



A fluorescence lifetime-based assay for serine and threonine kinases that is suitable for high-throughput screening

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ARTICLE INFO

Article history:

Received 11 December 2009

Received in revised form 4 March 2010

Accepted 10 March 2010

Available online 15 March 2010

Keywords:

Protein kinase

Fluorescence lifetime

High-throughput screening

ABSTRACT

We describe the development of a novel method for the assay of serine/threonine protein kinases based on fluorescence lifetime. The assay consists of three generic peptides (which have been used by others in the assay of >140 protein kinases in various assay formats) labeled with a long lifetime fluorescent dye (14 or 17 ns) that act as substrates for protein kinases and an iron(III) chelate that modulates the fluorescence lifetime of the peptide only when it is phosphorylated. The decrease in average fluorescence lifetime as measured in a recently developed fluorescence lifetime plate reader (Edinburgh Instruments) is a measure of the degree of phosphorylation of the peptide. We present data showing that the assay performs as well as, and in some cases better than, the “gold standard” radiometric kinase assays with respect to Z' values, demonstrating its utility in high-throughput screening applications. We also show that the assay gives nearly identical results in trial screening to those obtained by radiometric assays and that it is less prone to interference than simple fluorescence intensity measurements.

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During recent years, the catalytic phosphorylation and dephosphorylation of proteins by protein kinases and protein phosphatases, respectively, have been shown to participate in the regulation of nearly all aspects of cell life. It has also become apparent that abnormalities of protein phosphorylation are involved in many diseases and pathological conditions such as inflammatory diseases, cancer, diabetes, heart disease, and hypertension. This has led to an increase in interest in protein kinases and phosphatases as therapeutic targets [1].

A number of protein kinase and protein phosphatase inhibitors are currently in clinical use or clinical development. The immunosuppressant cyclosporin, which inhibits the protein phosphatase 2B calcineurin, has found widespread use in the field of organ transplantation [2]. An alternative immunosuppressant, rapamycin,

inhibits the protein kinase mTOR¹ (mammalian target of rapamycin) [3]. One of the first drugs to be developed by targeting of a specific protein kinase is Gleevec (STI 571), which is an inhibitor of the Abl tyrosine kinase. This drug has proven to be very effective in the treatment of chronic myelogenous leukemia, a disease that is caused by conversion of the Abl kinase to a constitutively active form by a chromosomal rearrangement [4]. These successes have greatly stimulated interest in protein kinases and phosphatases as potential drug targets.

After identification of a target enzyme, one of the first steps in the drug discovery process involves screening compound libraries

¹ Abbreviations used: mTOR, mammalian target of rapamycin; ELISA, enzyme-linked immunosorbent assay; FP, fluorescence polarization; FRET, fluorescence resonance energy transfer; HTS, high-throughput screening; TR-FRET, time-resolved FRET; ATP, adenosine triphosphate; TFA, trifluoroacetic acid; TIS, triisopropylsilane; RP-HPLC, reverse phase high-performance liquid chromatography; ESI-MS, electrospray ionization mass spectrometry; UV, ultraviolet; ACE-14, O-(N-succinimidyl)-6-(9-oxo-9H-acridin-10-yl)hexanoate; DTT, dithiothreitol; BSA, bovine serum albumin; PMA, phenylmalonic acid; PKB α , protein kinase B alpha; IMAC, immobilized metal affinity chromatography; CHK1, checkpoint kinase 1; PKA, protein kinase A; 9AA, 9-aminoacridine; SGK1, serum- and glucocorticoid-induced protein kinase 1; MSK1, mitogen- and stress-activated kinase 1; p70s6K, p70 S6 kinase; MAPKAP-K2, mitogen-activated protein kinase-activated protein kinase 2.

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to identify “lead” inhibitors of the protein kinase of interest. This requires the availability of a suitable assay for the target enzyme. The radiometric “gold standard” assay for protein kinases relies on the incorporation of radiolabeled phosphate into peptide and protein substrates. However, one of the major drawbacks of such radioactive assay formats is that, due to the large numbers of compounds in these libraries (>1 million compounds), high levels of radioactivity would be required to run these assays. Due to safety aspects and environmental considerations involved in the storage and disposal of radioactive waste, the development of alternative nonradioactive assay formats has become increasingly important. Several nonradioactive assay formats are currently available, including an enzyme-linked immunosorbent assay (ELISA)-type assay based on an antibody specific for a common phosphorylated epitope in a set of generic substrate peptides [5] that has been further adapted using fluorescently labeled peptides in a fluorescence polarization (FP) assay. Other formats are also available based on anti-phosphotyrosine antibodies using FP and fluorescence intensity (see reviews in Refs. [6–8] and references therein for details on different screening technologies). All of these assays rely on either antibodies (generic or specific), fluorescence intensity, FP, or fluorescence resonance energy transfer (FRET) [6]. These assays are susceptible to interference by the compounds in the chemical libraries being screened that can result in high rates of false positive and negative results; furthermore, some methods are unsuitable for high-throughput screening (HTS) due to the number of washing steps involved in the assay method.

The fluorescence lifetime of a fluorophore is defined as the time taken for the fluorescence intensity to decay to $1/e$ or approximately 36.7% of the original intensity. The application of this intrinsic fluorescence property as the reporting modality in biochemical and cell-based assays is attractive because it is independent of probe concentration and volume and is unaffected by autofluorescence, light scattering, and inner filter effects. In addition, because a lifetime decay is being measured, it enables background interference from short-lived fluorescent compound libraries to be minimized, leading to fewer false positives and negatives in drug screening applications [9].

The duration of this lifetime is dependent on the chemical environment of the fluorophore. For example, it has been shown that the proximity of tyrosine and tryptophan to long-lifetime dyes results in a reduction in lifetime that, in the case of tyrosine, can be reversed by phosphorylation of the tyrosine residue. In light of this fact, we have investigated the applicability of fluorescence lifetime to the assay of serine/threonine protein kinases based on the premise that the addition of a phosphate to a dye-labeled peptide could be used to induce a sufficient change in the environment of the dye to result in a change in fluorescence lifetime, thereby enabling the development of homogeneous serine/threonine protein kinase assays that use changes in fluorescence lifetime to report substrate phosphorylation and, hence, enzyme activity. A fluorescence lifetime assay for protein kinases that depends on the interaction of a dye-labeled derivative of the general protein kinase inhibitor staurosporine with the protein kinase of interest, resulting in a change in dye lifetime, has been reported [10]. Potential inhibitors are screened by competing for this binding, thereby reversing the lifetime change. A similar assay based on the same principles, but using time-resolved FRET (TR-FRET) as the readout, has also been reported [11,12]. However, neither of these assays measures enzyme activity or competition for adenosine triphosphate (ATP), and any IC_{50} values obtained for compounds of interest are dependent on the affinity of the labeled staurosporine for the enzyme rather than on the enzyme affinity for the natural ATP substrate.

Materials and methods

All general chemicals used were of Analar grade or better and obtained from Sigma–Aldrich Chemicals except where otherwise stated. The dye-labeled generic peptides RARTLSFAEPG (peptide 1), RRRLSFAEPG (peptide 2), KKLNRLLSFAEPG (peptide 3), and their phosphorylated forms were synthesized on Rink amide resin by Fmoc-based solid phase peptide synthesis (SPPS) and dye labeled on resin using 3-(9-aminoacridin-2-yl)propionic acid. The dye-labeled peptides were cleaved with trifluoroacetic acid (TFA)/ H_2O /triisopropylsilane (TIS) (92.5:5:2.5) for 4 h and lyophilized overnight. The crude peptides were purified by reverse phase high-performance liquid chromatography (RP-HPLC) using an acetonitrile/water gradient containing 0.1% TFA. The desired isolated peptides were shown to be more than 95% pure by analytical RP-HPLC and were confirmed as the correct mass by electrospray ionization mass spectrometry (ESI-MS). Purified peptides were quantified by ultraviolet (UV) based on the dye absorbance at 405 nm ($\epsilon = 8735 \text{ M}^{-1} \text{ cm}^{-1}$). Crosstide peptide was labeled with *O*-(*N*-succinimidyl)-6-(9-oxo-9*H*-acridin-10-yl)hexanoate (ACE-14, available as Puretime 14 from AssayMetrics) using a 2:1 molar excess of dye at 4 °C overnight in bicarbonate buffer at pH 8.5. In addition, peptide sequences labeled at the C terminus with ACE-14 were obtained from Alta Biosciences.

Radiometric assay

Protein kinase activity was estimated using incorporation of ^{33}P from ^{33}P - γ -ATP into peptides as described previously [13].

Lifetime assays

Protein kinase activity was estimated by incubation of various protein kinases at the required concentration with dye-labeled peptides, ATP, and MgCl_2 in Tris–HCl buffer (pH 7.5), 150 mM NaCl, 1 mM dithiothreitol (DTT), and 1 mg/ml bovine serum albumin (BSA). The concentrations of ATP, MgCl_2 , enzymes, and peptide substrates are detailed in the figure legends. Assays were terminated by the addition of a solution of equimolar amounts of iron(III) perchlorate and phenylmalonic acid (PMA) in acetic acid as detailed in the figure legends. Fluorescence lifetimes were measured on a Nanotaurus time-correlated single-photon counting fluorescence lifetime plate reader (Edinburgh Instruments). Standard settings used were a 100-ns time range with 512 channels (0.1953 ns/channel) and excitation by 405-nm laser at 5 MHz with a 438-nm emission filter, and data were collected to a peak of 10^4 counts, giving an average read time of 3 s per well unless otherwise stated in the figure legends (note that a 384-well plate can be accurately read in <5 min by changing the instrument settings). Data were analyzed using a double-exponential fit, and the average lifetime was calculated.

Results

Substrate viability

The first stage of the assay development was to determine whether or not the labeling of known substrate peptides with dye had any deleterious effect on their ability to act as substrates. In the first instance, this was trialed using the synthetic Crosstide peptide [13] (GRPRTSSFAEG) substrate for PKB α (protein kinase B alpha). The peptide was selectively labeled at the N terminus using the succinimidyl ester of the proprietary ACE-14 dye as described in Materials and methods. The results (Fig. 1) show that although

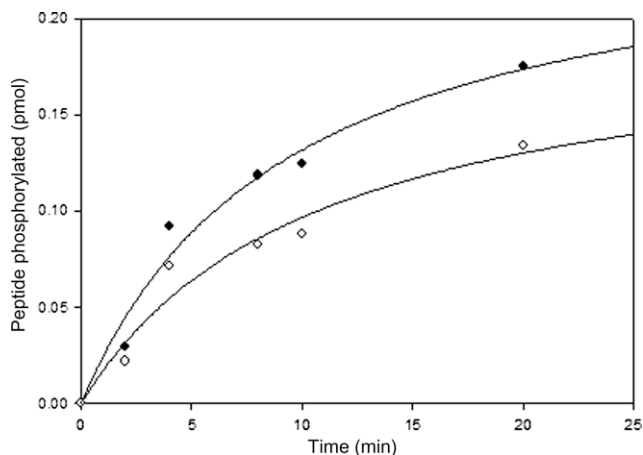


Fig. 1. Comparison of normal (◆) and ACE-14-labeled Crosstide (◇) as substrates for PKB α in a radiometric assay. The assays were carried out using 0.1 mU of PKB α incubated with 10 μ M Crosstide (either native or dye labeled) in the presence of 33 P- γ -ATP and incorporation of 33 P into peptide estimated as described previously [13].

there is a reduction in the reaction rate using the labeled peptide compared with unlabeled peptide, it is still a viable substrate.

Development of lifetime assay

Initial investigation into the effects of phosphorylation on fluorescence lifetime again made use of the synthetic Crosstide peptide. To gauge the direct effect of peptide phosphorylation on the fluorescence lifetime of the labeled substrate, the ACE-14-labeled Crosstide was assayed under the same conditions as described for Fig. 1. Analysis of the fluorescence lifetime showed that there was no observable change in fluorescence lifetime. The phosphorylation of the dye-labeled peptide was confirmed by mass spectrometry of duplicate assays (data not shown).

To obtain a change in fluorescence lifetime dependent on phosphorylation, the ability of two antibodies, a generic anti-phosphoserine antibody and the more target-specific phospho-GSK-3 α (Ser21)(27ES) monoclonal antibody (Cell Signaling) (because the substrate peptide is based on one of the GSK3 phosphorylation sites), to effect a lifetime change were tested, but again no effect on fluorescence lifetime was observed with either antibody.

Because no observable effects on fluorescence lifetime were observed in the above experiments, we investigated whether or not the distance of the phosphate from the dye was a factor in determining the ability of the phosphate to affect the lifetime of the dye. A series of synthetic phosphorylated peptides were obtained with a C-terminal lysine, and the ϵ -amino groups of the lysines were then labeled with the reporter dye of choice at increasing distances from the phosphorylated residue, as shown in Table 1. The other amino acids in the peptides were chosen at random because their only function was to serve as spacer residues.

Comparison of the fluorescence lifetime of the control peptide [A] containing no phosphate and the peptide with the phosphate

Table 1
Synthetic phosphorylated peptides.

Ac-GSPNANK	[A]	Ac-GPNS(Pi)ANK	[D]
Ac-GS(Pi)PNANK	[B]	Ac-GPNAS(Pi)NK	[E]
Ac-GPS(Pi)NANK	[C]	Ac-GPNANS(Pi)K	[F]

Note. Series of *N*-acetylated synthetic phosphopeptides labeled with ACE-14 dye on the C-terminal lysine. S(Pi) indicates the position of the phosphoserine residue. Letters in brackets are used in reference to the text.

adjacent to the labeled lysine [F] showed no difference in lifetime (data not shown). From this, we concluded that the proximity of a phosphate per se is not sufficient to change the lifetime of the dye. From these preliminary experiments, it was apparent that if phosphorylation-dependent changes in the fluorescence lifetime were to be induced, a different strategy would need to be employed.

Development of metal chelates

The high affinity of phosphates for trivalent metal cations, particularly those of aluminium, iron, and gallium, is well known and central to the purification of phosphopeptides using immobilized metal affinity chromatography (IMAC) [14]. Investigations were carried out to determine whether the use of iron, as well as other transition metals, in conjunction with a chelate could be used as an indicator of the presence of phosphate groups within a series of substrates. Having established that the presence of a phosphate group alone is insufficient to alter the fluorescence lifetime and that neither of the phospho-specific antibodies was capable of altering the lifetime of the labeled peptide, we investigated the effect of various ionic species on the dye lifetime. Of the salts tested, only iodide, nickel(II), copper(II), and iron salts had any significant effect on lifetime, with iodide eliminating the lifetime almost completely in a concentration-dependent manner. Significantly, sodium phosphate also failed to affect the fluorescence lifetime (data not shown).

It was found that iron(II) as ferrous sulfate on its own results in a lifetime change in both the phosphorylated and nonphosphorylated peptides in a concentration-dependent manner, as shown in Fig. 2. The effect on the nonphosphorylated peptide is probably mediated through the nitrogen-containing side chains of the peptide interacting with the iron, resulting in two or more peptides being brought into close proximity whereby the dye molecules could interact.

Iron(III) has a lower affinity for interaction with nitrogens, and its use was expected to reduce any effect on the nonphosphorylated peptide. It was also hoped that the presence of chelating groups containing aromatic substituents would increase the phosphate-specific effect on lifetime. Iron(III) alone had a similar effect on fluorescence lifetime to that obtained by iron(II) but, as anticipated, had a lesser effect on the fluorescence lifetime of the nonphosphorylated peptide. Combining the iron(III) with an aromatic chelator should have two advantages: the polymerization of iron(III) in aqueous solution can be ameliorated by chelation, and the presence of an aromatic group on the chelate has the potential to induce a greater lifetime change when brought into proximity with the dye via iron-mediated complexes. Exploiting the encour-

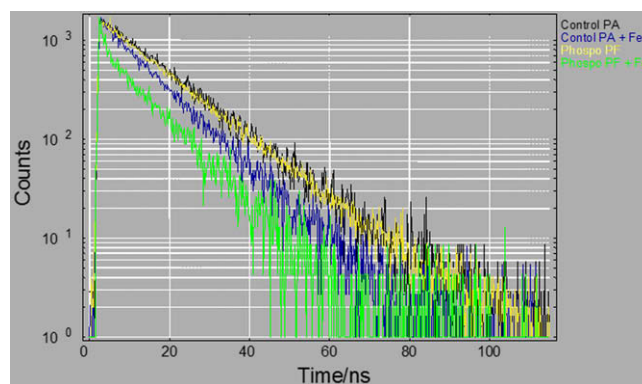


Fig. 2. Fluorescence decay curves for ACE-14-labeled peptides [A] and [F] (Table 1) in the presence and absence of iron(II) ions.

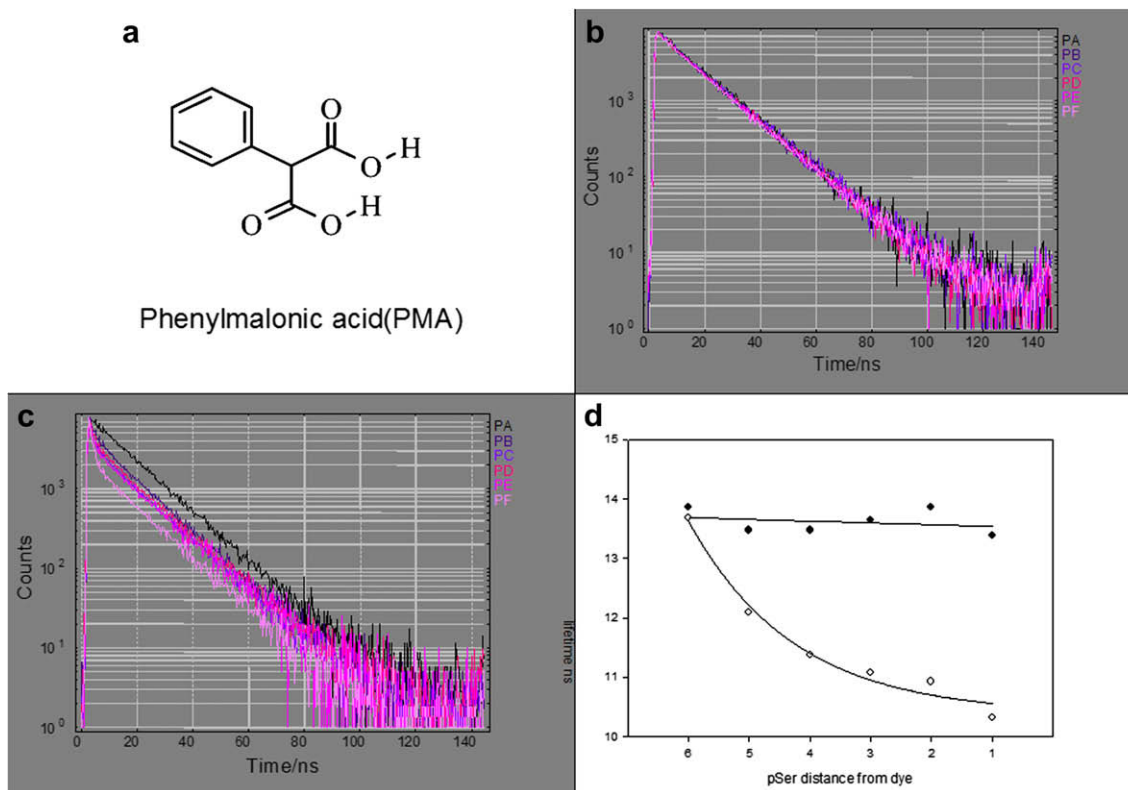


Fig. 3. Effect of PMA and iron(III) on fluorescence lifetime of ACE-14-labeled phosphopeptides (Table 1) and effect of phosphate distance from the chromophore. (A) Structure of PMA. (B) Fluorescence decay curves for peptides in the absence of iron and PMA. (C) Fluorescence decay curves for peptides in the presence of iron and PMA. (D) Plot of relationship between distance of the phosphate from the chromophore and fluorescence lifetime in the presence (○) and absence (●) of iron and PMA.

aging results above, several iron chelates were tested for their ability to affect the ACE-14 lifetime in a phosphate-dependent manner. The compound phenylmalonic acid (PMA) (Fig. 3A) in equimolar amounts with iron(III) was found to be effective in decreasing the lifetime of the dye in a phosphate-dependent manner. The distance over which the effect of the chelate could be observed was also investigated using the previously synthesized phosphopeptide series A to F (Table 1). The data presented in Fig. 3B and C show the lifetime decay curves for the peptide series in the absence and presence of iron chelate, respectively. A plot of the average lifetime against dye-labeled residue distance (Fig. 3D) shows a regular relationship between the fluorescence lifetime of the phosphorylated peptide and the distance of the phosphorylated residue from the dye (note that distance 6 corresponds to the control nonphosphorylated peptide).

To determine the sensitivity of the assay for phosphopeptides, a series of peptide mixtures containing the control nonphosphorylated Crosstide peptide and increasing mole fractions of phosphorylated Crosstide peptide were prepared and the lifetimes were measured on the addition of 16 mM Fe(III)/PMA solution in 10% acetic acid. The results show (Fig. 4A) that a 10% to 20% substrate conversion is easily detectable and, therefore, the detection sensitivity is in the range required for initial rate measurements of enzyme activity.

Trial assays using ACE-14 dye

Using the iron/PMA chelate described above, a trial PKB α assay was conducted using a substrate mix containing ATP, MgCl₂, and dye (ACE-14)-labeled Crosstide in a volume of 15 μ l that was added to 10 μ l of recombinant PKB α enzyme, giving final concentrations of 20 μ M ATP, 2 mM MgCl₂, and 20 μ M peptide. After incu-

bation at 25 °C for 20 min, the assay was stopped by the addition of 25 μ l of 2 mM iron chelate in 50% acetic acid. The data shown in Fig. 5A show a time course of the assay, and using the same reaction conditions, the linear dependency on protein concentration is shown in Fig. 5B.

To verify the applicability of this assay format to a wide range of protein kinases, we obtained three generic substrate peptides, RARTLSFAEPG (peptide 1), RRRLSFAEPG (peptide 2), and KKL

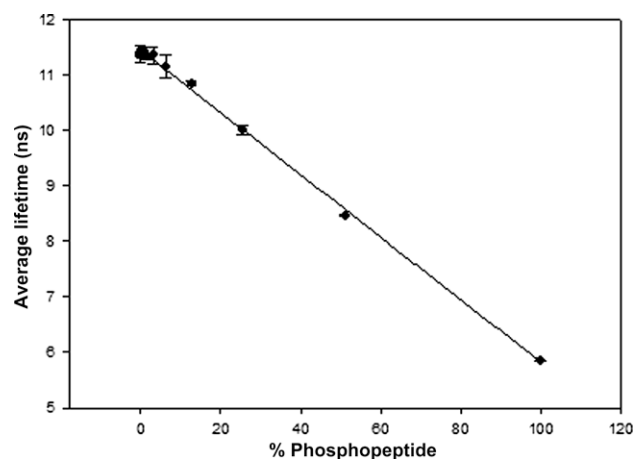


Fig. 4. Standard curve showing decrease in lifetime with increasing percentages of phosphopeptide. Peptides [A] and [F] (Table 1) labeled with ACE-14 dye were mixed to give a final concentration of 10 μ M with increasing percentages of phosphopeptide in a volume of 25 μ l of assay buffer (see Materials and methods). An equal volume of PMA/iron(III) at a concentration of 16 mM in 10% acetic acid was added and fluorescence lifetime was measured (see Materials and methods).

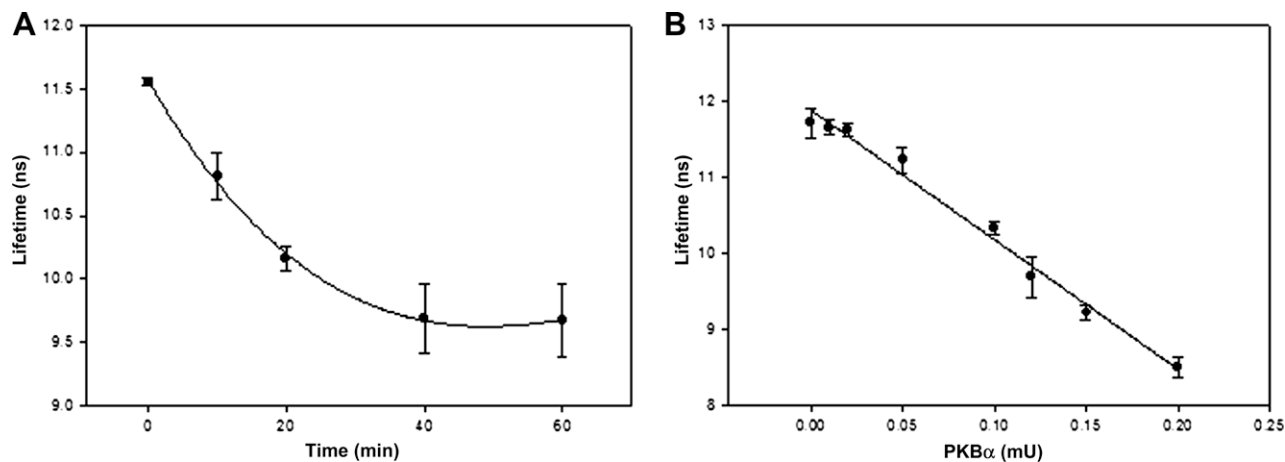


Fig. 5. Time course and protein dependence of PKB α activity. The time course (A) of PKB α activity was determined by incubation of ACE-14-labeled Crosstide at 20 μ M in assay buffer (see Materials and methods) containing 2 mM MgCl₂ and 20 μ M ATP with 0.1 mU of PKB α in a volume of 25 μ l. The assay was terminated by the addition of an equal volume of 16 mM PMA/iron(III) perchlorate in 50% acetic acid and fluorescence lifetime was measured (see Materials and methods). The protein dependence of the reaction (B) was determined as for the time course except that the amount of enzyme was varied as shown and the incubations were for 20 min in all cases.

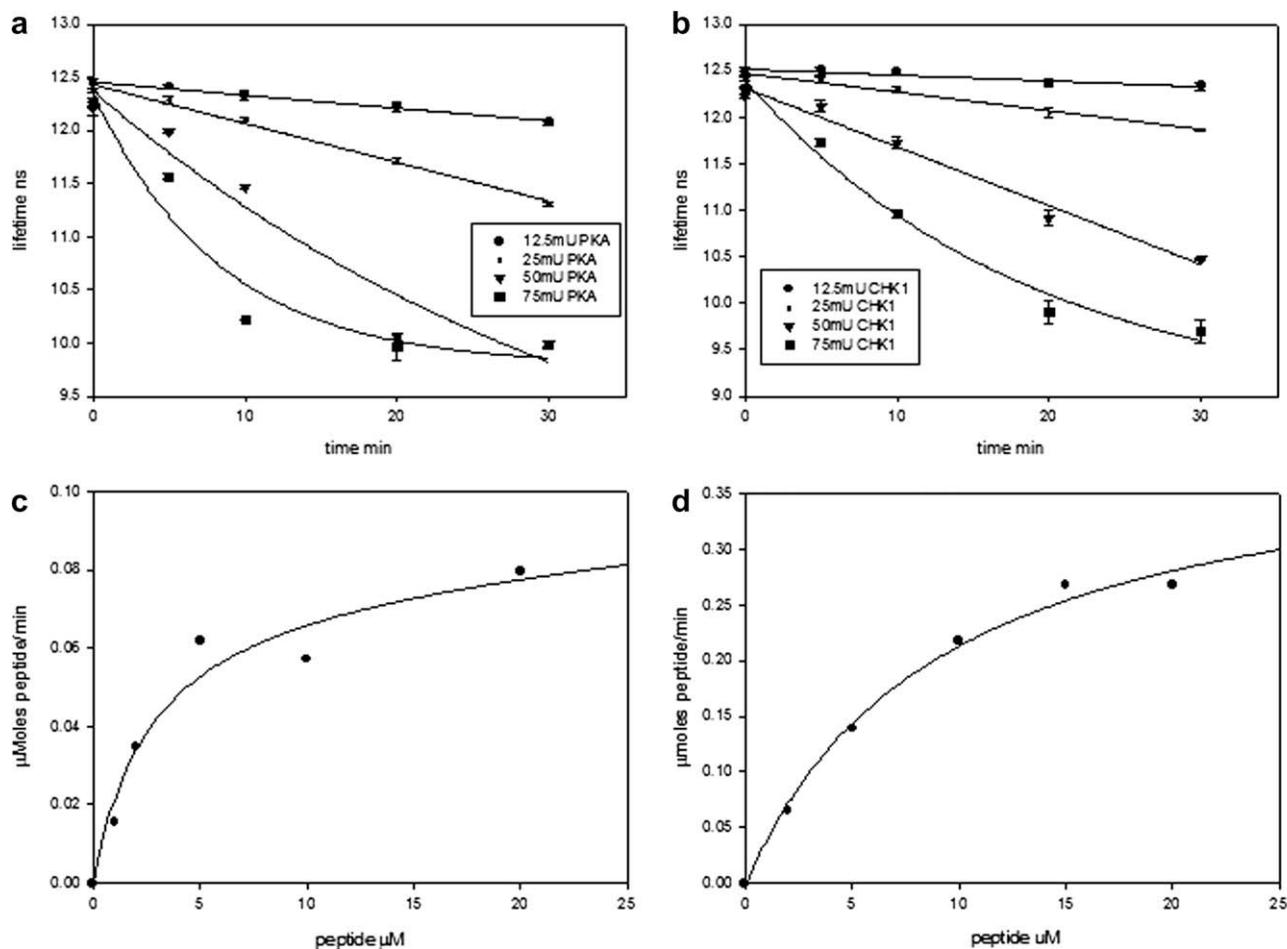


Fig. 6. (A and B) Time courses of PKA (A) and CHK1 (B) carried out at the various enzyme concentrations shown. These assays were conducted using generic peptides 2 (PKA) and 3 (CHK1) (see Materials and methods) labeled with ACE-14 dye as substrates. (C and D) Plots of initial rates of reaction dependent on substrate peptide concentration for estimation of K_m as described in the text. All assays were conducted with 5 mM MgCl₂ and 0.5 mM ATP in a volume of 25 μ l and were terminated by the addition of an equal volume of 16 mM PMA/iron(III) perchlorate in 25% acetic acid and fluorescence lifetime was measured (see Materials and methods).

NRTLFAEPG (peptide 3), labeled at the C terminus with ACE-14 dye. These peptides, first described by Ross and coworkers [5], were designed to act as substrates for several of the main groups of pro-

tein kinases that the authors anticipated would allow the assay of more than 100 protein kinases. After further optimization of the assay parameters with regard to ATP and chelate concentration, the

kinetic parameters for the assay were determined using the synthetic peptides described above with the protein kinases CHK1 (checkpoint kinase 1, peptide 3) and PKA (protein kinase A, peptide 2). The apparent K_m value for each peptide substrate was estimated, as demonstrated by the example shown in Fig. 6. Fig. 6A and B show data from time course experiments conducted at different enzyme

concentrations to determine linear reaction conditions for use in determining initial rates of reaction for K_m determinations. Fig. 6C and D show the results of K_m determinations for PKA and CHK1, respectively. The initial rates shown in the K_m plots were determined in triplicate from time courses of enzyme activity conducted at the peptide concentrations shown. The data are expressed as

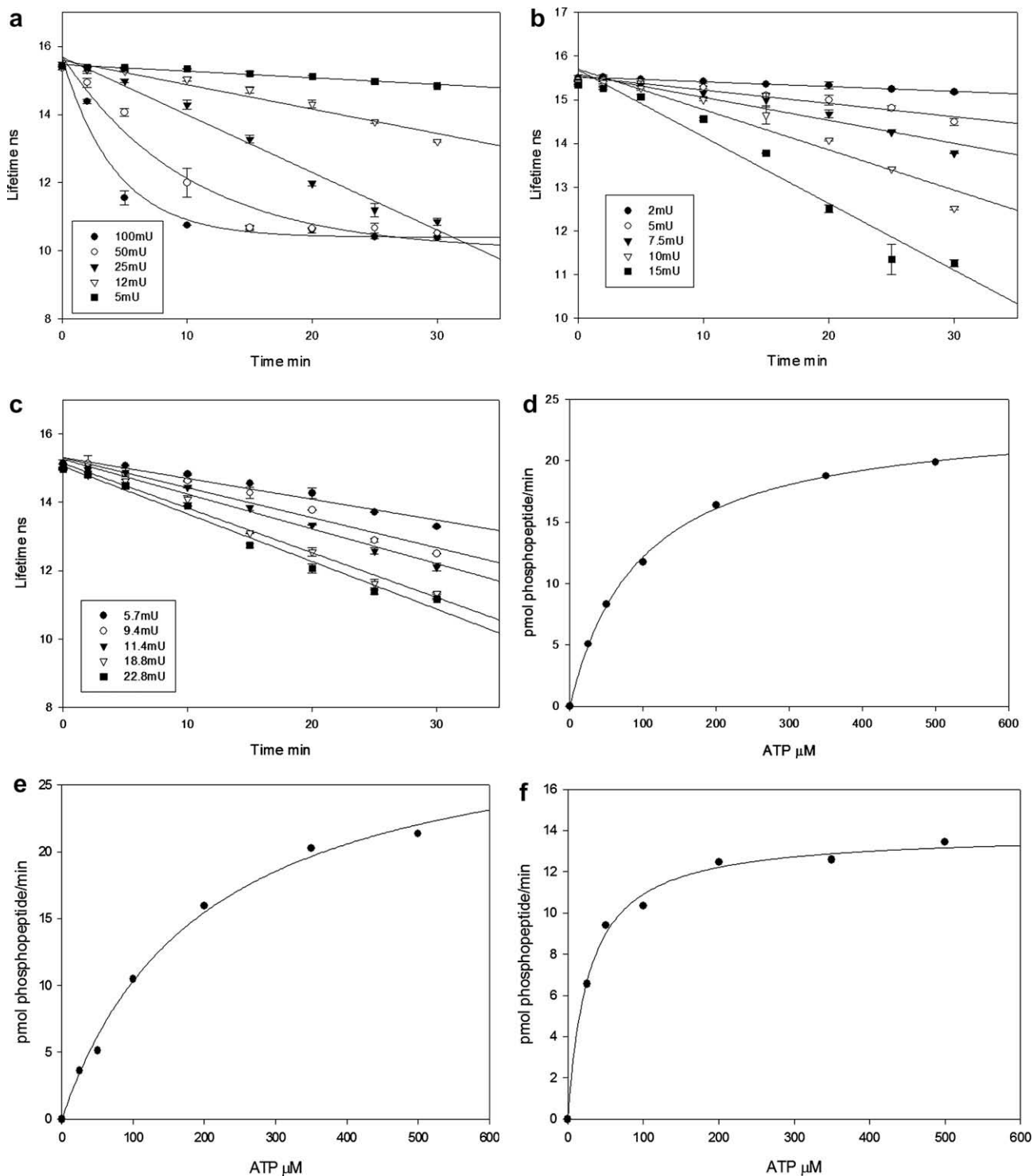


Fig. 7. (A–C) Example data obtained for enzyme linearization for PKB α (A), PKA (B), and CHK1 (C). (D–F) Estimation of ATP K_m values for PKB α (D), PKA (E), and CHK1 (F). Assays were conducted using generic peptides 1, 2, and 3 (see Materials and methods) labeled with 9AA dye, respectively. The substrate peptides were used at a concentration of 10 μ M, and the ATP concentration was varied as shown. All assays were conducted with 5 mM MgCl₂ in a volume of 25 μ l and were terminated by the addition of an equal volume of 16 mM PMA/iron(III) perchlorate in 25% acetic acid and fluorescence lifetime was measured (see Materials and methods). The data plotted are initial rates determined from time courses conducted at the different ATP concentrations as described in the text.

Table 2

Summary of kinetic data for exemplar enzymes using the 9AA-labeled generic peptides (derived as described in the text) in comparison with those determined by radiometric assay.

Peptide	Kinase	Peptide K_m (μM)		ATP K_m (μM)	
		Radiometric	Lifetime	Radiometric	Lifetime
3	CHK1	5	3	50	95
2	PKA	30	7	20	27
3	MAPKAP-K2	5	1	49	96
1	p70s6K	6	29	43	32
1	PKB α	2	4	140	197
3	PRAK1	40	3	20	25
1	SGK1	4	2	46	332
1	MSK1	2	8	55	256

micromole peptide phosphorylated per minute (μmol peptide phosphorylated/min) by reference to a standard curve constructed by mixing known amounts of phosphopeptide and nonphosphopeptide and measuring the fluorescence lifetime under assay conditions. The K_m values were determined by nonlinear regression fitting using the SigmaPlot software package with values of $2.8 \mu\text{M}$ for PKA and $9.1 \mu\text{M}$ for CHK1. Further experimental details are given in the figure legend.

Application of 9AA dye and estimation of assay kinetics

The lifetime of the dye used to label the substrate peptides may influence the dynamic range of the assay in that a longer lifetime dye should give the potential for a greater dynamic range. To this end, we have developed a novel fluorophore based on 9-aminoacridine (9AA) that has a lifetime of 17 ns (full details to follow). This derivative has both brightness and excitation maximum comparable to those of ACE-14 as well as a significantly longer lifetime and increased solubility. We prepared three generic substrate peptides, RARTLSFAEPG (peptide 1), RRRLSFAEPG (peptide 2), and KKLNRTLSFAEPG (peptide 3), labeled with 9AA dye. The lifetimes of these substrates were found to be 15.7 ns (peptide 1), 15.8 ns (peptide 2), and 15.6 ns (peptide 3), respectively, under stopped assay conditions, whereas the phosphorylated forms of the peptides were synthesized and, under the same conditions, the lifetimes were shown to be 10.6 ns (peptide 1), 11.3 ns (peptide 2), and 10.7 ns (peptide 3), respectively. This significant phosphorylation-dependent change in lifetime for all three peptides confirmed them as excellent reporters for our lifetime kinase assay technology. After further optimization of the assay parameters with regard to ATP and chelate concentration, the kinetic parameters for the assay were determined using the synthetic peptides described above

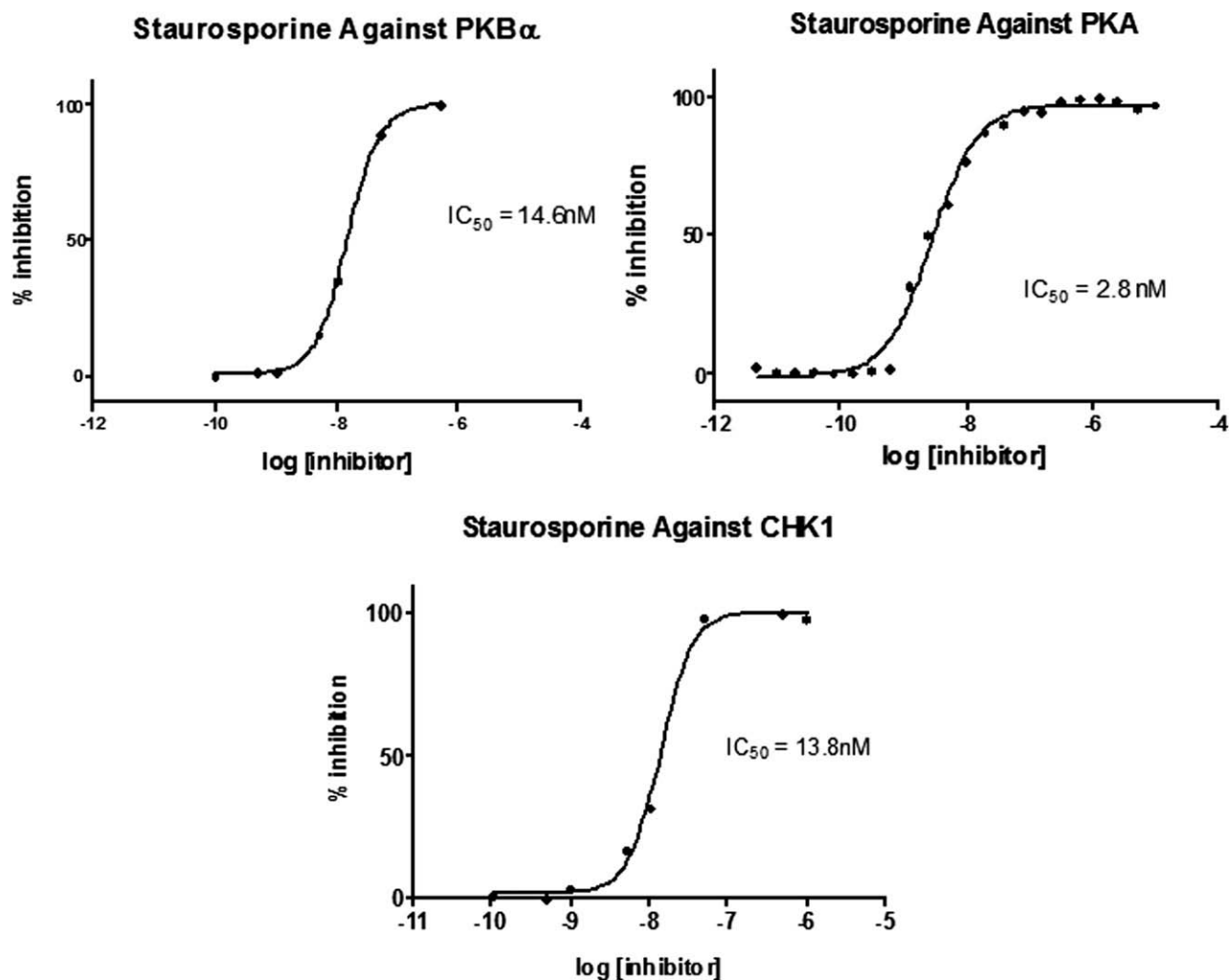


Fig. 8. Estimation of IC_{50} values for staurosporine inhibition of PKB α , PKA, and CHK1 activity using 9AA-labeled peptides. Assays were conducted using PKB α (25 mU/assay) with peptide 1 (20 μM), PKA (10 mU/assay) with peptide 2 (20 μM), and CHK1 (14.25 mU/assay) with peptide 3 (20 μM) to a final volume of 12.5 μl in the presence of increasing concentrations of staurosporine. The reactions were started by the addition of 1 mM ATP/2 mM MgCl_2 in 12.5 μl of assay buffer. After 30 min, the reaction was terminated by the addition of 16 mM PMA/iron(III) perchlorate in 25% acetic acid (25 μl) and fluorescence lifetime was measured (see Materials and methods). Average lifetime data were converted to percentage inhibition by reference to the standard curves for the peptide substrates.

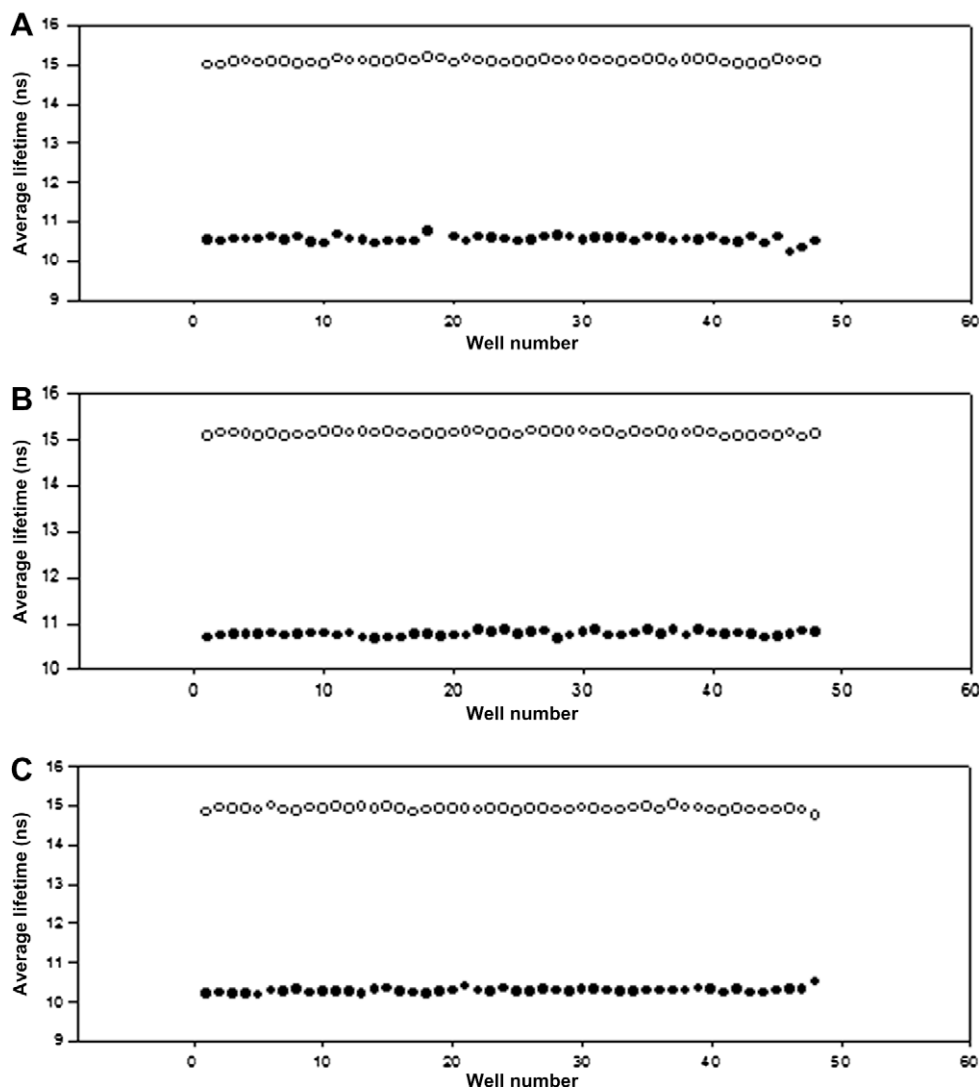


Fig. 9. Z' Factor estimation for PKB α , PKA, and CHK1. For each enzyme assay, mixes were prepared containing 20 μ M 9AA-labeled peptide and 30 mU of PKB α (peptide 1) (A), 10 mU of PKA (peptide 2) (B), or 14.25 mU of CHK1 (peptide 3) (C) in 12.5 μ l of assay buffer without ATP. This assay mix was added to wells of a 96-well plate, half of which contained 25 μ l of 16 mM PMA/iron(III) perchlorate in 25% acetic acid stop solution (\circ) and half of which were empty (\bullet). The assays were started by the addition of 1 mM ATP/2 mM MgCl₂ in 12.5 μ l of assay buffer to all wells and were incubated for 30 min before the addition of stop solution to the wells where not already present. Fluorescence lifetimes were then determined as described previously (see Materials and methods).

for the protein kinases PKB α , SGK1 (serum- and glucocorticoid-induced protein kinase 1), MSK1 (mitogen- and stress-activated kinase 1), and p70s6 K (p70 S6 kinase) (peptide 1); PKA (peptide 2); and PRAK1 (p38-regulated/activated protein kinase), MAPKAP-K2 (mitogen-activated protein kinase-activated protein kinase 2), and CHK1 (peptide 3). Using the enzymes listed and the 9AA-labeled peptides, the K_m values for the peptide substrates and ATP were estimated as described for PKA and CHK1 above. Fig. 7 shows example data obtained for enzyme linearization for PKB α , PKA, and CHK1 (Fig. 7A–C, respectively) and ATP K_m values for PKB α , PKA, and CHK1 (Fig. 7D–F, respectively). A summary of the K_m values obtained for both peptide and ATP is shown in comparison with the values obtained by radiometric assay in Table 2. The kinetic parameters, with respect to the peptides, for the radiometric assay are as published previously [5] and for ATP were provided by University of Dundee, Division of Signal Transduction and Therapeutics, Kinase Profiling Service. The kinetic parameters obtained by the two assay methods are in broad agreement with few exceptions. In some cases, the dye-labeled peptide has a higher, and occasionally lower, K_m value than that measured by radiometric assay. This may be a consequence of the dye altering the affinity

of the substrate, either positively or negatively, for the enzyme. However, in general, the kinetic parameters indicate that the assay is practicable for the enzymes tested.

Inhibitor testing

To verify that the assay can perform in inhibitor studies, assays were performed to determine the IC₅₀ value with the general kinase inhibitor staurosporine for the enzymes PKB α , PKA, and CHK1. The results shown in Fig. 8 demonstrate that the assay can be used in determining inhibitor kinetics giving IC₅₀ values for staurosporine inhibition of 14.6 nM (PKB α), 2.8 nM (PKA), and 13.8 nM (CHK1) at an ATP concentration of 0.5 mM. These values are in close agreement with previously published data, namely 1.5 nM (PKB α), 3.6 nM (PKA), and 9.5 nM (CHK1) at an ATP concentration of 10 μ M [15].

Z' determination

To be of use in inhibitor screening, the assay must fulfill other criteria in that it must be reproducible, give data comparable to

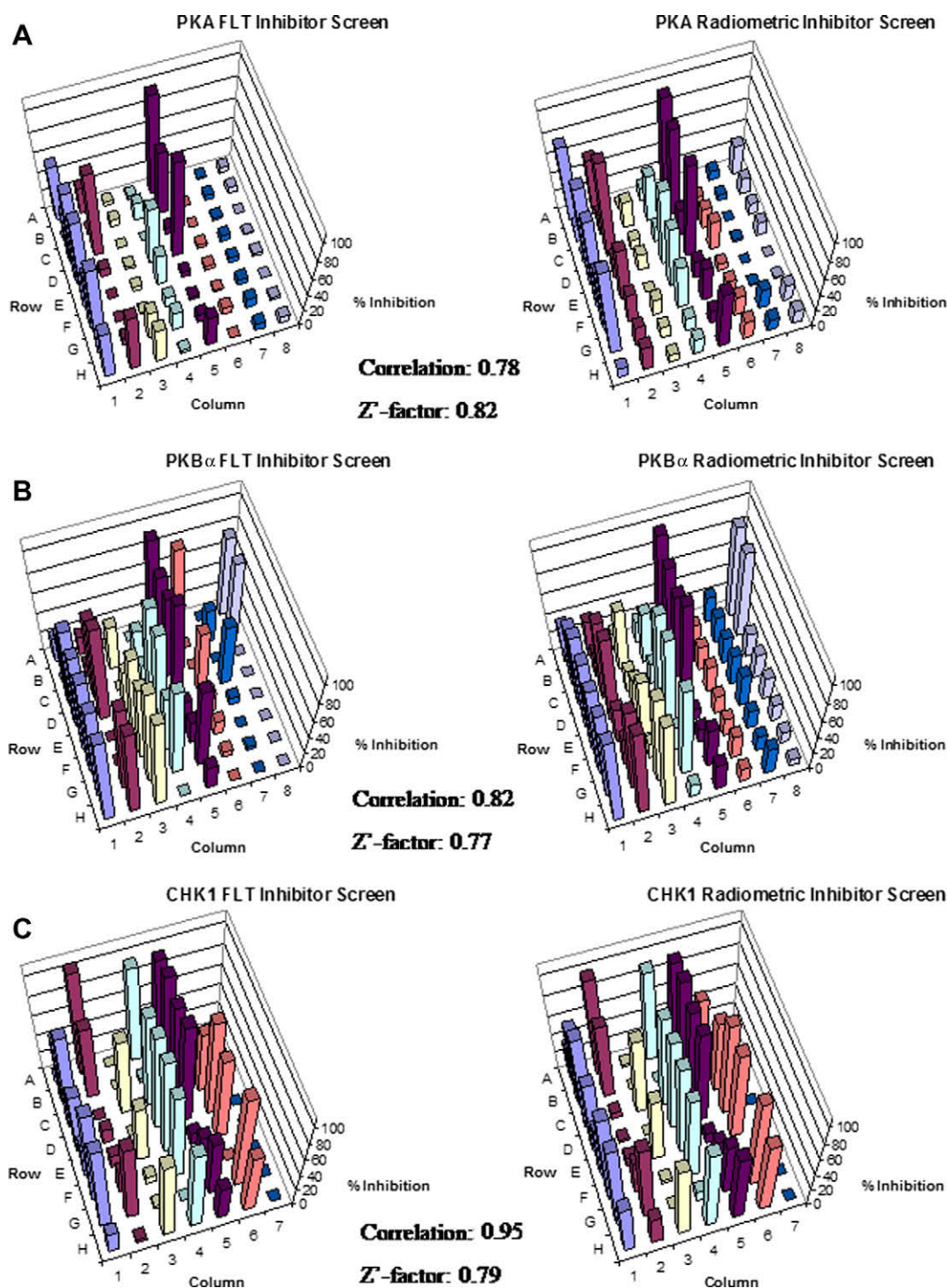


Fig. 10. Comparison of fluorescence lifetime (FLT) with radiometric assay in screening for PKA (A), PKB α (B), and CHK1 (C). The figure shows data from example plates used in an inhibitor screen that was conducted using a radiometric assay (University of Dundee, Division of Signal Transduction and Therapeutics, Kinase Screening Service) in comparison with data obtained using fluorescence lifetime. The fluorescence lifetime assays were conducted under conditions of linearity for reaction rates. For all three enzymes, the concentrations were 10 μ M peptide, 0.5 mM ATP, and 2 mM MgCl₂ in assay buffer. Enzyme and peptide substrates were added to wells containing 1 μ l of test compounds in dimethyl sulfoxide (DMSO) and were incubated for 10 min before starting the assay by the addition of ATP/MgCl₂. After 15 min, the reactions were terminated by the addition of an equal volume (25 μ l) of 16 mM PMA/iron(III) perchlorate in 25% acetic acid stop solution. Controls for 0% and 100% inhibition with 1 μ l of DMSO and stop added before the addition of ATP, respectively, were also included, allowing the calculation of Z' values as a quality control check.

the gold standard radiometric assay, and be free of interference from test compounds. To address these points, the Z' values for the assay, the standard measure of reproducibility and signal-to-noise discrimination, were determined [16]. The Z' value is obtained by conducting multiple sets of assays consisting of controls and assay points. In this case, the controls were assays stopped be-

fore the addition of ATP. All other parameters were as described in the figure legend. The data shown in Fig. 9 for PKB α give a calculated Z' factor of 0.92, for PKA give a Z' factor of 0.94, and for CHK1 give a Z' factor of 0.94 (Fig. 9A–C, respectively). These excellent scores indicate that this assay is viable for use in high-throughput screening [16].

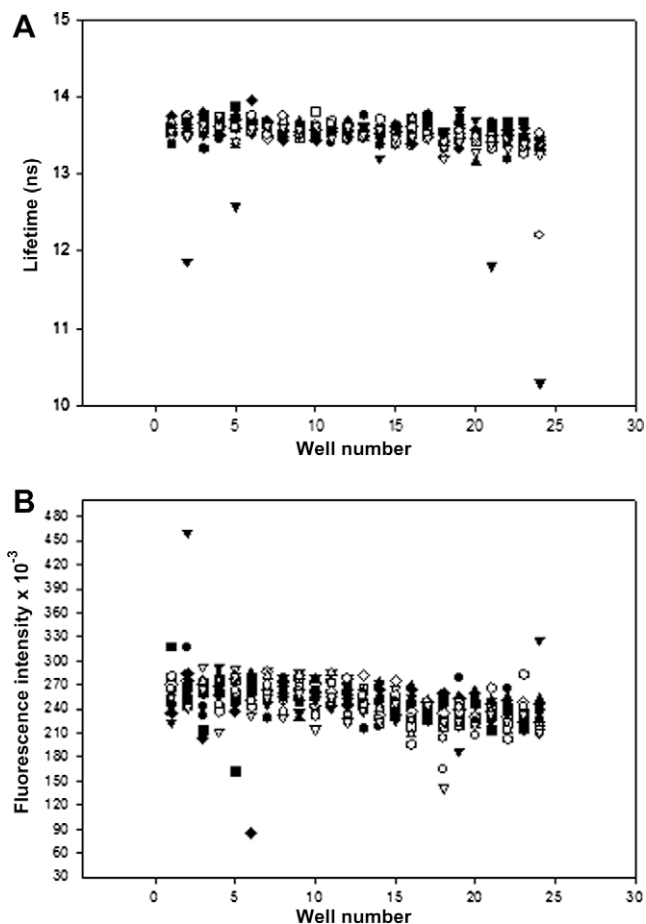


Fig. 11. Comparison of fluorescence intensity and fluorescence lifetime susceptibility to compound interference: (A) fluorescence lifetime readings; (B) fluorescence intensity readings. Details of the plate setup are given in the text.

Comparison with radiometric screening

Comparisons of data obtained with each of the peptide substrates with those obtained with an independent radiometric assay conducted by the University of Dundee, Division of Signal Transduction and Therapeutics, screening service were carried out. The results presented in Fig. 10 for the enzymes PKB α , PKA, and CHK1 (Fig. 10A–C, respectively) using 9AA-labeled substrates show that the lifetime assay gives results comparable to those obtained by the radiometric assay with Z' values of approximately 0.8 and correlation coefficients of 0.78–0.95 when compared with the radiometric assay values.

Resistance to interference

With regard to the potential of compounds to interfere with the assay readout, a preliminary test was carried out by mixing 9AA-labeled phosphopeptide and nonphosphopeptide in a 40:60 ratio in assay mix to simulate an assay with 40% substrate conversion. This was then added to 384-well plates containing 1408 randomly selected compounds from a compound library. The lifetime values for all wells should be identical in the absence of compound interference. Examples of lifetime and fluorescence intensity for the same plate are shown in Fig. 11A and B, respectively. Defining off-target readings as greater than 1 standard deviation from the plate mean, the data from all of the test plates indicate that the lifetime readings give 1% off-target readings, whereas the fluorescence intensity readings give 4% off-target readings.

Discussion

We have presented data showing that fluorescence lifetime is an excellent format for protein kinase assays in that it provides a simple homogeneous format that is amenable to full automation and scalable to any assay volume required because the lifetime readout is a concentration-independent parameter.

The assay, when compared with the gold standard radiometric formats for protein kinases, gives comparable Z' values and in actual screening trials gave nearly identical results to those of the radiometric assay with a correlation of 0.85 or better. The assay also gives comparable kinetic parameters for peptide substrate and ATP K_m values with a few exceptions that can probably be accounted for by the difference in substrate structure caused by the addition of the reporter dye to the peptides.

The assay has an advantage over the radiometric assay that, in principle, it can be carried out at any ATP concentration without the loss in sensitivity that occurs in the radiometric assay with the reduction in specific activity of the labeled ATP. There are further advantages over other kinase assay formats in that the assay does not require any expensive antibodies or washing steps. Further advantages include the low cost of the reagents and elimination of costs for the storage or disposal of radioactive waste.

One of the main advantages of fluorescence lifetime as a readout is the relative freedom from interference by exogenous test compounds. This is shown by the results presented in Fig. 11 comparing the 1% off-target results in the lifetime readings compared with 4% in the fluorescence intensity readings. This compares favorably with the findings of other workers, notably Fowler and coworkers [17], who found that lifetime-discriminated fluorescence polarization gave a hit rate of 2.84% compared with 9.1% for standard fluorescence polarization and 0.54% for radiometric assay at 50% inhibition threshold. Results of a study by Moger and coworkers [18] indicated that fluorescence lifetime measurements had the potential to reduce the incidence of false positives due to compound aggregation that the authors estimated may be responsible for up to 70% of false positives and that the 30% due to fluorescence could be resolved due mostly to the very short lifetimes of the interfering compounds. We would point out that in this study, we used novel long lifetime dyes compared with the above studies that will increase the efficacy of this discrimination.

Using this approach, fluorescence lifetime assays have now been successfully developed for a variety of different serine/threonine kinases, and compatibility with a wide range of ATP and substrate concentrations has also been demonstrated (data not shown). This homogeneous, antibody-free, fluorescence lifetime-based protein kinase assay technology shows excellent promise as a generic, cost-effective, and robust approach for screening this therapeutically important class of proteins. In addition, fluorescence lifetime assays targeting other enzyme families are currently in development through application of 9AA-labeled peptide reporters.

Acknowledgments

We acknowledge Edinburgh Instruments for provision of the NanoTaurus lifetime plate reader and Dmitry Gakamsky for reader setup advice. Funding for this project was provided by the Technology Strategy Board under the Fluorescence Lifetime-based Assays and Sensors for Healthcare (FLASH) project TP M1537F.

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