ORIGINAL RESEARCH

An alternative kinase activity assay for primary cultures derived from clinical isolates

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Abstract

Purpose: The measurement of protein kinase activity is central to understanding the signaling pathways that regulate cellular proliferation and apoptosis in virtually all disease processes. These measurements typically involve either indirect, time consuming assessment methods that require large amounts of sample, such as western immunoblotting, or the use of high maintenance, specialized equipment not typically available to a small clinical research facility. The purpose of this project was to determine if a benchtop Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) unit could be used to detect and directly assess kinase activity of the serine/threonine kinase Akt.

Method: Biotinylated substrate peptides, predicted to be recognized and phosphorylated by Akt to varying extents, were incubated in crude lysates of primary cells derived directly from clinical isolates. Streptavidin-coated chips were then used to isolate the substrate peptides from the lysates after incubation. Finally SELDI-TOF-MS was used to detect the peptide substrates and identify any changes in mass resulting from phosphorylation.

Results: The biotinylated peptide substrates were readily detected and a simple, rapid procedure that allows direct measurement of Akt activity in less than 1 µg of cell lysate in a 2µL volume was developed. Further, a linear correlation between native to phospho-peptide ratios and SELDI-

TOF-MS output demonstrated that this approach is semi-quantitative.

Conclusion: This assay avoids many of the pitfalls associated with the currently available kinase protocols as well as labour-intensive mass-spectrometry analysis by specialist laboratories. We propose that this approach may be a viable alternative for clinical research laboratories aiming to measure the activity of kinases in clinical isolates.

The human genome encodes approximately 500 protein kinases, which function to transmit chemical signals through the activation/deactivation of their target effector molecules¹. As kinases are components of most, if not all, cellular processes, they clearly have potential as therapeutic targets in human disease. It is therefore desirable to be able to precisely measure their activity and assess their contribution to a particular disease process.

A variety of technologies has been developed in the last few decades to measure kinase activity either directly or indirectly: each has inherent disadvantages that limit or complicate their use. In this report, we describe a proof-of-principle study demonstrating the

use of streptavidin-coated PS-20 activated chips to capture biotinylated peptide substrates from complex kinase reaction mixtures. These mixtures can be subsequently analyzed using a benchtop SELDI-TOF-MS unit to measure kinase activity through the direct assessment of the phosphorylation-induced change in peptide mass. While the method described can be theoretically adapted to measure the activity of any kinase, we chose Akt, also known as Protein Kinase B (PKB), or RAC (related to A and C Kinase) due to its sequence homology to the AGC kinase superfamily members, Protein Kinase A and Protein Kinase C², as a suitable focus for the development of this assay. Akt is a 57 kDa serine/threonine kinase that is the cellular homolog of the Akt8 retroviral transforming protein v-Akt.³ While this kinase was chosen based on its established roles in cellular proliferation⁴, apoptosis⁵, cell motility⁶, protein synthesis⁷ and glucose metabolism⁸, we anticipate that the approach described here may be adapted to the concurrent measurement of multiple kinases in clinical isolates or small volume primary cultures.

Materials and Methods

Cell Culture and Protein Isolation

Primary Fibroblast cultures, treated with or without Platelet Derived Growth Factor (PDGF) to induce Akt activity, were utilized for the optimization of this method. The routine procedures used for cell culture, protein extraction and assessment by Western Immunoblotting are detailed in the Appendix.

Synthetic Peptide Substrates

The Akt substrate peptides designed in this study were based on the target sequence of the endogenous Akt target GSK-3 β . Based on previous studies by Alessi et al., 1996¹¹, Akt Substrate Peptide 1 was predicted to be efficiently phosphorylated by Akt and a variety of other kinases including, but not limited to, P70S6 Kinase and Mitogen-Activated Protein Kinase-Activated

Protein (MAPKAP) Kinase 1. Akt Substrate Peptide 2 was predicted to be more specifically, but less efficiently, phosphorylated by Akt relative to Akt Substrate Peptide 1. Substrate Peptide 3 was predicted to be unsuitable as an Akt substrate and was included as a negative control for Akt kinase activity. All 3 Substrate peptides were synthesized by the Don Rix Protein Identification Facility at the University of Western Ontario (London, Ontario, Canada) with an N-terminal biotin moiety.

GSK-3 β (residues 1-14)
(MSG **RPRTTSF** AESC)

Akt Substrate Peptide 1:
Biotin – GGSG **RPRTTSF** AESA-COOH,
MW 1706 Da

Akt Substrate Peptide 2:
Biotin – GGSG **RPRAATF** AESA-COOH,
MW 1660 Da

Akt Substrate Peptide 3:
Biotin – GGSG **RPRAATE** AESA-COOH,
MW 1642 Da

In Vitro Kinase Reactions

In vitro kinase reactions were performed using human recombinant active Akt1, P70S6 Kinase (BioSource, Invitrogen – MediCorp, Montreal, QC, CA), MAPKAP Kinase 1 (K1) and SGK (Δ 1-59, S422D, Upstate Cell Signaling Solutions – Millipore, Billerica, MA, USA). Recombinant Akt1 was diluted in enzyme dilution buffer (20 mM Tris-HCl, pH 7.2, 1 mM EDTA, 0.01% Brij – 35, 5% glycerol, 0.1% 2 β – Mercaptoethanol and 1mg/mL Bovine Serum Albumin (BSA, BioShop, Burlington, ON, CA)) to 5000 ng/mL and stored in 5 μ L aliquots at -80 °C. Recombinant P70S6 Kinase, MAPKAP Kinase 1 and SGK were diluted in a similar enzyme dilution buffer (20 mM MOPs, pH 7.2, 1 mM EDTA, 0.01% Brij – 35, 5% glycerol, 0.1%

2 β – Mercaptoethanol and 1mg/mL BSA) to 5000 ng/mL, 500 ng/mL and 1650ng/mL respectively and stored in 5 μ L aliquots at -80°C. MAPKAP K1 and SGK were further diluted 10 fold and 3 fold respectively to normalize their activity per mg of enzyme to both Akt1 and P70S6 Kinase prior to assay.

Recombinant enzyme kinase reactions were adapted from Suresh Babu et al., 2005⁹. In brief, reactions were set up in PCR tubes (Progene, St. Laurent, QC, CA) in 20 μ L reaction volumes containing 10 x Mg/ATP solution (100 mM MOPs (pH 7.2), 375 mM MgCl₂, 2.5 mM ATP (Invitrogen, Carlsbad, CA, USA), 25 mM EGTA, 25 mM β -glycerol phosphate, 5 mM Na₃V0₄, 5 mM DTT), 10 x kinase reaction buffer (80 mM Tris-HCl (pH 7.2), 2 mM EDTA), the substrate peptides and recombinant enzyme. Final concentrations of the enzymes in reaction were 1000 ng/mL for Akt1 and P70S6Kinase, 100 ng/mL for MAPKAP Kinase 1, and 330 ng/mL for SGK. Final substrate peptide concentrations were 10 ng/ μ L. Kinase reactions were prepared on ice and performed in a P x 2 thermal cycler (Thermo Scientific, Inc. – Fisher Scientific, Inc., Ottawa, ON, CA) at 30 °C for 1 hour. Reactions were stopped by boiling the samples at 100 °C for 5 minutes and then either kept on ice for subsequent SELDI analysis or stored at -20 °C.

In vitro kinase reactions using whole cell lysate were performed as above except that 10 μ g of whole cell lysate was used in place of recombinant enzyme. In addition, final substrate peptide concentrations in the reactions were reduced to 0.33 ng/ μ L and peptide was diluted in Akt enzyme dilution buffer prior to addition to the reactions. Finally reactions with cell lysate were performed for 2 hours at 30° in 30 μ L volumes.

PS20 Array Chip Preparation and SELDI-TOF MS Analysis

PS20 chips are pre-coated with free epoxide groups by the manufacturer (CIPHERGEN Biosystems, Inc. – Biorad Laboratories Inc., Hercules, CA, USA). PS20

chips were loaded into the Ciphergen Bioprocessor sample reservoir to accommodate the addition of substance volumes of up to 400 μ L to the 8 sample spots on up to 12 array chips. Array spots were coated in excess with 10 μ L of 20 ng/ μ L (200 ng per spot) of Immunopure Streptavidin (Pierce Biotechnology, Rockford, IL, USA) in Phosphate Buffered Saline (PBS). Streptavidin was added by micropipetting into the Bioprocessor wells then covering the unit with Parafilm and placing the Bioprocessor on a MicroMix 5 (DPC – Inter Medico, Markham, ON, CA) microplate shaker for 1 hour at room temperature with the Form set to 20 and the Amplitude set to 3.

Subsequent to incubation, spots were washed twice with 50 μ L of PBS-T (1 x PBS with 0.1% Triton X-100 (Biorad Laboratories Inc., Hercules, CA) per spot using a multi-channel micropipette to pipette the wash up and down. The Bioprocessor was then MicroMixed for 5 minutes with the Form set to 20 and the Amplitude set to 5. This process was performed 3 times with vacuum aspiration using a 5 $\frac{3}{4}$ inch Pasteur Pipette capped with a disposable 200 μ L pipette tip to remove and discard the waste wash buffer.

To ensure that no free epoxide groups were available to react on each PS20 spot after coating with Streptavidin, spots were blocked with 2 mg/mL Bovine Serum Albumin (BSA) (BioShop, Burlington, ON, CA) in 1M Tris (pH 8) with 0.1% Triton X-100 (Biorad Laboratories Inc., Hercules, CA,)). One mL aliquots of Triton X-100 free blocking buffer were frozen at -20 °C, thawed and Triton X-100 was added just prior to use. The blocking buffer was added at 50 μ L per spot, and placed on the MicroMix 5 with the Form set to 20 and the Amplitude set to 5 for 30 minutes at room temperature. After blocking, spots were washed as described previously.

Kinase reaction samples containing Akt Substrate Peptide 1, 2 or 3 were then free spotted (i.e. without the use of a Bioprocessor) onto the Streptavidin coated and blocked PS20 spots in duplicate using a micropipette. 2 μ L volumes were free spotted, equating to 20 ng of substrate peptide in total per spot for

recombinant enzyme reactions and 0.67 ng for lysate reactions. PS20 array(s) were then put into a humidity chamber for 1 hour at room temperature. At the end of the incubation, the chip was returned to the Bioprocessor, and each spot was washed 3 times with 100 μ L of wash buffer consisting of 50 mM Ammonium Bicarbonate (Sigma – Aldrich, St. Louis, MO, USA) in 20% Methanol, followed by shaking on the Micromix for 5 min with Form 20, Amplitude 5.

α -Cyano-4-Hydroxy-Cinnamic Acid (CHCA) (Sigma – Aldrich, St. Louis, MO, USA) was used as the energy absorbing matrix (EAM) for this assay. Immediately prior to use, a saturating quantity of CHCA was added to a 1.5 mL microcentrifuge tube (Progene, St. Laurent, QC, CA) containing EAM diluent (50% Acetonitrile, 0.25% Trifluoro-Acetic Acid (TFA) (Sigma – Aldrich, St. Louis, MO, USA)). The mixture was vortexed for 2 min to ensure saturation and centrifuged at 13 000 rpm for 10 min at room temperature to remove un-dissolved CHCA. Saturated CHCA in EAM Diluent was then diluted 5 fold with more EAM diluent to a 20% saturated solution. PS20 arrays were prepared for matrix addition by free spotting 2 μ L of Milli-Q H₂O onto each spot as a final wash to remove salts and other contaminants that may interfere with MALDI-TOF MS. The H₂O was then removed and a 20% CHCA matrix solution was added by free spotting a 1 μ L volume on to each spot. Chips were left to air dry for 10 minutes and analyzed by matrix-assisted laser desorption ionization (MALDI)-TOF MS.

SELDI-TOF Mass Spectrometry Analysis

PS20 arrays were analyzed using a Ciphergen Biosystems PCS4000 TOF-MS Personal Edition mass spectrometer and Ciphergen Express Software, Version 3.05.014 (Ciphergen Biosystems, Inc. – Biorad Laboratories Inc., Hercules, CA, USA). The protocol and parameters are outlined in Table 1. Each spot of each chip was divided equally by pixel number and location into 2 equal and representative partitions for analysis. Results and spectra were therefore generated from TOF data encompassing 50% of the pixels contained in each spot. Any baseline lifting was corrected for using the Baseline Subtraction function. Peaks of interest were highlighted and labeled using the Centroid function and the peak information of choice (mass to charge ratio (m/z), intensity and signal to noise ratio) was exported for analysis into Excel spreadsheet files (Microsoft, Redmond, WA, USA).

The phospho:native (P:N) peptide ratio for each sample was calculated by dividing the numeric intensity value of the peak representing the phospho-peptide by the numeric intensity value of the peak representing the native peptide and averaging these values between duplicate spots. Peak intensities with signal to noise ratios of less than 5 were not analyzed.

Results

Akt Substrate Peptides 1, 2 and 3 were individually incubated on Streptavidin coated PS20 arrays in water and subjected to SELDI-TOF MS analysis. As shown in Fig. 1A, the resulting spectrum for each peptide contains one single, cleanly resolved peak with a m/z ratio within 5 Da of the predicted molecular weight of the substrate. To determine if substrate peptide phosphorylation could be resolved using this protocol, all 3 Akt substrate peptides were added to *in vitro* kinase reactions at 10 ng/ μ L with or without human recombinant active Akt1 and the kinase reactions containing substrate peptides were assayed using SELDI-TOF MS. As shown in Fig. 1B, the resulting spectra from Substrate Peptides 1 and 2 contained peaks that were

TABLE 1.

SELDI Parameter	Parameter Value/Mode
Acquisition Mode	Positive Ions – Source 25 kV
Mass Range Assessed	0 – 10 000 Da
Matrix Attenuation	500 Da
Sampling Rate	400 MHz
Acquisition Method	SELDI Quantitation
Number of Warming Shots	5
Number of Data Shots	10
Laser Intensity	750 nJ

representative of a peptide 80 Da heavier than their respective non-reacted native peptide, indicating that these two substrates were phosphorylated by Akt, and that these phosphorylated substrate peptides could be resolved using this technique. Addition of recombinant Akt did not alter the mass of Substrate Peptide 3, consistent with this substrate peptide being unsuitable for Akt phosphorylation.

To determine if the peak intensities generated by SELDI-TOF-MS analysis correlated with the degree of substrate peptide phosphorylation, known amounts of each phosphorylated and native substrate peptide were mixed in fixed ratios and subjected to SELDI-TOF MS analysis in duplicate (Fig. 2A). The ratio of the phosphorylated peak intensity to the native peak intensity (P:N) obtained from each replicate of two independent trials, performed in duplicate, were averaged and plotted against the predicted P:N ratios for each peptide. While the phosphorylated form of Substrate Peptides 1 (Fig. 2B) and 2 (Fig. 2C) were less readily ionized and desorbed than their corresponding native forms, a linear and reproducible relationship was evident between the measured and predicted phosphorylated to native peptide intensity ratios.

To determine if this assay could sensitively detect endogenous kinase activity directly from protein lysate, cultured primary human fibroblasts were treated with 100 ng/mL Platelet Derived Growth Factor (PDGF), a known activator of Akt.¹⁰ In parallel, Akt activation was independently assessed by Western Blot analysis of total protein lysate samples, derived from both the PDGF and vehicle treated cells, for the active form of Akt, (Threonine 308 (T-308) phosphorylated Akt¹¹). PDGF treatment resulted in readily detectable phospho-Threonine 308 Akt in the primary fibroblasts while little to no phospho-Threonine 308 Akt was detected in vehicle treated cell lysates (Fig. 3A). The spectra generated by SELDI-TOF MS analysis of these reaction mixtures containing Substrate Peptide 1 indicated that the substrate was phosphorylated during the reaction by the lysate derived from

cells that were PDGF treated, and not the vehicle treated cells (Fig. 3B).

To determine the specificity of the peptide substrates for Akt phosphorylation relative to other serine threonine kinases, Substrate Peptides 1, 2 and 3 were incubated with recombinant Akt, P70S6 Kinase, MAPKAP K1 and SGK and assessed by SELDI-TOF-MS analysis. As shown, Substrate Peptide 1 was phosphorylated *in vitro* by recombinant Akt, P70S6 Kinase, MAPKAP K1 and SGK as predicted (Fig. 4A). Substrate Peptide 2 was not phosphorylated by recombinant P70S6 Kinase or MAPKAP K1 as predicted but was efficiently phosphorylated by SGK (Fig. 4B). Substrate Peptide 3 was also phosphorylated by SGK (Fig. 4C).

Discussion

The aim of this study was to develop an easy-to-use and sensitive alternative assay system to measure kinase activity in the small sample volumes typically available from clinical isolates. The proof-of-principle experiments performed in this study demonstrate that the assay system is robust, reproducible and sufficiently sensitive to detect enzyme activity in less than 1 µg of cell lysate in a 2µL volume. During the process of matrix-assisted laser desorption ionization (MALDI), molecules that differ chemically will be desorbed and ionized to a different extent by the laser, as the physical chemistry of each peptide plays a role in these processes. As such, a quantitative comparison of SELDI-TOF MS calculated peak intensities between the chemically different species of phosphorylated and non-phosphorylated substrate peptides required validation. While MS is not widely perceived to be suitable for quantitative assessment, our data indicate that P:N ratios derived from SELDI-TOF-MS analysis could be used in conjunction with a linear correlation equation to allow for relative quantitation of phosphorylated to native peptide between samples. This ratio is an internally normalized value representing the amount of substrate phosphorylation in a par-

A **B**

FIGURE 1. Substrate Peptide Phosphorylation and Resolution with SELDI-TOF MS.

(A) The 3 Akt substrate peptides were resolved within < 5 Da of their predicted molecular weight.

(B) All 3 substrate peptides were either incubated with recombinant human Akt1, or Akt dilution buffer alone. Upper spectra depict substrate peaks that have undergone kinase reactions, lower spectra depict the reactions that have been vehicle treated.

FIGURE 2. Validation of Assay Quantitation. (A) 8 spots of the PS20 SELDI array indicating the ratio of phospho:non-phosphorylated peptide assessed. Representative spectra using Akt Peptide Substrate 1 are shown as; (i) –75% Phospho : 25 % Native, (ii) – 50:50, (iii) – 25:75, (iv) – 10:90. Phospho-peak intensity to the native-peak intensity (P:N). A line of best fit for Substrate Peptides 1 (B) and 2 (C).

A **B**

FIGURE 3. Substrate Peptide Phosphorylation and Resolution from Kinase Reactions Using Protein Lysate. (A) Human primary fibroblasts were treated with PDGF or vehicle. (B) In vitro kinase reactions.

ticular sample. Theoretically, the ratio of P:N peptide peak intensities should not vary between replicates and in practice displayed less than 5% variance in our hands. P:N peptide ratios were compared between samples as an indicator of the amount of phosphorylation the substrate underwent during kinase reactions to yield a semi-quantitative indicator of the relative amount of Akt activity present between the samples.

The most commonly used indirect measurement of enzyme activity is western immunoblotting with phospho-specific antibodies. This approach requires relatively large quantities of sample, can be complicated by the existence of multiple phospho-forms of

kinases where the relationship between multiple phosphorylation status and activity is unclear, and the possibility that a kinase may operate through a gain of function mutation that inherently alters the relationship between its phosphorylation status and activity. In contrast, recent advances have seen the rapid development of kinase methods based on a diverse array of technologies including time-resolved fluorometry, FRET, TR-FRET, Fluorescence polarization and many more (for a comprehensive review, see ¹²). Mass spectrometry-based assays have been used widely for enzyme analysis (for review, see ¹³). However, these analyses require specialized mass spectrometry

FIGURE 4. Determining Akt Substrate Peptide Specificity for Direct Analysis from Protein Lysate. (A) Akt, P70S6 Kinase, MAPKAP K1 and SGK with Substrate Peptide 1. (B) Akt, P70S6 Kinase, MAPKAP K1 and SGK with Substrate Peptide 2. (C) Akt, P70S6 Kinase, MAPKAP K1 and SGK with Substrate Peptide 3.

equipment supported by personnel dedicated to the maintenance of these complex systems. The method described here is faster than routine western immunoblotting, requires much less sample and results in a direct, rather than indirect, measurement of kinase activity. The benchtop SELDI-TOF-MS unit, while inferior in scope and resolution to large MS units, is relatively small, inexpensive and simple to use and maintain.

One of the primary goals in the development of this assay was to assess kinase activity directly from whole cell protein lysates without prior enzyme extraction, such as immunoprecipitation. To achieve this, it was necessary to design substrate peptides that were specific for the enzyme of interest. While the ability to generate a substrate peptide that is specific for an enzyme of interest greatly simplifies this type of analysis, in practice this may not be achievable when some degree of functional duplication occurs with other kinases. In this assay, Akt and SGK were found to phosphorylate substrate peptide 2, which was designed to be specific for Akt. This result is consistent with published data indicating that, while the kinase domain of SGK shares homology to Akt, SGK generally displays less stringency in its substrate preference.¹⁴ This relative laxity in substrate preference correlates with our finding that Substrate peptide 3, designed as a negative control, was also readily phosphorylated by SGK. Thus, in its present form, the combined profiles of all three substrate peptides can be compared to discern if Akt, SGK or other serine/threonine kinases such as P70S6 Kinase or MAPKAP K1 are contributing to serine/threonine phosphorylation in a cell lysate.

As biotinylated substrates for different kinases can readily be created with different molecular weights, the phosphorylation of each separate substrate can be specifically resolved by MS, allowing for the simultaneous assessment of the activity of multiple kinases from the same sample. We have recently adapted our SELDI-TOF-MS-based Glycogen Synthase Kinase (GSK)-3 β assay¹⁵ to this streptavidin-biotin protocol

and are currently determining if the GSK-3 β substrate peptides and the substrate peptides described in this report can be concurrently purified from cell lysates and assessed. This approach has the potential to unravel complex signaling networks in cell systems and therefore provide a better understanding of specific or aberrant signal transduction in that system. For example, we are currently using this technology to assess Akt and SGK activity in minute clinical isolates of phenotypically normal palmar fascia collected with patient consent during surgical resection of Dupuytren's Disease cord (unpublished data). We envisage this approach to be particularly amenable to punch or needle biopsy samples where tissue quantities are limited.

Conclusion

This report describes a simple and rapid SELDI-TOF-MS based alternative that utilizes streptavidin to purify biotinylated peptide substrates. TOF analysis using a small benchtop SELDI unit allows for the rapid, sensitive and direct measurement of kinase activity in small volumes of cell lysates. Further, the assay is potentially adaptable to the concurrent measurement of multiple kinases to assess the contributions of specific kinases to disease processes.

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