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A homogeneous and nonisotopic assay for phosphatidylinositol 4-kinases

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Abstract

Phosphatidylinositol 4-kinases (PI 4-kinases) catalyze the conversion of phosphatidylinositol to phosphatidylinositol 4-phosphate (PtdIns4P). The four known mammalian PI 4-kinases, PI4KA, PI4KB, PI4K2A, and PI4K2B have roles in intracellular lipid and protein trafficking. PI4KA and PI4KB also assist the replication of several positive-sense RNA viruses. The identification of selective inhibitors of these kinases would be facilitated by assays suitable for high-throughput screening. We describe a homogenous and nonisotopic assay for PI 4-kinase activity based on the bioluminescent detection of the ADP produced by kinase reactions. We have evaluated this assay with known nonselective inhibitors of PI 4-kinases and show that it performs similarly to radiometric assay formats previously described in the literature. In addition, this assay generates Z-factor values of > 0.7 for PI4KA in 384-well format, demonstrating its suitability for high-throughput screening applications.

Keywords

Phosphoinositides; Phosphatidylinositol 4-kinase; Kinase assay; High-throughput screening assays

Introduction

Phosphoinositides are biologically active membrane lipids with a diverse range of cellular signaling functions. The temporal and spatial regulation of phosphoinositide metabolism is carried out by lipid kinases and phosphatases that interconvert the seven distinct phosphoinositide species among one another. Phosphatidylinositol (PtdIns¹) 4-phosphate is generated from PtdIns by PI 4-kinases. Four different mammalian PI 4-kinases have been identified (reviewed in [1]), which have been categorized into types II (PI4K2A and

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¹Abbreviations used: HTS, high-throughput screening; PtdIns, phosphatidylinositol; PtdIns4P, phosphatidylinositol 4-phosphate;

PI4K2B) and III (PI4KA and PI4KB) based on their *in vitro* sensitivities to wortmannin and adenosine. There are a number of known PtdIns4P effectors, including the coat adaptor AP-1 [2] and lipid transfer proteins such as OSBP1 and CERT (reviewed in [3]). More recently, a critical role has been demonstrated for the type III PI 4-kinases PI4KA and PI4KB (also known as PI4KIII α and PI4KIII β , respectively) in the replication of hepatitis C virus (HCV) and enteroviruses, respectively [4; 5; 6; 7]. Evidence suggests that these PI 4-kinases play a central role in the formation of altered host membrane structures upon which these viruses replicate.

Elucidating the functions of PI 4-kinases and PtdIns4P would be greatly facilitated by selective pharmacologic inhibitors of these lipid kinases. Moreover, selective PI 4-kinase inhibitors are expected to have antiviral activity against HCV and other viruses that depend on these kinases for their lifecycle. The IC₅₀ of wortmannin against type III PI 4-kinases is roughly 100-fold higher than for PI 3-kinases [8], suggesting that it should be possible to identify inhibitors that are selective for type III PI 4-kinases versus PI 3-kinases. However, such selective inhibitors have not yet been identified. Another inhibitor, PIK93, displays selectivity for PI4KB over PI4KA, but also potently inhibits several PI 3-kinases [9] and therefore cannot be used to discriminate among PI4KB and PI 3-kinases in complex systems. The identification of selective PI 4-kinase inhibitors has been hampered by the lack of PI 4-kinase assays suitable for high-throughput screening of compound libraries. The methods currently used for the assay of PI 4-kinases have relied on the incorporation of [³²P] followed by separation of the unreacted [³²P]ATP from the product [³²P]PtdIns4P, typically using an organic solvent extraction step [8; 10].

In recent years, however, several technologies have been developed that measure kinase activity through detection of the product ADP. While such methods have been applied to PI 3-kinases [11; 12], none have been reported to date for PI 4-kinases. Here we describe a homogeneous, nonisotopic PI 4-kinase assay based on the ADP-Glo assay technology, which detects the ADP generated in kinase reactions.

Materials and Methods

Reagents

Anti-FLAG monoclonal antibody (clone M2), wortmannin and bovine liver phosphatidylinositol were obtained from Sigma-Aldrich (St. Louis, MO). PIK-93 [9] was purchased from Symansis (Washdyke, New Zealand).

Cell culture

293T (GenHunter, Nashville, TN) and COS-7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 μ g/mL of streptomycin.

PI 4-kinase expression constructs

The constructs encoding full-length human PI4KA and PI4KB cDNAs tagged with an N-terminal 3XFLAG epitope have been previously reported [4]. FLAG-PI4KB was generated by cloning the Flag sequence between EcoRV and Xho I sites at the N-terminus of human PI4KB in the pcDNA3.1 plasmid (generously provided by Gordon Polevoy and Julie Brill, The Hospital for Sick Children, Toronto, Canada). The kinase-inactive PI4KA mutant D1899A contains a mutation in the conserved lipid kinase catalytic domain corresponding to the kinase-inactivating mutation D656A for PI4KB reported by Godi *et al.* [13] and was introduced into PI4KA by overlap extension PCR. The amplified region was completely sequenced to confirm that the D1899A mutation had been introduced without any unwanted

mutations. Constructs encoding full-length human PI4K2A and PI4K2B cDNAs tagged with an N-terminal 3XFLAG epitope were constructed by PCR amplification from full-length cDNAs obtained from Open Biosystems (Huntsville, AL) and subcloning into the pFB retroviral vector (Stratagene; La Jolla, CA). Primer sequences and a detailed cloning strategy will be provided upon request.

Expression and affinity purification of PI 4-kinases

293T or COS-7 cells were transfected with plasmids encoding FLAG-tagged PI 4-kinase constructs using FuGENE HD (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. 24 hours post-transfection, cells were washed with PBS and then lysed in 20 mM Tris-HCl pH 7.5, 100 mM NaCl, 1% NP-40, 10% glycerol, 1 mM EDTA, 0.5 mM DTT, and Halt protease inhibitor cocktail (Pierce, Rockford, IL) for 15 minutes on ice. The lysate was centrifuged for 15 minutes at maximum speed in a microcentrifuge at 4°C to remove nuclei and insoluble material. The supernatant was then mixed with protein G-conjugated Dynabeads (Invitrogen, Carlsbad, CA) prebound with anti-FLAG monoclonal antibody (clone M2, Sigma-Aldrich) for 1 hour at 4°C.

The Dynabeads were then washed 3 times with lysis buffer (including protease inhibitors) and then once with kinase buffer (40 mM Tris-HCl pH 7.5, 20 mM MgCl₂, 1 mM EGTA, 0.2% Triton X-100) supplemented with 0.5 mM DTT and 0.1% bovine serum albumin (BSA). Bound kinase was eluted with kinase buffer supplemented with 2 mM DTT, 0.1% BSA, and 500 µg/mL 3XFLAG peptide (Sigma-Aldrich) for 30 minutes at 4°C. The elution was repeated twice more; the eluates were pooled, aliquoted, snap-frozen in liquid nitrogen, and stored at -80°C for subsequent use.

ADP-Glo PI 4-kinase assay

White low-volume 384 well polystyrene plates (ProxiPlate-384 Plus, PerkinElmer, Waltham, MA) were used for the ADP-Glo assay. PI dissolved in chloroform was dried in Eppendorf brand microcentrifuge tubes under a nitrogen stream in a fume hood, resuspended to 2.5 mM in kinase buffer containing 0.2% Triton X-100, and then sonicated by four 15-second pulses with a Branson Sonifier microtip (Danbury, CT) until the suspension became translucent. 2.5 µL of kinase were mixed with 2.5 µL of kinase buffer/2 mM DTT/0.1% BSA with PI micelles (800 µM final concentration) and ultrapure ATP (Promega, Madison, WI). Enzyme concentrations were chosen so that the reactions of both PI4KA and PI4KB were linear during the 15-minute incubation, and all reactions were carried out in triplicate. Blank wells lacked enzyme but did include kinase buffer, substrate, and ATP. The plates were covered and the reactions were carried out at room temperature for up to 60 minutes. Reactions were stopped with the addition of 5 µL ADP-Glo reagent (Promega). After a 40 minute incubation at RT, 10 µL of Kinase Detection Reagent (Promega) were added and the plates were incubated for another 40 minutes at RT (60 minutes for ATP concentrations greater than 100 µM). Plates were read on a BioTek Synergy 2 plate reader (Winooski, VT) with a sensitivity of 150 and an integration time of 1 second per well. Data were analyzed using Prism 5.0 software (GraphPad, La Jolla, CA).

For kinase reactions involving inhibitors, 2 µL of kinase were preincubated with 1 µL of inhibitor at varying concentrations in kinase buffer for 10 minutes at RT prior to addition of 2 µL of kinase buffer with PI (800 µM final concentration), ATP (100 µM final), and inhibitor. Wortmannin stocks were prepared in DMSO and were diluted immediately before use due to its instability in aqueous solutions. The percent inhibition was calculated relative to an enzyme control without inhibitor. IC₅₀s were calculated by four-parameter nonlinear regression using Prism 5 software (GraphPad Software, La Jolla, CA).

High-throughput PI4KA assay

For Z-factor determination, 4 μ L of purified PI4KA in kinase buffer containing 1 mM PI micelles were dispensed into Corning 3674 low-volume 384-well plates using a Multidrop Combi dispenser (Thermo Scientific, Barrington, IL). One column was left without enzyme to measure background luminescence. 100 nL of DMSO were dispensed into 11 columns using a Biomek FX pin tool dispenser (Beckman Coulter, Brea, CA), while 100 nL of 500 μ M wortmannin in DMSO (10 μ M final concentration) were dispensed into 12 columns using a Mosquito dispenser (TTP LabTech, Cambridge, MA). After a 10 minute preincubation, kinase reactions were started by adding 1 μ L of 500 μ M ATP using the Multidrop Combi dispenser. Kinase reactions were carried out for 15 minutes at RT. Reactions were stopped with the addition of 5 μ L ADP-Glo reagent. After a 40 minute incubation at RT, 10 μ L Kinase Detection Reagent was added and the plate was incubated for another 40 minutes at RT. Plates were read on a Pherastar plate reader (BMG LABTECH, Cary, NC).

Radiometric PI 4-kinase assay

Radiometric assays for PI 4-kinase activity were performed as described in [8]. In brief, 5 μ L of purified PI4KA or PI4KB was added to 40 μ L of kinase buffer (50 mM Tris-Cl pH 7.5, 20 mM $MgCl_2$, 1 mM EGTA, 1 mM PI, 0.4% Triton X-100, 0.5 mg/mL BSA) and preincubated with inhibitors for 10 minutes. Reactions were started with 5 μ L of 1 mM [$\gamma^{32}P$]ATP and incubated at RT. Reactions were stopped after 30 minutes by the addition of 3 mL $CHCl_3/CH_3OH$ /concentrated HCl (200:100:0.75, v/v) followed by mixing and then adding 0.6 mL of 0.6 M HCl. After centrifugation, the aqueous phase containing unreacted [$\gamma^{32}P$]ATP was removed, and the organic phase containing PtdIns4P was reextracted with 1.5 mL of $CHCl_3/CH_3OH$ /0.6 M HCl (3:48:47, v/v). The lower organic phase was transferred to scintillation vials, dried, and the product [$\gamma^{32}P$]PtdIns4P was quantified by liquid scintillation.

Results and Discussion

Expression and affinity purification of full-length PI4KA and PI4KB

In order to develop a kinase assay for type III PI 4-kinases, we needed to express and purify PI4KA and PI4KB. Attempts at purifying full-length or N-terminal truncated PI4KA in *E. coli* were limited by very low levels of full-length protein (data not shown), which may be due to the relatively high molecular weight of this kinase at 230,000. We then sought to determine whether full-length human PI4KA and PI4KB could be expressed in cultured cells in quantities sufficient for *in vitro* kinase assays. 293T human embryonic kidney cells or COS-7 were transiently transfected with an expression vector encoding N-terminal epitope-tagged full-length PI4KA or PI4KB. FLAG-tagged kinases were then affinity purified by magnetic beads coated with anti-FLAG antibody and competitively eluted using a 3XFLAG peptide to avoid the use of harsh elution conditions (e.g. low pH) that could adversely affect enzyme activity.

Colloidal Coomassie Blue staining of purified 3XFLAG-PI4KA and FLAG-PI4KB demonstrated bands migrating at the expected positions for these two kinases (Figure 1A, lanes 2 and 3), while no bands were seen in anti-FLAG immunoprecipitates from untransfected 293T cells (Figure 1A, lane 1). Although PI4KA has a predicted molecular weight of 230,000, it migrates on SDS-PAGE with an apparent mass of approximately 200–210 kDa. Immunoblotting with an anti-FLAG antibody revealed that both of the protein bands seen in the 3XFLAG-PI4KA and FLAG-PI4KB preparations were immunoreactive (Figure 1B, lanes 2 and 3). No anti-FLAG reactive bands were observed in immunoprecipitates from untransfected 293T cells (Figure 1B, lane 1).

Characterization of the PI4KA and PI4KB ADP-Glo kinase assays

The ADP-Glo kinase assay technology was selected for development of a homogeneous, nonisotopic type III phosphatidylinositol kinase assay. This method is based on a two-step bioluminescent detection of the ADP produced by a kinase reaction [14]. In the first step, ADP-Glo Reagent is added at the end of the kinase reaction to both stop the kinase reaction and deplete the remaining ATP. In the next step, the ADP produced in the kinase reaction is regenerated into ATP, which in turn is converted to a luminescence by a luciferin/luciferase reaction. The magnitude of the luminescence signal is a linear function of the ADP produced and hence of the kinase activity. Another general approach to the detection of PI kinase activity employs protein domains (e.g. pleckstrin homology domains) that selectively bind to the phosphoinositide reaction products; this interaction can be detected, for example, by Alphascreen or by time-resolved fluorescence resonance energy transfer [15]. However, limitations of this approach include the need for the PI substrate and/or reaction product to be conjugated to molecules such as biotin or fluorescent tracers, and the requirement for a protein sensor domain of the correct specificity. The ability of the ADP-Glo assay to use unmodified PI as a substrate reduces costs and potentially increases flexibility if multiple PI kinases are to be assayed in parallel, such as in inhibitor profiling applications.

As seen in Figure 2, addition of either purified PI4KA or PI4KB to a kinase reaction mixture containing PI micelles and ATP resulted in a strong luminescence signal with signal:background ratios of 9.6 and 10.2, respectively. The concentrations of PI4KA and PI4KB used were chosen to maintain linearity of the kinase reactions during the 15-minute incubation period (data not shown). No luminescence above background was detected from anti-FLAG immunoprecipitates from untransfected 293T cells. Similarly, no luminescence above background was detected when PI4KA or PI4KB was incubated with ATP in the absence of PI micelles, demonstrating the absence of other contaminating non-PI kinases and the absence of substrate-independent ATP hydrolysis by purified PI4KA or PI4KB. Finally, we expressed and purified a mutated PI4KA(D1899A) mutant predicted to lack kinase activity. This mutant was also inactive in the ADP-Glo kinase assay, which suggests that no contaminating PI kinases were present in our purified PI4KA preparation.

We then compared the ATP K_m of PI4KA and PI4KB, determined using the ADP-Glo assay, to previously published K_m values for ATP. A limitation of the ADP-Glo assay is that the maximum ATP concentration is 1 mM; as the concentration of ATP added to the kinase reaction increases, the background also increases, most likely due to trace amounts of contaminating ADP. As a result, the signal:background ratio of the PI 4-kinase ADP-Glo assay decreases at high ATP concentrations. This limitation notwithstanding, we found that PI4KA had an apparent ATP K_m of 209 μ M (Figure 3), in line with previous determinations of 250 and 300 μ M using radiometric assays [16; 17]. Using the ADP-Glo assay, PI4KB had an apparent ATP K_m of 120 μ M, while it has been reported to be 128 μ M using a radiometric assay (Invitrogen, [http://tools.invitrogen.com/content/sfs/manuals/PI4KB_\(PI4Kbeta\)_Adapta.pdf](http://tools.invitrogen.com/content/sfs/manuals/PI4KB_(PI4Kbeta)_Adapta.pdf)).

Inhibitor profiling of PI4KA and PI4KB using an ADP detection assay

A characteristic of the type III PI 4-kinases that distinguishes them from type II kinases is their relative insensitivity to inhibition by adenosine [18]. PI4KB was not inhibited by adenosine in the ADP-Glo assay at concentrations up to 200 μ M (Figure 4), while PI4KA activity was inhibited by 22% at 200 μ M adenosine. Similar mild inhibition of PI4KA at this concentration of adenosine was also observed in [18].

While there are no known selective inhibitors of PI4KA, wortmannin has been used as an inhibitor of both PI4KA and PI4KB [16; 19; 20]. As seen in Figure 5A, the wortmannin IC_{50}

for PI4KA was 192 nM in the ADP-Glo assay, which corresponds closely to a value of 200 nM measured by Gehrmann et al. using a radiometric assay [16]. The wortmannin IC₅₀ for PI4KB was 298 nM (Figure 5B), which agrees with an IC₅₀ of 320 nM determined by a radiometric assay [21].

In contrast to wortmannin, the small molecule PIK93 selectively inhibits PI4KB over PI4KA, though it also inhibits several PI 3-kinases with nanomolar potency [9]. We compared PIK93 dose-response curves against PI4KA and PI4KB using the luminescent assay compared to a radiometric assay [8]. As seen in Figure 6, the PIK93 dose-response curves for the two assays were comparable for both enzymes. The PIK93 IC₅₀ for PI4KA was 921 nM in the ADP-Glo assay and 1620 nM in the radiometric assay, while the PIK93 IC₅₀ for PI4KB was 17.5 nM for the ADP-Glo assay and 28.0 nM for the radiometric assay. The PIK93 IC₅₀ measurements were 1.6-1.75 fold higher for the radiometric assay compared to the luminescent assay, perhaps reflecting in part batch-to-batch variability in PIK93 potency or other technical variables between the two assays. However, the calculated selectivity ratios of PIK93 for PI4KB over PI4KA were similar between the two assay formats, being 57.9 using the radiometric assay and 52.6 using the ADP-Glo assay.

Adapting the ADP-Glo PI 4-kinase assay to high-throughput screening methodology

The ADP-Glo assay has several advantages over traditional radiometric lipid kinase assays that make it suitable for high-throughput screening (HTS) applications: namely, the assay is homogeneous, nonisotopic, and can be carried out in 384-well plates. We first determined the sensitivity of the PI4KA and PI4KB ADP-Glo assays to DMSO, as this is the solvent typically used for compound libraries. PI4KA activity was insensitive to DMSO at concentrations up to 1%, and only minimally affected by 2% DMSO (Figure 7A). PI4KB activity, on the other hand, was not affected by DMSO concentrations up to 2%.

We then proceeded to determine the suitability of the PI4KA ADP-Glo assay for HTS by measuring the Z-factor [22]. The Z-factor is a widely used statistical parameter for evaluating a HTS assay that incorporates the dynamic range and variability of the assay; a Z-factor of > 0.5 is typically considered a robust assay. We measured PI4KA activity in a low-volume 384-well plate in which 192 wells were treated with 10 μ M wortmannin, 176 with DMSO as a negative control, and 16 had no enzyme to measure the background luminescence. As seen in Figure 7B, the dynamic range of the PI4KA assay is high in this assay format. The dotted lines above and below the DMSO negative control data points represent 3 standard deviations above and below the mean of the negative control wells. The wide separation of the mean-3SD line from the wortmannin-treated positive control wells indicates that the false positive rate of this assay is expected to be very low. In two independent experiments, we obtained Z-factors of 0.72 and 0.74, demonstrating the suitability of this assay for HTS in 384-well format. The high Z-factors obtained even before extensive assay optimization suggest that acceptable Z-factors could be achieved in 1536-well format.

Inhibitor profiling of the type II PI 4-kinases PI4K2A and PI4K2B

While our development and validation of the ADP-Glo PI 4-kinase assay has focused mostly on the type III kinases due to our interest in their roles in viral replication, it was of interest to determine whether this assay could be generalized to include the type II PI 4-kinases PI4K2A and PI4K2B. In contrast to the type III kinases, the type II kinases are inhibited by adenosine and are insensitive to wortmannin [23]. We found that affinity-purified PI4K2A and PI4K2B were both active in the ADP-Glo PI 4-kinase assay (Figure 8). Of note, the identical assay methodology was used for all four PI 4-kinases; no modifications to the assay reagents or protocol were necessary.

Both PI4K2A and PI4K2B were inhibited by adenosine at concentrations that do not significantly inhibit PI4KA and PI4KB activity (Figure 8A). Wortmannin, on the other hand, had no effect on either PI4K2A or PI4K2B activity at concentrations up to 3 μ M (Figure 8B). These findings indicate that a single assay can be used to measure the activity of all four human PI 4-kinases, which should facilitate PI 4-kinase inhibitor profiling studies.

In conclusion, we have developed and characterized a homogenous and nonisotopic assay for PI 4-kinases that is suitable for high-throughput screening applications. Moreover, this assay does not require any organic solvent extraction steps, as opposed to conventional radiometric lipid kinase assays. An important limitation of this assay, shared by other ADP-detection technologies, is its inability to measure PI 4-kinase activity in the presence of other kinases or enzymes with ATPase activity, such as in cell lysates or tissues. As a result, the ADP-Glo based PI 4-kinase assay is limited to purified enzymes. On the other hand, a major strength of this assay is that it should be readily applicable to inhibitor profiling across multiple PI 3- and 4-kinases in parallel, in comparison to assays that rely on specific protein sensor domains and covalently modified reaction products. We expect that this and other suitable PI 4-kinase assay technologies will be used to screen chemical libraries for selective PI 4-kinase inhibitors, which in turn will greatly advance our understanding of PI 4-kinase functions *in vitro* and in living cells.

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Fig. 1.

Expression and affinity purification of PI4KA and PI4KB in 293T cells. (A) 293T human embryonic kidney cells were transfected with 3XFLAG-tagged PI4KA (middle lane), FLAG-PI4KB (right lane), or left untransfected (left lane). Tagged PI 4-kinases were affinity-purified by anti-FLAG antibody-conjugated magnetic beads followed by elution with 3XFLAG peptide. Eluted proteins were separated by SDS-PAGE and visualized by colloidal Coomassie Blue staining. (B) The purified PI 4-kinases were also characterized by immunoblotting with an anti-FLAG antibody.

Fig. 2. Characterization of purified PI4KA and PI4KB in the ADP-Glo kinase assay. Affinity-purified PI4KA and PI4KB were incubated in a reaction mixture containing 100 μ M ATP and 800 μ M PtdIns (columns 4 and 7) or in a mixture containing 100 μ M ATP and no PtdIns (columns 3 and 6). Columns 1 and 2 show the ADP-Glo luminescence signal from reactions lacking enzyme or from reactions with anti-FLAG immunoprecipitates from untransfected 293T cells, respectively. Column 5 shows the ADP-Glo signal from a mutant D1899A PI4KA lacking kinase activity. The data are means $\pm$ SD from quadruplicate measurements in a representative experiment.

Fig. 3.

Determination of ATP K_m for PI4KA and PI4KB. Kinase reactions were performed with purified PI4KA (circles; solid line) and PI4KB (squares; dashed line) and varying concentrations of ATP. Blank reactions were measured for each ATP concentration tested, as the background luminescence is a function of ATP concentration. Data are plotted as means $\pm$ SD from triplicate measurements in two independent experiments ($n=6$).

Fig. 4.

Demonstration of PI4KA and PI4KB adenosine insensitivity using the ADP-Glo assay. Purified PI4KA (solid bars) and PI4KB (empty bars) were assayed with the indicated concentrations of adenosine. The measured blank-subtracted luminescence values were normalized to reactions lacking adenosine. Data are plotted as means $\pm$ SD from triplicate measurements in two independent experiments (n=6).

Fig. 5. Determination of IC₅₀ values of wortmannin for type III PI 4-kinases. (A) Purified PI4KA and (B) PI4KB were assayed with the indicated concentrations of wortmannin, which is a known inhibitor of type III PI 4-kinases. The measured blank-subtracted luminescence values were normalized to reactions lacking inhibitor. The reactions were performed at 100 μ M ATP and 800 μ M PtdIns. Data are plotted as means $\pm$ SD from triplicate measurements in three independent experiments (n=9).

Fig. 6.

Comparing the ADP-Glo PI 4-kinase assay with a radiometric assay for the determination of PIK93 IC₅₀ values. Assays were performed as described in Materials and Methods. The measured blank-subtracted luminescence values were normalized to reactions lacking inhibitor. Data are plotted as means $\pm$ SD from triplicate measurements in three independent experiments (ADP-Glo assay) or from quadruplicate measurements in two independent experiments (radiometric assay).

Fig. 7.

DMSO sensitivity of PI 4-kinase assays and Z-factor measurement for PI4KA. (A) Purified PI4KA (solid bars) and PI4KB (empty bars) were assayed with the indicated concentrations of DMSO. Data are plotted as means $\pm$ SD from triplicate measurements in two independent experiments (n=6). (B) PI4KA kinase reactions were carried out in 5 μ L volumes in low-volume 384-well plates. Wells containing 10 μ M wortmannin (diamonds) were compared to negative control reactions containing 2% DMSO (solid circles). 16 of the wells contained no enzyme (open circles). The solid line indicates the mean luminescence for the negative control reactions, while the dashed lines indicate the mean $\pm$ 3 SD for the negative controls. The data shown are plotted from a single 384-well plate; two independent experiments were performed.

Fig. 8. Inhibitor profiling of the type II PI 4-kinases PI4K2A and PI4K2B using the ADP-Glo assay. (A) Affinity-purified PI4K2A (solid bars) and PI4K2B (empty bars) were assayed with the indicated concentrations of adenosine. The measured blank-subtracted luminescence values were normalized to reactions lacking adenosine. Phosphatidylinositol was omitted from the reactions shown in columns 1. Data represent triplicate measurements from a representative experiment and are plotted as means $\pm$ SD. (B) Affinity-purified PI4K2A (solid bars) and PI4K2B (empty bars) were assayed with the indicated concentrations of wortmannin. Data are normalized to control reactions lacking wortmannin and are plotted as means $\pm$ SD of triplicate measurements from a representative experiment.