COVER SHEET

TITLE: Methods for Identifying Substrates of Atypical Mitochondrial Kinases

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ABSTRACT

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Mitochondria are chiefly tasked with the production of ATP. Reversible phosphorylation is postulated to be a central regulatory mechanism in mitochondrial biology, and yet the mitochondrial kinases that perform the phosphorylation events are largely uncharacterized[1]. One group of atypical mitochondrial kinases, the ADCKs (aarF-domain containing kinases), has garnered interest because of the role one of its members play in human disease. Here, we designed and validated a radiolabeling-based approach to identify substrates of atypical mitochondrial kinases. To detect labeled proteins, we optimized SDS-PAGE; to detect labeled lipids, we optimized thin layer chromatography (TLC). By manipulating kinase reaction conditions, we reduced background phosphorylation of proteins and lipids by endogenous kinases. From there, we established a high-throughput, *in vitro* kinase assay to validate kinase-substrate pairs. Our next step is to use this approach to identify substrates of the ADCK family to elucidate their roles in human health and disease.

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Methods for Identifying Substrates of Atypical Mitochondrial Kinases

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Senior Honors Thesis

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ABSTRACT

Mitochondria are chiefly tasked with the production of ATP, which is the cell's form of chemical energy. Reversible phosphorylation is postulated to be a central regulatory mechanism in mitochondrial biology, and yet the mitochondrial kinases that perform the phosphorylation events are largely uncharacterized[1]. One group of atypical mitochondrial kinases, the ADCKs (aarF-domain containing kinases), has garnered interest because of the role one of its members play in human disease. ADCK3 has been implicated in ARCA2, a recessive cerebellar ataxia associated with Coenzyme Q deficiency. The identification of the endogenous substrate of a member of this family of kinases would provide important information about the biological function of this kinase family. A key step toward this goal would be the optimization of kinase assays for atypical mitochondrial kinases. Here, we designed and validated a radiolabeling-based approach to identify substrates of atypical mitochondrial kinases. To detect labeled proteins, we optimized Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE); to detect labeled lipids, we optimized thin layer chromatography (TLC). By manipulating kinase reaction conditions, we reduced background phosphorylation of proteins by endogenous kinases and detected cytosolic phosphorylation by exogenous Protein Kinase A (PKA). We also created mitoliposomes, which present the mitochondrial lipidome to kinases without endogenous kinases. From there, we established a high-throughput, *in vitro* kinase assay to validate kinase-substrate pairs that will be identified through the radiolabeling approach. Our next step is to use this approach to identify substrates of the ADCK family to elucidate their roles in human health and disease.

INTRODUCTION

Mitochondria are chiefly tasked with the production of ATP, which is the cell's form of chemical energy. To accomplish this task, mitochondria completely oxidize carbon sources to carbon dioxide using the TCA cycle. Then, the reduction of O_2 with these electrons is coupled to proton pumping across a membrane to create a proton motive force, which is harnessed to create ATP by ATP Synthase.

Reversible phosphorylation is postulated to be a central regulatory mechanism in mitochondrial biology[1]. Reversible phosphorylation constitutes a key post-translational modification regulating protein activity, evidenced by the fact that 30% of proteins are phosphorylated during their lifetime[1]. The presence of a phosphate group can have profound effects on a protein's function. For example, pyruvate dehydrogenase complex (PDC), a well-characterized mitochondrial complex that converts pyruvate into acetyl CoA for entry into the Krebs cycle, is under tight regulation by a kinase-phosphatase pair which alters the phosphorylation state of PDC depending on the cell's metabolic status[2]. Another example from our group shows differential phosphorylation between lean and obese mice in over 100 mitochondrial proteins (unpublished data). Clearly, substantial regulation by reversible phosphorylation occurs in mitochondria, but many of the kinases that are responsible for mitochondrial phosphorylation events are uncharacterized.

One group of atypical mitochondrial kinases, the ADCKs (aarF-domain containing kinases), has garnered interest because of the role one of its members play in human disease. ADCK3 has been implicated in ARCA2, a recessive cerebellar ataxia associated with Coenzyme Q deficiency[3]. The identification of the endogenous substrate of a member of this family of kinases would provide important information about the biological function of

this kinase family. A key step toward this goal would be the optimization of kinase assays for atypical mitochondrial kinases. However, the identification of substrates for atypical kinases is uniquely challenging, since even the class of this substrate is unknown (Figure 1).

Here, we designed and validated a radiolabeling-based platform to identify the substrates of atypical mitochondrial kinases (Figure 2). Briefly, mitochondrial lysates are labeled with ^{32}P-ATP by the purified atypical kinases. Labeled proteins are analyzed by SDS-PAGE; labeled lipids and small molecules are analyzed by TLC (thin-layer chromatography). After identification of phosphorylated substrates by mass spectrometry, kinase-substrate pairs are validated using a high-throughput, luminescent kinase assay. In the future, we plan to use this method to identify the substrate class of the ADCK members. From there, we will conduct a targeted approach to isolate the specific endogenous substrates for these kinases and use this information to elucidate the biological function of these kinases in human health and disease.

METHODS

Purification of mitochondria

Mitochondria were purified from bovine heart muscle using differential centrifugation. Briefly, small cubes of tissue were cut into fine mince and rinsed. The mince was homogenized by three vigorous strokes in a Potter-Elvehjem homogenizer. To produce whole cell lysate, homogenate was centrifuged (800 x g, 12 min, 4 °C) to produce a pellet rich in nuclei and cell membranes. The supernatant was decanted through cheese-cloth and centrifuged (15000 x g, 15 min, 4 °C) to produce a mitochondrial pellet, which was resuspended and sonicated to yield mitochondrial lysate. To isolate mitochondrial lipids, a classic Bligh & Dyer organic extraction was performed.

Creation of liposomes

PC (phosphatidylcholine) and PC/PIP_2 (phosphatidylinositol 4,5-bisphosphate) liposomes were prepared for use as substrates in *in vitro* kinase assays. Lipids suspended in organic solvent were mixed and dried under $Ar_{(g)}$ to form a dry lipid cake. The cake was resuspended in 1x reaction buffer and vortexed vigorously. Liposomes were incubated at 37°C for 1 hour and extruded through a 100 nm filter 19 times. Liposome diameters were analyzed by DLS (Differential Light Scattering).

Creation of mitoliposomes

Mitochondrial lipids were organically extracted from mitochondrial lysate by the addition of $CHCl_3$/MeOH (2:1, v/v) and glass beads. After vigorous vortexing, beads were removed and phase separation was effected through centrifugation. The organic layer was collected and dried under $Ar_{(g)}$. Lipids were resuspended in 1x reaction buffer buffer and

vortexed vigorously. Mitoliposomes were incubated at 37°C for 1 hour and sonicated in a bath sonicator for 10 minutes before use in kinase reactions.

Radiolabeling

Kinase reactions were performed using the desired substrate (whole cell lysate, mitochondrial lysate, or mitoliposomes), the exogenous kinase (PKA or PI(3)K), and ^{32}P-ATP. The addition of ATP commenced the reactions, which proceeded with shaking at 30°C for 40 minutes. Reactions were quenched by the addition of 1N HCl. Samples of each reaction were taken for SDS-PAGE. For reactions undergoing TLC, organic extractions were performed.

Analysis of labeled proteins by SDS-PAGE

Reactions were run on SDS-PAGE in 10% Bis-Tris gels[4]. Gels were stained with Coomassie Brilliant Blue stain and destained. Gels were then incubated in gel drying solution and were dried. Wrapped gels were used to expose a phosphor screen, which were visualized by phosphorimaging.

Lipid extraction from radiolabeled reactions

Lipids were extracted from radiolabeled reactions by organic extraction. $CHCl_3$/MeOH (1:1, v/v) was added to the reactions, which were vortexed vigorously. Phase separation was effected via centrifugation, and the aqueous phase was removed. The organic phase was washed twice with MeOH/1N HCl (1:1, v/v).

Thin-Layer Chromatography

Silica gel 60A TLC (thin-layer chromatography) plates (Merck, 20 x 20 cm or 20 x 10 cm, 0.5 mm thick layer) were treated with 1% oxalate and dried in a fume hood. Prior to spotting, plates were baked at 60°C for 20 minutes. Lipids dissolved in

organic solvent were spotted on plates, and the plate was developed. In 1D-TLC, plates were developed using Acidic Buffer ($CHCl_3$/acetone/MeOH/AcOH/H2O 39:28:11:11:11 v/v/v/v/v). In 2D-TLC, plates were first developed with Acidic Buffer and dried for 2 hours in fume hood. Then, plates were turned 270° and developed with Basic Buffer ($CHCl_3$/MeOH/NH_4OH/H_2O 50:40:8:2 v/v/v/v). For non-radioactive samples, plates were stained with CAM. For radioactive samples, phosphor screens were exposed to plates and visualized by phosphorimaging.

In vitro kinase assays

Kinase assays were performed using the ADP-Glo kit from Promega in 384-well Costar plates in reaction volumes of 5 uL. This kit measures ATP consumption by coupling the stoichiometric generation of ADP with a luciferin-luciferase assay. Briefly, addition of kinase to the buffer and substrates initiated the reactions. After 30 minutes of incubation at room temperature, reactions were quenched with 5 uL of ADP-Glo reagent and incubated for 40 minutes at room temperature. Kinase Detection Reagent was added (10 uL), and reactions were incubated for 40 minutes. Relative Luminescence Units (RLU) of each reaction were measured and compared with a standard curve to calculate the %ATP generated.

RESULTS

Heterogeneous liposomes can be phosphorylated by lipid kinases

Following extrusion through 100nm filters, heterogeneous liposomes were analyzed by Dynamic Light Scattering (DLS) to determine the size distribution of liposome species (Figure 3). The median diameter of PC (phosphatidylcholine) and PC/PIP_2 (phosphatidylinositol 4,5-bisphosphate) liposomes was around 150nm, which is similar to previously reported data.

To assess the ability of kinases to phosphorylate substrates in heterogeneous liposomes, radiolabeling studies were performed using PI(3)K (phosphatidylinositol-3 kinase) (Figure 4). PI(3)K revealed specific phosphorylation of PIP_2 in PC/PIP_2 liposomes, but no phosphorylation of PC liposomes.

TLC can separate resolve phosphorylated lipids

In order to visualize lipid separation by TLC, various lipid species were spotted on TLC plates. However, many buffer conditions offer inadequate separation of the highly polar phosphorylated lipids species that our radiolabeling approach hopes to visualized. After trying numerous TLC systems, we found a particular system that permitted excellent separation of phosphorylated lipids. Here, oxalate-treated TLC plates were developed using an acidic buffer (Figure 5a). Using PIP_2 as a standard for the species we would expect to see in radiolabeling studies, we found that this system yielded a R_f value for PIP_2 of 0.45. Less polar species such as PC and PS (phosphatidylserine) had R_f values over 0.75. Little to no ATP migration was found in these conditions. When extracted mitochondrial lipids were separated, most species migrated with R_f values over 0.75, which was expected based on previous reports.

To increase the resolution of TLC, we adapted 2D-TLC for our work. This method permits separation of lipid species not only by polarity, but also by the pK_a of the lipid's head group. Mitochondrial lipids were spotted on TLC plates and developed with both an acidic and a basic buffer. This plate was visualized by CAM stain (Figure 5b). More species were resolved in 2D-TLC than in 1D-TLC.

Radiolabeling of lysates allows detection of protein phosphorylation

The main challenge with radiolabeling is that active, endogenous kinases are present in lysates and can both generate false positives and mask phosphorylation by an exogenous kinase. This challenge is illustrated in Figure 6a, where endogenous kinases provide considerable background. To address this issue, numerous approaches were taken to maximize the signal-to-noise ratio. Background was minimized by reducing the amount of lysate used in the reactions and by both minimizing the ATP concentration and using ^{32}P-ATP with a very high specific activity. Signal was maximized by adding saturating amounts of an exogenous kinase. Here, we incubated PKA (Protein Kinase A), a promiscuous cytosolic kinase, with whole cell lysate and ^{32}P-ATP and analyzed labeled proteins by SDS-PAGE. By optimizing the conditions, we minimized background phosphorylation to low levels and detected considerable phosphorylation of proteins in the lysate by PKA (Figure 6b).

Radiolabeling of mitoliposomes permits mitochondrial lipid phosphorylation

As noted above, phosphorylation by endogenous kinases provides the greatest source of background, and this holds true for analysis of phosphorylated lipids as well (Figure 7a). When analyzing the protein fraction, these kinases cannot be easily removed without disturbing potential protein substrates. However, lipids can be isolated from endogenous kinases.

Here, we created mitoliposomes to use as substrates for lipid kinases. Following organic extraction of mitochondria, mitochondrial lipids are dried and reconstituted into liposomes to create mitoliposomes. The utility of these species is that they present the mitochondrial lipidome without the endogenous kinases. For example, when mitoliposomes were incubated with PI(3)K and ^{32}P-ATP, specific phosphorylation of lipids by PI(3)K were detected, while little to no background was detected (Figure 7b).

High-throughput kinase assays as a means to validate kinase-substrate pairs

Molecules identified as atypical kinase substrates through the radiolabeling study must be validated. For instance, the identified substrate of the exogenous kinase could be a kinase itself (or a lipid that activates a kinase) that causes downstream phosphorylation of other compounds that show up as positive hits in our approach. However, these secondary substrates are not *bona fide* substrates for the exogenous kinase.

To firmly establish kinase-substrate pairs, we optimized a high-throughput *in vitro* kinase assay called ADP-Glo which couples ATP depletion to a luciferin-luciferase assay. Here, we report dose-dependence using saturation curves of a lipid kinase, a protein kinase, and a carbohydrate kinase (Figure 8). Furthermore, this phosphorylation is specific to the kinases' preferred substrates, demonstrating the utility of this assay in established kinase-substrate pairs.

DISCUSSION

The identification of substrates for atypical, mitochondrial kinases is a challenging endeavor, particularly in the absence of additonal information about the biological role of the kinase. Here, we optimized a radiolabeling-based approach for detecting substrates of atypical kinases, as well as protein/lipid kinases. We were able to detect cytosolic substrates of PKA by modifying the reaction conditions to optimize the signal-to-background ratio. Additionally, we report the creation of mitoliposomes which can be used to present the mitochondrial lipidome without background phosphorylation by endogenous kinases. As a proof-of-principle, we detected PI(3)K-mediated phosphorylation of lipids within mitochondria with little to no detectable background.

We have also optimized a high-throughput, *in vitro* kinase assay to validate the kinase-substrate pairs identified in the radiolabeling experiments. We report sufficient dose-dependence in the assay for protein, lipid, and carbohydrate kinases that was selective for the kinases' preferred substrates.

The next steps to optimize this method are to use mitochondrial kinases in place of the protein kinase (PKA) and lipid kinase (PI(3)K) used in this study. Furthermore, we plan to perform chromatography on the mitochondrial lysate prior to radiolabeling of proteins. Chromatography would allow for simplification of the mitochondrial lysate presented to the exogenous kinase and could separate endogenous kinases from their substrates, which would reduce background phosphorylation.

In the meantime, we will use this radiolabeling system with the ADCK kinases, a few of which we have recently purified, to identify substrates for these kinases. Indeed, melting curves of purified ADCK3 revealed typical melting of a folding protein as well as dose-

dependent stabilization of the kinase with increasing ATP concentration, suggesting that the purified ADCK3 contains an intact ATP-binding domain and may be active. The identification of the substrates, or even the substrate class of these kinases would represent a great stride in elucidating the biological function of these kinases in human health and disease.

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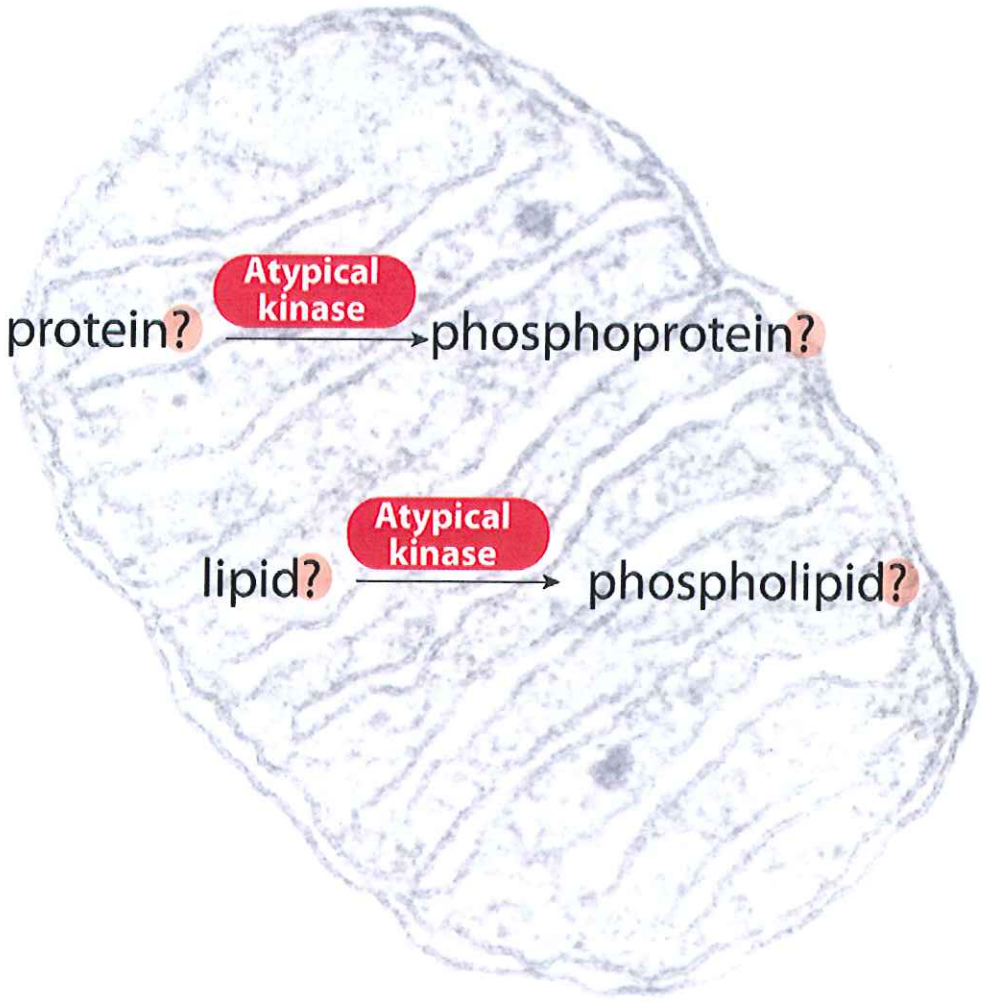

Figure 1: Identifying substrates of atypical kinases is challenging because the class of the substrate is unknown. Since the analysis of proteins and lipids differs considerably, the overall approach to identify the substrate of an atypical kinase must be broad and should include methods designed to detect both phosphorylated proteins and lipids.

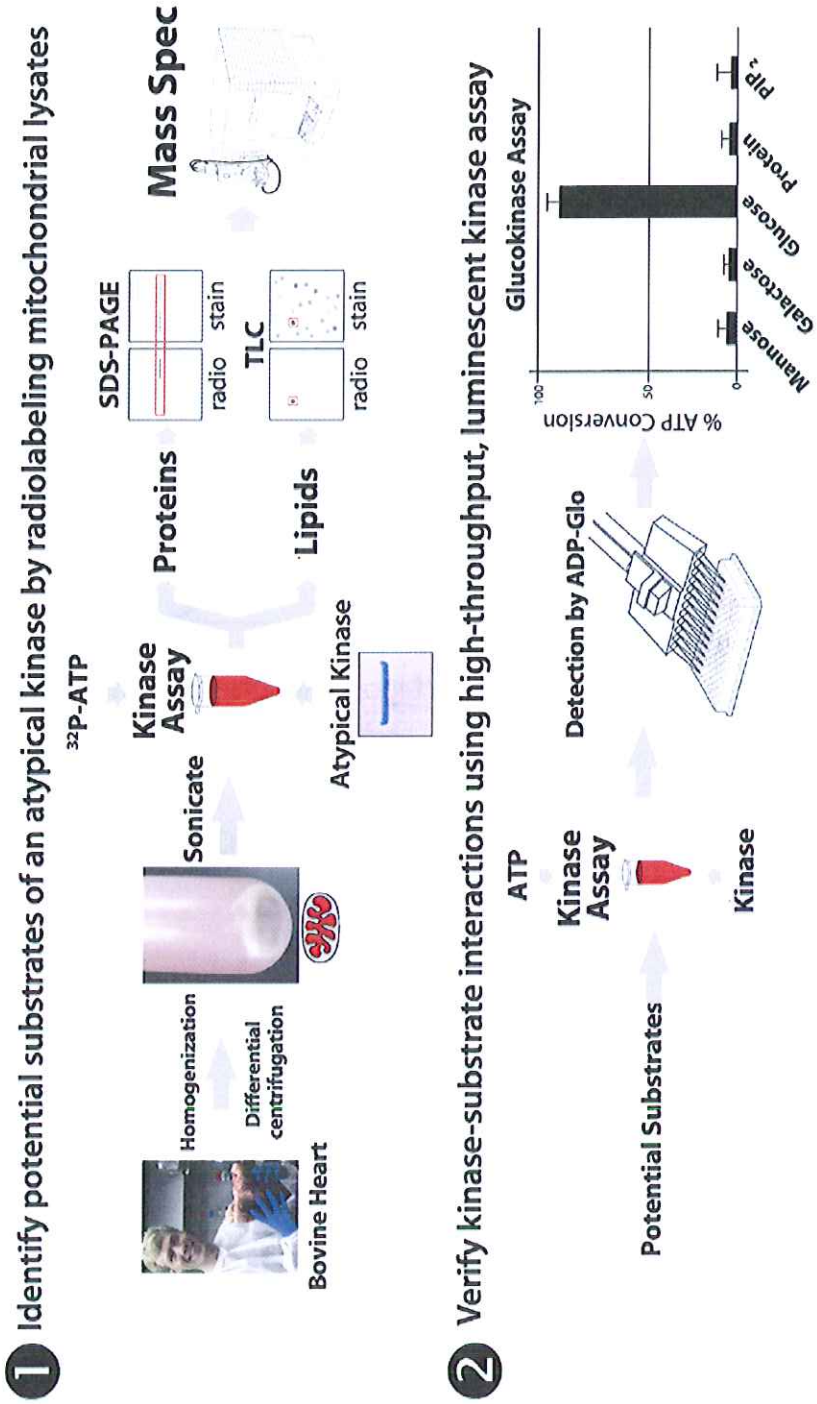

Figure 2: Flow diagram of our method to identify the substrates of atypical mitochondrial kinases. In step 1, potential substrates are detected using a radiolabeling-based approach. In step 2, identified substrates are validated using a high-throughput, luminescent kinase assay.

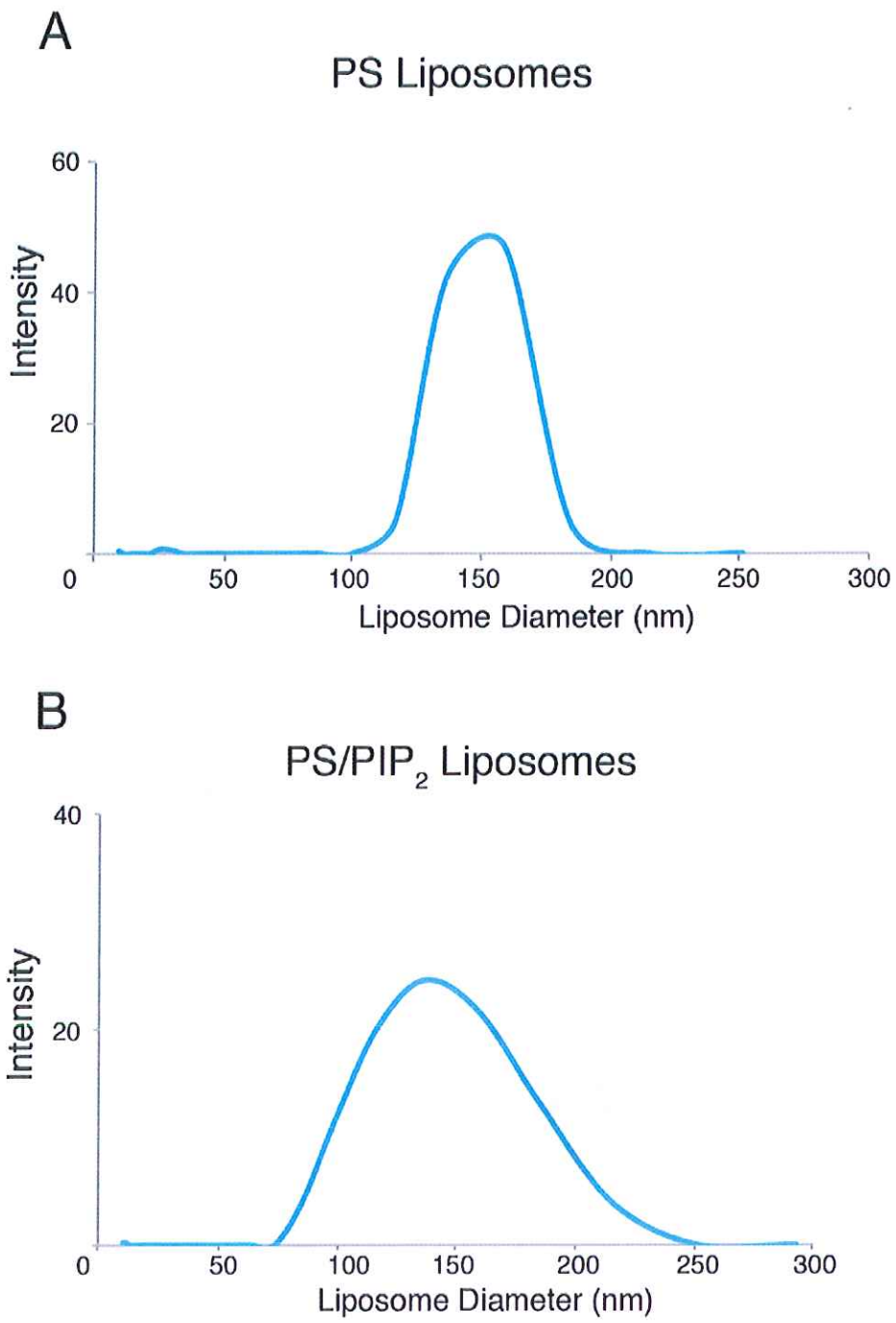

Figure 3. Liposome analysis by DLS. Liposome sizes of PC (**A**) and PC/PIP_2 (**B**) were determined by dynamic light scattering (DLS). In both cases, average liposome diameter was roughly 150 nm.

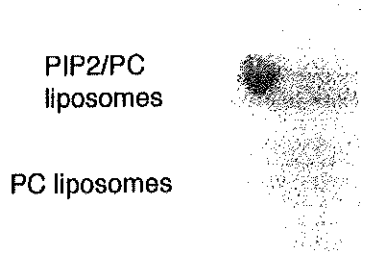

Figure 4. Dot-Blot assay of lipid reactions by PI(3)K. PI(3)K kinase reactions with different lipid substrate performed with ^{32}P-ATP and analyzed by a dot-blot assay on PVDF, which binds lipids but not ATP.

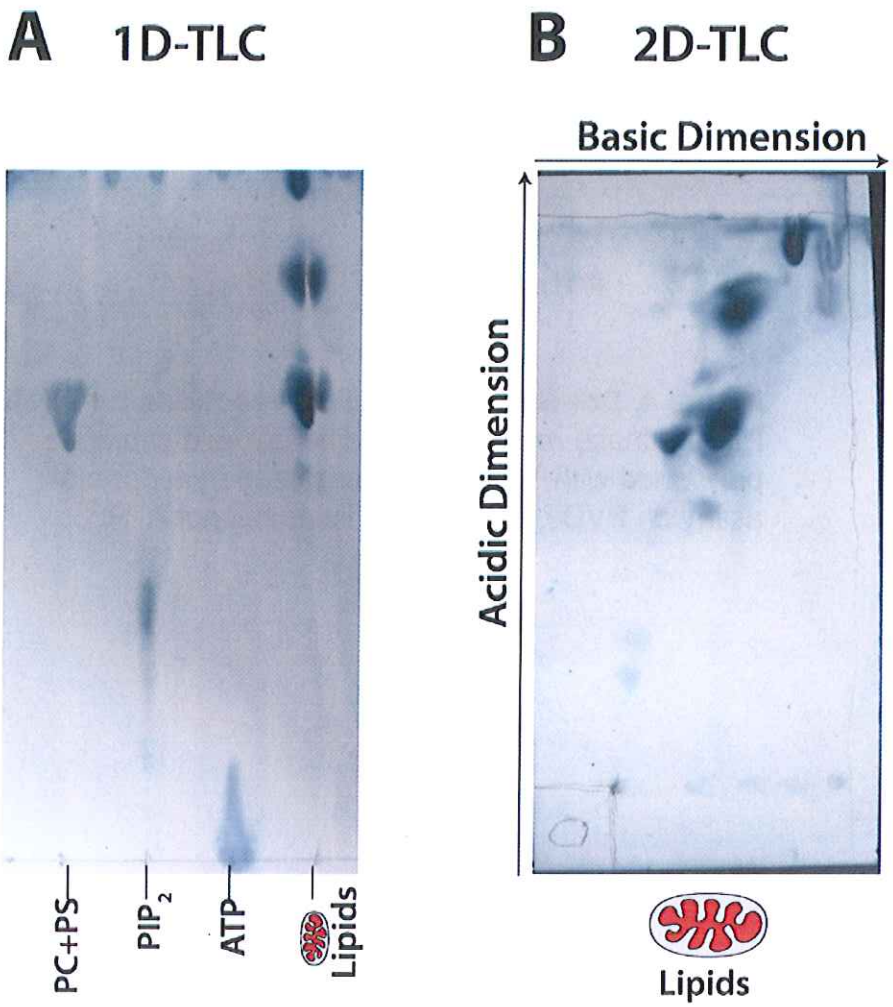

Figure 5: Optimization of TLC conditions in order to visualize polar mitochondrial phospholipids. **(A)** Numerous standards and extracted mitochondrial lipids were developed by TLC and visualized by CAM staining. PIP_2, a doubly phosphorylated lipids, was separated from baseline, demonstrating the ability of this method to resolve highly polar phospholipids. **(B)** Mitochondrial lipids were separated by 2D-TLC, which increases resolution.

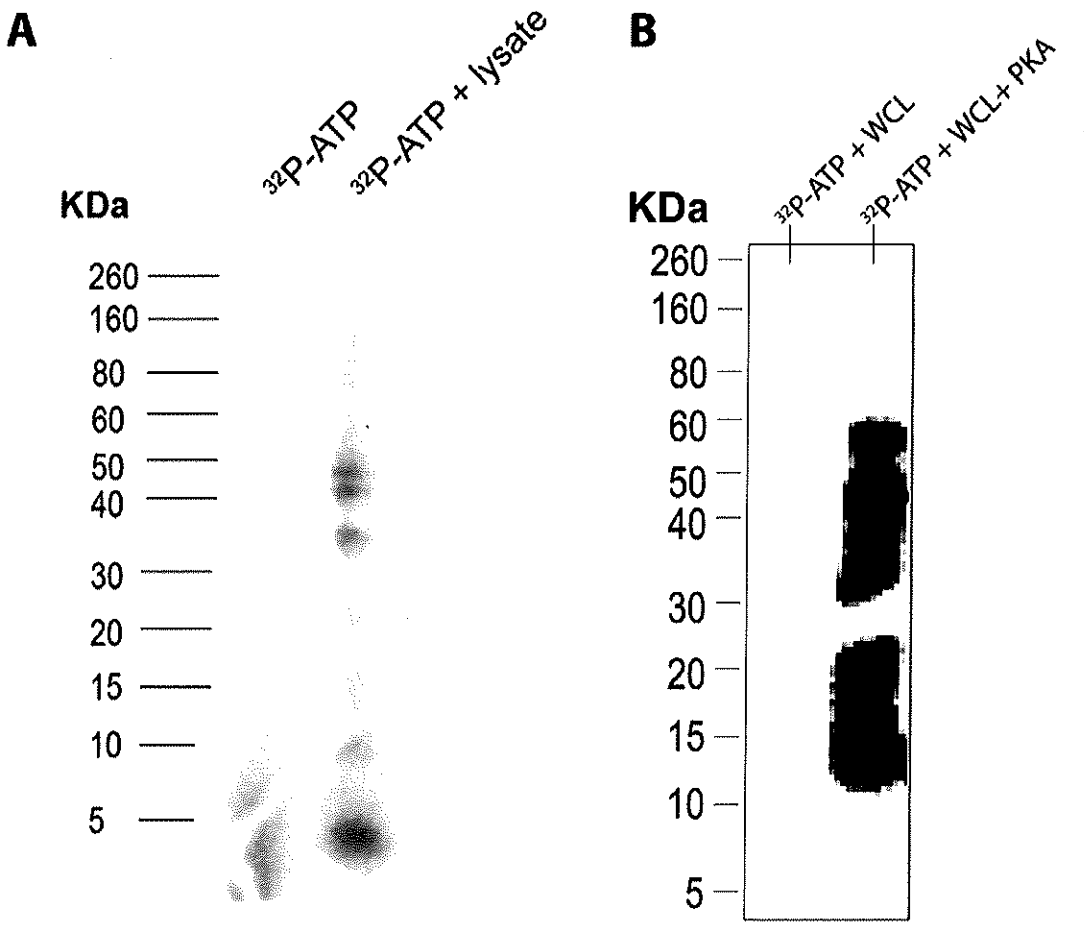

Figure 6: Radiolabeling reactions analyzed by SDS-PAGE. (A) Kinase reactions were loaded onto SDS-PAGE and visualized by phosphorimaging. **(B)** By optimizing reaction conditions in order to minimize background activity, we can detect proteins phosphorylated by an exogenous kinase.

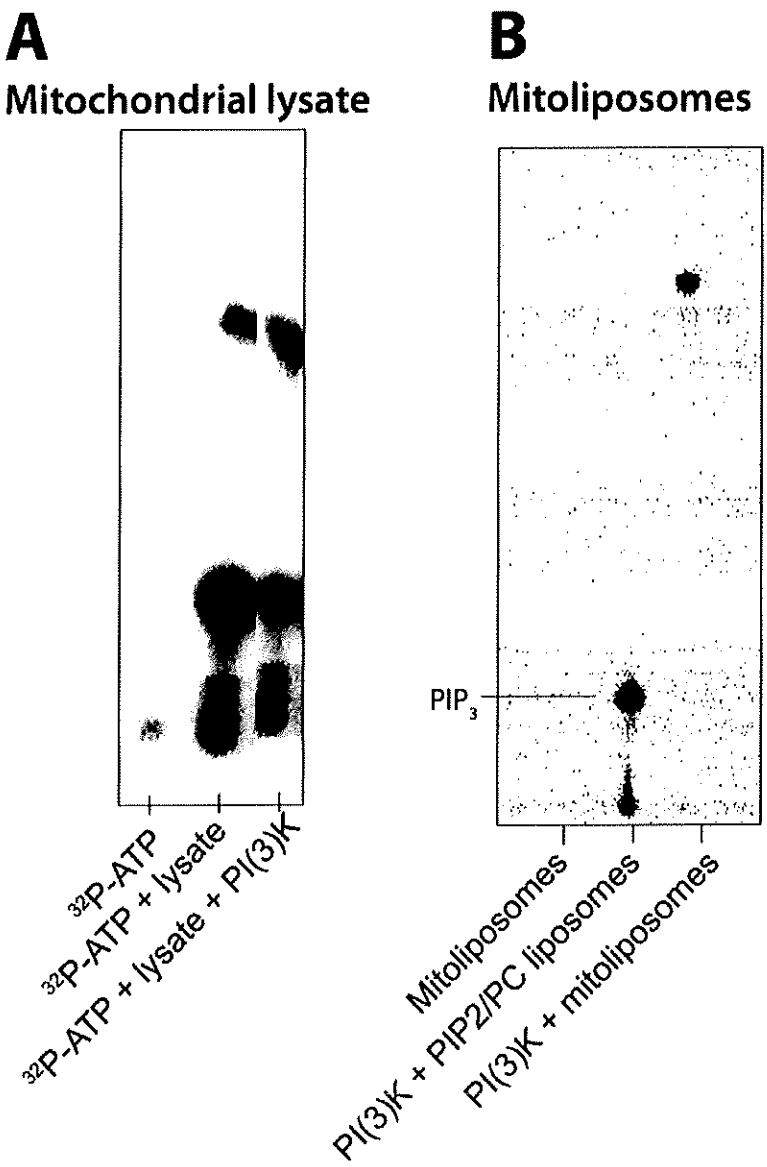

Figure 7: Radiolabeling reactions analyzed by TLC. (A) Organic extractions of kinase reactions containing mitochondrial lysate were developed by TLC and visualized by phosphorimaging. Considerable background from endogenous kinases is present. **(B)** Organic extractions of kinase reactions containing mitoliposomes (mitochondrial lipids reconstituted into liposomes) were developed by TLC. The use of mitoliposomes reduced background phosphorylation and allowed for the identification of substrates phosphorylated by an exogenous kinase.

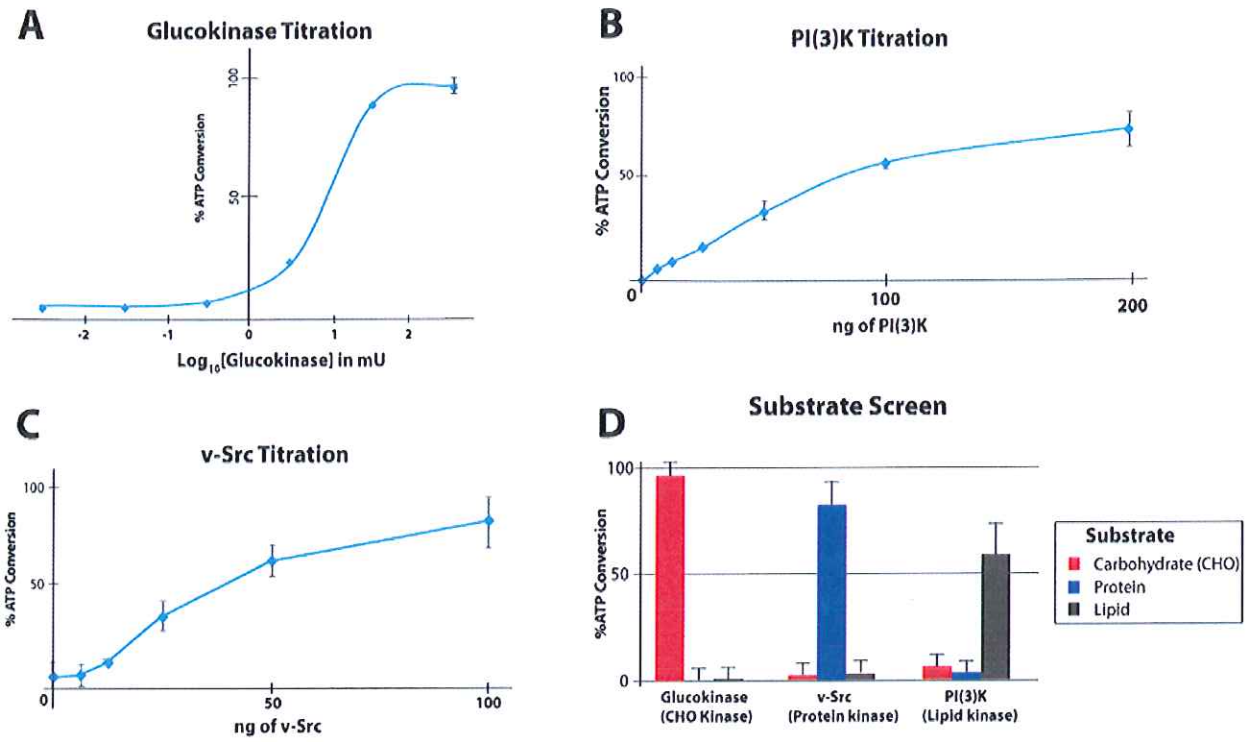

Figure 8: High-throughput, luminescent kinase assay optimized for validating kinase substrate pairs. Panels A, B, and C show saturation curves of a small molecule kinase **(A)**, a lipid kinase **(B)**, and a protein kinase **(C)**. In **D**, the results of a kinase assay demonstrating the specificity of these kinase classes is shown. The use of a high-throughput assay allows for validation of substrates and identification of false positives.