the presence of 1 μM inhibitor against a panel of selected 60 kinases. In addition, PF-5168899 was also submitted to a smaller in-house kinase panel
and showed $K_i$ values > 1 μM against mTOR, Akt1, 6K, and PI3Kα [21].

Production of polyHis-tagged PDK1 (aa 51–359) kinase domain

A nucleotide sequence encoding amino acids 51–359 of human PDK1 was cloned into a custom baculovirus transfer vector that appended the cloned fragment with an N-terminal polyhistidine purification tag (MIYVMYHHHHHHDDYGPITENLYFQAL). Recombinant baculovirus was prepared using the Bac-to-Bac method (Invitrogen) and used to infect S9 insect cells (at moi = 1). Infected cells were harvested after 48 h and stored at ~80 °C. The insect cell pellet was lysed in 50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 0.25 mM TCEP, containing one “EDTA-free” protease inhibitor tablet (Roche) per 75 mL buffer. The suspension was centrifuged at 5000g for 1 h and the target bound to ProBond resin (5 mL; Invitrogen). The resin was washed overnight with 50 mM Tris-HCl, pH 7.4, 400 mM NaCl, 250 mM imidazole-HCl, pH 7.4, 1 mM TCEP, and the bound PDK1 step-eluted by using 50 mM Tris-HCl, pH 7.4, 400 mM NaCl, 250 mM imidazole-HCl, pH 7.4, 1 mM TCEP. PDK1 was concentrated to 2 mL by using an Amicon Ultracel 10K (Millipore) centrifugal concentrator and passed through a BioSep S-3000 gel filtration HPLC column (Phenomenex) equilibrated with 25 mM Tris-HCl, pH 7.4, 250 mM NaCl, 1 mM TCEP. The peak fractions were pooled and the PDK1 concentrated to 2.6 mg/mL. Protein concentration was determined by using the Coomassie Plus Protein Reagent (Pierce) with BSA (2 mg/mL; Pierce) as standard.

Complex formation and organizational activation of PDK1 enzyme activity by TDA 2.0 protein assembly reagent

The activity of PDK1 (full-length and catalytic domain) was measured with and without TDA 2.0 in 50 mM Tris buffer, 10 mM MgCl₂, 0.01% Tween 20, pH 7.4 with 5% DMSO and 1 mM ATP. PDK1 (50 nM) with and without TDA 2.0 (5 μM, initial concentration) was serially diluted 2-fold and added to Tris buffer. 5FAM-labeled PDK1 peptide (5 μM) was added in the reaction media in a 96-well V-bottom plate. The enzymatic reaction was initiated on addition of ATP (1 mM). An aliquot of the assay mixture was then transferred to a low volume 384-well black plate for determination of the relative amounts of substrate peptide and product phosphopeptide using a Caliper EZ-reader from which the rate of turnover was calculated (micromolar per minute). The substrate and product were separated on the basis of charge using upstream and downstream voltages of ~2250 and ~300 V, respectively, and a screening pressure of ~1.2 psi.

**Akt activation in the presence of mTOR and PDK1**

Activations of Akt1 and Akt2 were conducted in a similar Tris buffer (see previous section) with 2% DMSO. The reaction was conducted with 25 nM inactive Akt1 (1 and 2), 25 nM mTOR, 25 nM PDK1, and 2.5 PM TDA 2.0. The reaction was initiated with 5 μM Akt substrate and 1 mM ATP and the rate of substrate conversion was measured on a LabChip EZ-Reader. The instrument was set up to collect aliquots from the assay mixture at regular intervals. The upstream, downstream voltages and the pressure intervals were set to ~2800 and ~380 V, and 0.8 psi, respectively.

Determination of the apparent Michaelis–Menten constants, $k_{\text{app}}^{\text{m}}$ and $k_{\text{cat}}^{\text{app}}$ for PDK1

$k_{\text{app}}^{\text{m}}$ and $k_{\text{cat}}^{\text{app}}$ values for ATP were determined in the presence of 25 nM FL-PDK1 and 2.5 PM TDA 2.0 in 50 mM Tris buffer, 10 mM MgCl₂, 0.01% Tween 20, pH 7.4, with 5% DMSO. The enzyme was incubated for 10 min in the assay buffer in the presence of 5 μM 5FAM-PDK1 peptide in a 96-well V-bottom plate. The reaction was then initiated by the addition of various concentrations of ATP. Product phosphopeptide was determined as previously described (see previous section). $k_{\text{app}}^{\text{m}}$ and $k_{\text{cat}}^{\text{app}}$ values for 5FAM-labeled peptide were determined using the same experimental conditions in the presence of 1 mM ATP and various concentrations of peptide.

**Enzyme inhibition**

Inhibition studies were conducted using two assay formats, Omnia and Caliper. For the Omnia assay, $K_{\text{app}}^{\text{m}}$ studies were conducted in the presence of 20 nM KD-PDK1, 50 μM ATP, and 3 μM Sox peptide in a 50 mM Hepes, 5 mM MgCl₂, 0.01% Brij-35, 1 mM DTT assay buffer at pH 7.4. The increase of fluorescence ($\Delta F_{\text{ex}} = 360$ nm and $\Delta F_{\text{em}} = 485$ nm) was recorded continuously using a Safire TECAN plate reader. For the Caliper assay, the $K_{\text{app}}^{\text{i}}$ constant for FL-PDK1 alone was determined in the presence of 25 nM enzyme. For Akt1, the reaction was conducted with 25 nM inactive Akt1, 25 nM mTOR, 2.5 mM FL-PDK1. Both sets of Caliper inhibition studies were conducted with 2.5 μM TDA 2.0, 1 mM ATP, and 5 μM peptide in 50 mM Tris buffer, 10 mM MgCl₂, 0.01% Tween 20, pH 7.4, with 5% DMSO. The enzyme, the peptide, and various amounts of inhibitor were preincubated for 15 min, prior to addition of ATP to the reaction media.

$K_{\text{app}}^{\text{m}}$ and $K_i$ were calculated by fitting the experimental data to the following equations [22]

$$V_i = V_0 \left(1 - \frac{|E|_0 + |I|_0 + K_{\text{app}}^{\text{m}}}{|E|_0^2} \right)$$

(1)

where $K_{\text{app}}^{\text{m}} = K_i (1 + |A|_0 / K_m)$. (2)

$|E|_0$ and $|I|_0$ are the total active enzyme and inhibitor concentrations, respectively; $K_i$ is the inhibition binding constant; $V_i$ and $V_0$ are the rates of peptide phosphorylation in the presence or in the absence of inhibitor, respectively; $|A|_0$ is the total ATP concentration.

**Western blots**

Enzyme concentrations for Western analysis were as follows 200 nM Akt1 or Akt2, 200 nM mTOR, 20 nM FL-PDK1, and 20 pM TDA 2.0. Samples from kinase reactions were analyzed by SDS–PAGE (4–20% acrylamide tris-glycine, Lonza) using standard methods. Antibodies used were anti-His (Clontech), Phospho-AKT (Thr308) (C31E5E) (Cell Signaling), Phospho-AKT (Ser473) (D9E) (Cell Signaling), anti-GST (91G1) (Cell Signaling), goat anti-rabbit IgG-AP (Vector Labs), goat anti-mouse IgG-AP (Jackson ImmunoResearch). Immunoreactive bands were visualized using Western Blue stabilized substrate. Western blots were quantitated using ImageJ software (v1.43, http://rsbweb.nih.gov/ij/) (see Supplemental Fig. S1).

**High content cellular assay**

PDK1-CHO cells were plated out at 3000 cells/well in 384-well plates (Aurora Biosciences, San Diego, CA). After 24 h the cells were washed 3 times with Ham’s F12 containing 1% penicillin-streptomycin, 5 mM Hepes 0.1% FBS, and 0.01% BSA, and cultured for 2 h. Compounds containing 0.3% DMSO final were added in a 4X volume in assay media (i.e., Ham’s F12 containing 1% penicillin-streptomycin, 5 mM Hepes, and 0.1% BSA) and incubated for 2 h. Assay media with or without 1 mg/mL (4X) recombinant human IGF-1 were added to the cell culture using a Janus liquid handler with a 384-well head from Perkin Elmer. The supernatants were mixed by pipetting and allowed to incubate for 4 min at ambient room
temperature. After incubation, the cells were fixed with the addition of an equal volume of a neutral buffered 10% formalin solution (Sigma-Aldrich). Following fixation, cells were permeabilized with 0.5% Triton X-100 in Dulbecco’s PBS without calcium and magnesium for 30 min and blocked with 1.0% BSA in PBS for 2 h. Primary antibodies were added in a staining buffer (i.e., PBS containing 0.3% Triton X-100 and 0.5% FBS) overnight at 4°C in a humidified chamber. Plates were washed thoroughly using a Biotek Elk405 plate washer. Secondary antibodies in staining buffer were added and incubated 2 h at room temperature. Cells were washed in a solution containing 0.5 μg/ml Hoechst and 2 μg/ml HCS CellMask Deep Red. The plates were imaged using a Perkin Elmer Opera equipped with a UV light source, 488, 532, and 633 nm lasers. Analysis of the images was completed using Acapella algorithms custom designed for each assay.

Results

**PKD1 and AKT1/AKT2 activity in the presence of TDA 2.0**

PKD1 activity was measured using a small 14-mer 5FAM-labeled peptide (for peptide sequence see Materials and methods) in the presence and in the absence of TDA 2.0. As illustrated in Fig. 2a and b, the addition of lipid-based particles in the assay buffer boosts the PKD1 enzyme activity by ~4- to 5-fold for the catalytic domain and 20-fold for the full-length enzyme as compared to the enzyme alone. Additionally, data in Fig. 2c show that the activation occurs only in the presence of His-tagged PKD1. The actual effect of these artificial vesicles on the PKD1 activity remains to be fully understood; however, TDA 2.0 contain Ni²⁺-chelating moieties creating a template which directs the assembly of purified His-tagged proteins which are normally membrane associated; this approach has been utilized by several research groups with a broad range of protein classes [23]. Therefore, we speculate that it promotes assembly of relevant membrane-associated conformation, stimulating the trans-autophosphorylation and subsequently the trans-activation of PKD1 via protein colocalization, thus replicating the normal cellular effect of PIP₂, recruitment of PKD1 to the membrane via its PH domain. Further kinetic analysis was conducted with FLPDK1 and TDA 2.0 to determine a $K_{\text{cat}}^{\text{app}}$ and $K_{\text{m}}^{\text{app}}$ values of 13.6 ± 2.7 μM and 0.72 ± 0.024 min⁻¹ for ATP, respectively, and 25.5 ± 5.7 μM and 1.8 ± 0.18 min⁻¹ for the 5FAM-peptide. Unfortunately, we were unable to measure and compare these same constants in the absence of TDA 2.0 due to the lack of significant PKD1 activity toward the peptide substrate.

The effect of TDA 2.0 was also evaluated on the activation of AKT1 and AKT2 by FL-PKD1 and mTOR. As illustrated in Fig. 3a and b, AKT is readily activated when FL-PKD1, mTOR, and TDA 2.0 are simultaneously present in the reaction media. Interestingly, our experimental data showed that a brief burst of AKT2 activity was also recorded only in the presence of PKD1 and TDA 2.0 (Fig. 3b); however, the activity of AKT2 plateaued very rapidly, within 20 min, suggesting that enzyme stability is negatively affected when mTOR is absent from the assay buffer. These results are in agreement with previous studies conducted by Facchinetti et al. [24] that identify mTOR as a key enzyme responsible for the folding and the stability of AKT.

**Western blot analysis**

Western blot analysis of phospho-specific antibodies of samples from kinase assays indicates that addition of mTOR and PKD1 with AKT1 increases the level of phospho-Ser473 and phospho-Thr308 (Fig. 4). Addition of TDA 2.0 significantly increases phosphorylation on these residues as well. Surprisingly, Western blot analysis also showed that AKT1 and AKT2 seem to autophosphorylate on Ser473 when TDA 2.0 is present in the reaction media and that mTOR can phosphorylate both residues, Ser473 and Thr308. Lastly, residue Thr450 on AKT1 and AKT2 appears to be already phosphorylated prior to addition of mTOR and PKD1 to the media.

**PKD1 and AKT1 inhibition**

A few inhibitors from the CAP series (see list Table 1) were evaluated against FL-PDK1. The mechanism of inhibition of these inhibitors has been resolved by previous crystallography studies [21] which showed these compounds competing with the ATP at the kinase hinge region. $K_i$ values for these compounds are reported in Table 1. One of these compounds, PF-5168899 (Fig. 1), was further evaluated to prevent the activation of AKT1. While the initial data set showed that the inhibitor can effectively inhibit the PKD1 activity in the nanomolar range at high concentrations of ATP (i.e., $K_{i}^{\text{app}}$ and a $K_i$ values of 31.5 ± 2.4 and 0.31 ± 0.04 nM were determined, respectively), the compound is considerably less effective in preventing the activation of AKT1 when used in a cascade assay (i.e., $K_{i}^{\text{app}}$ = 1.53 ± 0.66 μM, a ~70-fold increase as compared to the $K_i^{\text{app}}$ for PKD1; see Fig. 5).

**PKD1 and FoxO3a translocation and phosphorylation of AKT Thr308 in CHO cells**

The PKD1 inhibitor PF-5168899 was also evaluated in cells for its ability to modulate the insulin-like growth factor-1 (IGF-1)-dependent translocation of PKD1 to the cell membrane and the phosphorylation of Thr308-AKT. For these experiments, a high content cell-based assay was developed using CHO cells that were

![Fig. 2](image-url) The presence of TDA 2.0 liposomes in the reaction media boosts the activity of PKD1 activity. As illustrated in panel a, the activity of KD-PDK1 (catalytic domain) alone increases by 4- to 5-fold in the presence of these lipid-based vesicles (○) as compared to the reaction in the absence of it (●). A greater effect (~20-fold) was observed in the presence of FL-PDK1 (full-length) when mixed with the same lipid (○) as compared to the reaction in the absence of it (●) (b). As a control study, panel c shows that the increase of PKD1 enzyme activity can only be observed when the enzyme possesses a His tag. The last experiment was conducted in the presence of 50 nM enzyme while peptide, TDA 2.0, and ATP concentrations are similar to the rest of this study (see experimental section).
engineered to express GFP-PDK1. On stimulation with IGF-1, GFP-PDK1 migrated to the inner surface of the cell membrane (Fig. 6a, upper left panel). Prior treatment of the cells with PF-5168899 (50 μM) reduced the ratio of membrane-associated versus cytosolic GFP-PDK1 (Fig. 6b) after IGF-1 stimulation. A concentration-dependent effect was observed for the effect of PF-5168899 on the membrane/cytosol levels of GFP-PDK1 after IGF-1 stimulation with an IC50 value of 2.23 ± 0.56 μM (Fig. 7a). Given the high selectivity for PF-5168899 for inhibition of PDK1 activity, it is likely that PF-5168899 is able to modulate an autophosphorylation step that is required for either translocating PDK1 to the membrane and/or

---

**Table 1**

<table>
<thead>
<tr>
<th>PDK1 inhibitors</th>
<th>K_i (nM, Omnia)</th>
<th>K_i (nM, Caliper)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PF-4350677</td>
<td>2.3 ± 1.4 (n = 2)</td>
<td>10.6 ± 0.5 (n = 2)</td>
</tr>
<tr>
<td>PF-5168899</td>
<td>2.5 ± 1.1 (n = 3)</td>
<td></td>
</tr>
<tr>
<td>PF-5168961</td>
<td>0.2 ± 0.02 (n = 1)</td>
<td>0.6 ± 0.1 (n = 2)</td>
</tr>
<tr>
<td>PF-5201534</td>
<td>1.25 ± 0.55 (n = 4)</td>
<td>1.8 ± 0.1 (n = 2)</td>
</tr>
</tbody>
</table>

The K_i was converted into K_ion using Eq. (2) for competitive inhibitor.

* Data from Murphy et al. [19].
maintaining PDK1 at the membrane. This is consistent with previously published reports that PDK1 is autoactivated by dimerization and trans-phosphorylation at the plasma membrane [25]. In the same cells, IGF-1 induced the phosphorylation of Thr308-AKT which was blocked when the cells were cotreated with PF-5168899 (Fig. 6c and d). The modulation of IGF-1-stimulated phosphoryl-

**Fig. 5.** Inhibition of PDK1 and AKT1 by PF-5168899. PF-5168899 is a selective and potent inhibitor of PDK1 (see Supplementary data) with $K_{app}^{in}$ in the nM range (○). Surprisingly, the inhibitor has a limited effect downstream, on the AKT activity, and exhibits a $K_{app}^{out}$ in the μM range (●) against this enzyme.

Discussion

On activation by RTKs, the recruitment of PDK1 to the membrane triggers a cascade of events that includes the autoactivation of PDK1. In turn, PDK1 phosphorylates and activates several downstream kinases such as AKT, SGK3, and S6K. As described by Wick et al. [25], PDK1 is autoactivated through a series of well-coordinated events that requires the dimerization of the enzyme through the PH domain and trans-autophosphorylation in the activation loop (i.e., A-loop). Several studies have revealed that docking sites such as the PIF domain located on the PDK1 N-terminal domain can also play a critical role in the regulation of the enzyme activity [26,27]. In particular, the interactions between either large peptides or small ligands with these docking sites induce changes in the protein conformation and lead to an increase of enzyme activity [28–30]. Interestingly, we have also been able to enhance the enzyme activity by adding TDA 2.0, in the reaction media. These vesicles were added in order to mimic the cellular environment and to reproduce the cascade of events that leads to the PDK1 activation. As reported in this study, a 4- to 5-fold and 20-fold increases of enzyme activity were observed in the presence of a small artificial peptide (14-mer) with either the catalytic domain or the full-length PDK1, respectively. Although the mechanism of activation of this enzyme remains unclear, it is likely that PDK1 binds to TDA 2.0 through the His-tag and establishes dimers, or higher order oligomeric structures. The dimerization of this enzyme would be followed by trans-autophosphorylation and autoactivation.

The effect of TDA 2.0 was also studied using a more intricate biochemical assay that was designed specifically to study the activation of inactive AKT by PDK1 and mTOR kinases. As reported in Fig. 3a and b, the presence of these artificial vesicles significantly boosted the activation of AKT1 and AKT2 activity. Both AKT enzymes showed a burst of activity that quickly plateaued if coupled with PDK1 alone. However, AKT displayed a greater and more
linear rate level of activity when both enzymes, PDK1 and mTOR, were added to the assay (Fig. 3b). Conversely, these two enzymes have very limited impact on the AKT activation in the absence of these lipids vesicles. To further understand this mechanism of activation, a Western blot analysis was conducted in order to identify the phosphorylation state of the key amino acid residues that have been reported to regulate the enzyme activity. The results generated are in agreement with previous studies [31], which show that PDK1 phosphorylates residue Thr308 in the A-loop of AKT. The phosphorylation of this amino acid residue alone is sufficient to activate AKT to a limited extent; however, the full activation of this enzyme requires the phosphorylation of additional residues such as Ser473 in the C-terminal hydrophobic motif and Thr450 in the turn motif by mTOR and other kinases. As previously reported by Facchinetti et al. [24], the phosphorylation of residues Thr450 and Ser473 plays an important role in the stability of the enzyme which seems to be consistent with our kinetic and data. Also and similar to Facchinetti’s group, the present study shows that AKT autophosphorylates its own Ser473 residue. Surprisingly, the last piece of data provided by the Western blot analysis suggests that mTOR has the ability to phosphorylate both residues Ser473 and Thr308 on AKT (Fig. 4a and b, columns 7 and 8). The data generated with these liposomes indicate that we have been able to reproduce, to a limited extent and in a chemically defined in vitro assay, the cascade of events that lead to the in vivo activation of AKT. In agreement with recent studies [32], these data also suggest that the presence of PIP3 and the PH domain are not needed for activation of PDK1 or AKT. Therefore, we propose that AKT activation is initiated on binding to TDA 2.0 which provides a critical membrane context that leads to the exposure of the A-loop and the hydrophobic motif of the C-terminus, conformationally altering AKT to become an optimal substrate for PDK1 and mTOR. However, since the bic motif of the C-terminus, conformationally altering AKT to be-

### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2011.03.013.
References

[19] GraphPad Prism, version 5.01, GraphPad Software Inc., San Diego.