Published in final edited form as:

Anal Biochem. 2008 April 15; 375(2): 265–271.

A fluorescent plate reader assay for ceramide kinase

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Abstract

Ceramide kinase and its product ceramide 1-phosphate have been implicated in cellular proliferation and survival, activation of cytosolic phospholipase A₂, mast cell degranulation, and phagocytosis. Current assays for ceramide kinase activity employ [³²P]-ATP, with separation of labelled product from excess ATP by organic extraction and thin layer chromatography. We have developed a fluorescent plate reader assay for ceramide kinase, which uses commercially available C6-NBD ceramide. Our assay is based on the differential partitioning of substrate and product following a single chloroform/methanol extraction. The product, which partitions into the aqueous phase at physiological pH, is quantitated directly in a plate reader. The substrate may be delivered using either fatty acid free albumin or detergent/lipid mixed micelles, and we have found that the use of albumin rather than detergent micelles allows one to detect lipid interactions with the enzyme that might otherwise go unnoticed. Our method is useful for assaying ceramide kinase activity both *in vitro* and in cultured cells, and offers several advantages over the conventional assay, including greater speed, the ability to run a larger number of assay replicates at one time, and the elimination of environmental and safety issues associated with the use of radioactive materials.

Keywords

Ceramide kinase; CerK; C6-NBD ceramide; fluorescent; assay

Introduction

Ceramide kinase (CerK)¹ (EC 2.7.1.138) catalyses the phosphorylation of ceramides, forming ceramide 1-phosphate (Cer1P), a signaling lipid that has been implicated in mast cell functions and phagocytosis, as well as cellular proliferation and survival. Ceramides are diacyl lipids of the sphingolipid family that are formed via *de novo* synthesis from palmitoyl coenzyme A and serine, or breakdown of the abundant membrane lipid sphingomyelin. Ceramides may in turn be deacylated, yielding the 18 carbon sphingolipid backbone, sphingosine, or glycosylated, giving rise to complex glycolipids and gangliosides [1,2].

CerK is capable of phosphorylating ceramides of varying N-acyl chain lengths, but not sphingosine or diacylglycerol [3–6]. *In vivo*, the substrates for CerK range from C16 to C24 ceramide species, and it has recently been suggested that CerK preferentially phosphorylates

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¹Abbreviations used: BSA, bovine serum albumin; Cer1P, ceramide 1-phosphate; CerK, ceramide kinase; CerKL, ceramide kinase-like protein; DMS, dimethylsphingosine; MBP, maltose binding protein; C6-NBD ceramide, N-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-D-erythro-Sphingosine; TLC, thin layer chromatography

C16, C18 and C20 ceramides, which are delivered to the Golgi by the ceramide transporter, Cert [7]. *Cerk* knockout mice are viable, and do not show a reduction in their brain Cer1P level, suggesting alternative enzymes and pathways for Cer1P formation [8]. A closely related protein, ceramide kinase-like protein (*CerkL*), has been identified, but to date no enzymatic activity has been described for *CerkL* [9]. However, an amber mutation in the *CERKL* open reading frame gives rise to the inherited blindness disorder, retinitis pigmentosa [10].

Cer1P appears to be important for cell survival, as loss of *Cerk* promotes apoptosis in mammalian cells [11] and plants [12]. When added to cells, Cer1P promotes cell proliferation and inhibit apoptosis [11,13,14]. *Cerk* binds to and activates phospholipase A₂ and is therefore involved in prostaglandin synthesis [15–17]. *Cerk* is also involved in mast cell degranulation [18,19], and may play a role in phagocytosis, by increasing the potential for plasma membrane/vesicle fusion [20].

The conventional assay for *Cerk* activity measures transfer of labelled phosphate from [³²P]-ATP to ceramide. Four extraction steps are required to remove excess labelled ATP, and the product is then resolved by thin layer chromatography (TLC) and quantitated using either a Phosphorimager or scintillation counting [3,4,21]. Ceramides carrying a fluorescent NBD group on the N-acyl chain have been reported to act as substrates for ceramidase [22], and have been used as a *Cerk* substrate in a few publications, with visualisation of product by TLC [9, 16]. In this paper we have used commercially available C6-NBD ceramide as the substrate for a plate reader *Cerk* assay, involving a simple extraction protocol. Our assay is verified with the characterisation of *Cerk* inhibition by dimethylsphingosine (DMS).

Materials and Methods

Preparation of recombinant human *Cerk*

Cerk was purified as a C-terminal fusion to maltose binding protein (MBP). *CERK* cDNA was amplified from Human Umbilical Vein Endothelial Cell cDNA using primers: gaattcgagatgggggcgacgggggc (forward) and ggatcctcagctgtgtgagcttgctgcg (reverse). The PCR product was ligated into pCR-TOPO 2.1 vector (Invitrogen) and sequenced, then cloned as an *EcoRI* fragment into pMAL-c2e (New England Biolabs) or pIRES-EGFP (Clontech). MBP-*Cerk* was purified on amylose resin, from 200 mL bacterial cultures, as described in the product manual for pMAL-c2e. Expression was induced with 0.3 mM isopropyl β-D-1-thiogalactoside (IPTG). After purification, enzyme was concentrated to 1 mg/mL and dialysed into 20 mM Tris/10 mM KCl, pH 7.4, using Amicon Ultra spin columns (Millipore). Protein concentration was assayed with BCA reagent (Pierce), relative to bovine serum albumin (BSA) standards. Purified enzyme was stored at –20 °C, after addition of glycerol to 50% (v/v).

Cell culture

HEK293 and J774 cell lines were cultured in RPMI1640 medium containing 10% fetal bovine serum, 2 mM Glutamax, and Penicillin/Streptomycin. Cell culture reagents were purchased from Invitrogen, except serum, which was from Hyclone.

Ceramide kinase assay

NBD labelled ceramides were purchased from Avanti Polar Lipids; ATP and cardiolipin were from Sigma Aldrich; TLC plates, TritonX-100, and all solvents were from Fisher Scientific; and dimethylsphingosine was from Biomol. Mixed micelle assays with TritonX-100 and cardiolipin were performed as described previously [3], but also in a lower detergent format: micelles containing 1.25 mM (0.125%) TritonX-100, 0.2 mM cardiolipin, and 50 μM C6-NBD ceramide were prepared by drying lipids from ethanol stocks, then sonicating in reaction buffer (20 mM Hepes pH 7.4, 10 mM KCl, 15 mM MgCl₂, 1 mM DTT). The micelles were diluted

5-fold with reaction buffer, and 1 mM ATP was added. The 100 μ L reactions were started with the addition of 0.5 μ g MBP-Cerk, and reacted at 35 °C for 20 min in the dark. Silica Gel 60 TLC plates were spotted with 1 μ L of the reaction mix directly after the reaction, and resolved in butanol/acetic acid/water (3:1:1). Fluorescence was detected with a Typhoon flatbed scanner (GE Healthcare). Extractions were performed as detailed below.

For the assays using fatty acid free BSA (purchased from SAFC Biosciences) to solubilise the substrate, the reaction buffer consisted of 20 mM Hepes pH 7.4, 10 mM KCl, 15 mM $MgCl_2$, 10% glycerol, 1 mM DTT, 1 mM ATP, 0.2 mg/mL fatty acid free BSA, and 10 μ M C6-NBD ceramide, added directly from a 10 mM ethanol stock. The 100 μ L reactions were started with the addition of 0.5 μ g MBP-Cerk or 25 μ g J774 cell lysate, and incubated at 35 °C for 20 min in the dark. The reactions were stopped with 250 μ L chloroform/methanol (2:1), then vortexed, and centrifuged at 21800g for 30s to separate the phases. 100 μ L of the upper aqueous phase, containing the C6-NBD Cer1P, was transferred to a black 96-well plate, and 100 μ L dimethylformamide was added before reading NBD fluorescence in a fluorescent plate reader. Blanks consisted of reaction mix without enzyme, but including substrate. C6-NBD Cer1P fluorescence was converted to molar amounts relative to known amounts of C6-NBD ceramide. To prepare cell lysates for Cerk assays, cells were washed twice with PBS, then lysed with three freeze thaw cycles in 20 mM Hepes, pH7.4, 150 mM NaCl, 1 mM DTT, and complete protease inhibitor cocktail (Roche), at a density of 10^8 cells/mL.

Influence of pH on reaction rate and product extraction

To test the effect of pH on reaction rate, a series of reaction buffers were prepared containing 10 mM KCl, 15 mM $MgCl_2$, 10% glycerol, 1 mM ATP, 10 μ M C6-NBD ceramide, and 0.2 mg/mL fatty acid free BSA, with 50 mM Hepes-HCl, pH 6.5 or 7.0, or 50 mM Tris-HCl, pH 7.5, 8.0, 8.5, or 9.0. Reactions were started with 0.5 μ g MBP-Cerk, and incubated for 20 min at 35 °C, then transferred to ice. Reaction mix (1 μ L) was spotted directly onto a Silica Gel 60 plate, and resolved as described above. To test the influence of pH on C6-NBD Cer1P extraction, a 250 μ L reaction was run as described above, but using 20 mM Hepes, pH 7.0 as the pH buffer. After 20 min at 35 °C, 50 μ L of the reaction mix was added to 50 μ L of 0.1 M buffer at pH 7.0, 7.5, 8.0, 8.5, or 9.0. pH indicator sticks were used to confirm that extractions were carried out at the desired pH. Reactions were then extracted with 0.25 mL chloroform/methanol (2:1), and 100 μ L aqueous phase was combined with 100 μ L dimethylformamide before measuring the fluorescence in a plate reader.

Cerk assay for living cells

HEK293 cells in 6-well plates were transfected with 10 μ L lipofectamine 2000 and 4 μ g Cerk expression vector (pIRES-EGFP) per well, in Optimem I serum free growth medium. 24 h after transfection the cells were split into 24-well plates, and at 48 h after transfection cells were loaded for 20 min with 5 μ M C6-NBD ceramide, which was delivered from a 250 μ M solution in PBS with 0.3 mg/mL fatty acid free BSA. Immediately after loading, cells were moved onto ice and transferred into eppendorf tubes, then pelleted at 4 °C. The cell pellets were resuspended in 250 μ L chloroform/methanol (2:1), to which was added 100 μ L of 50 mM Tris, pH 8.5. Extracts were briefly sonicated in a sonicating water bath to break up the cell pellet, then centrifuged at 21800g for 30s to separate the phases. The fluorescence of 100 μ L of the aqueous phase was measured in a fluorescent plate reader, in the presence of 100 μ L dimethylformamide. Blanks were derived from cells to which the C6-NBD ceramide was added immediately prior to centrifugation. TLC was used to confirm that the major product formed was C6-NBD Cer1P, by comparing the migration of the major product spot to a standard.

Results

In vitro ceramide kinase assay

As an initial test of NBD labelled ceramides as substrates for CerK, we monitored time-dependent product formation using TLC. Human CerK was fused to the C-terminus of maltose binding protein (MBP-CerK) and purified on amylose resin, and substrate was delivered using TritonX-100/cardiolipin micelles [3]. Under these conditions, a reaction product was seen with both C6-NBD and C12-NBD ceramides (Figure 1A). No product was seen in the absence of ATP (not shown). As was previously observed with short chain ceramides [4], C6-NBD ceramide could be solubilised with fatty acid free BSA for use in the CerK assay. We found that unlike longer chain ceramide phosphates, including C12-NBD Cer1P, C6-NBD Cer1P partitioned into the aqueous phase following chloroform/methanol extraction of the reaction mix (Figure 1B). The aqueous phase fluorescence could then be read in a plate reader. Two extractions of the organic phase resulted in essentially complete extraction of the Cer1P at pH 7.4, but a single extraction was effective for quantitation. In published assays, C2 and C6 Cer1P are detected in the organic phase following chloroform/methanol extraction. We thought that the difference may derive either from the use of a high KCl aqueous buffer in the published assay [21], or from increased solubility due to the NBD group. When we added KCl at 0.8M prior to extraction, the C6-NBD Cer1P was efficiently extracted into the aqueous phase (Figure 1C), indicating that either the C6-NBD Cer1P is more soluble than C6 Cer1P, or that in the published method much of the short chain Cer1P is lost during extraction, in the discarded aqueous phase.

Using 0.4 mg/mL (7 μ M) BSA to solubilise the C6-NBD ceramide, the substrate was phosphorylated with a K_m of 1.0 μ M and V_{max} of 11 nmoles/min/mg enzyme (Figure 2A). The K_m for ATP was 28 μ M (Figure 2B). We also found that increasing concentrations of BSA and TritonX-100, which are commonly used to solubilise ceramide substrates, slowed the reaction (Figure 2C and 2D), indicating that the concentration of these reagents should be kept as low as possible. Inhibition of CerK with detergents at or above the critical micelle concentration has been reported previously [21]. However, when TritonX-100 is combined with cardiolipin in mixed micelle assays, the reaction rate improves. In our hands optimal reaction efficiency with C6-NBD ceramide as substrate was achieved using 0.2 mg/mL BSA for delivery, and the rate was $\leq 50\%$ of this when mixed micelles containing 0.25 mM TritonX-100 were used. Addition of 10% glycerol to the assay buffer enhanced the reaction rate by 70% ($n=3$) when BSA was used to solubilise the substrate, but addition of Ca^{2+} did not boost the reaction rate when 10 mM $MgCl_2$ was present.

Influence of pH on reaction rate and Cer1P extraction

We tested the activity of recombinant CerK over the pH range 6.5 to 9, and found that activity decreased as the pH increased (Figure 3A), in agreement with previous observations [5]. Partitioning of Cer1P into the aqueous phase should improve at higher pH, resulting from an increased charge on the phosphate group. We tested this by running the reaction at pH 7.0, and adjusting the pH after the reaction and before extraction. Aqueous extraction of Cer1P increased between pH 7.5 and 8.5, although the signal/background was sufficiently good at pH 7.0 – 7.5 to warrant running reactions and extractions at a single pH (Figure 3B). The increase in C6-NBD Cer1P solubility between pH 7.5 and 8.5 correlates with the pK_a2 value for the closely related glycerolipid, phosphatidic acid (data taken from Avanti Polar Lipids website, www.avantilipids.com/PhospholipidPKA.html). Note that the background signal derived from C6-NBD ceramide in the aqueous phase remained low at all pH values tested.

Assaying CerK activity in cell lysates and cultured cells

Lysates of the murine macrophage cell line J774 phosphorylated C6-NBD ceramide at a rate of 12.5 pmoles Cer1P/min/mg lysate protein, and TLC was used to confirm that C6-NBD Cer1P was the principal reaction product in the aqueous phase following extraction (Figure 4A and B). The assay was also suitable for measuring CerK activity in lysates of HEK293 cells (data not shown). To test the utility of C6-NBD ceramide for measuring CerK activity in living cells, HEK293 cells were transiently transfected with a CerK expression plasmid, and 48 h after transfection the cells were incubated with 5 μ M C6-NBD ceramide for the indicated times. The cells were extracted with chloroform/methanol/0.1M Tris, pH8.5 (2:1:1.2), and the fluorescence of the aqueous phase was measured (Figure 4C). As expected, there was a time-dependent increase in the Cer1P level, in CerK transfected cells. There was also a time-dependent increase in extracted Cer1P in untransfected HEK293 cells, when the cells were incubated over a 2 h time frame (data not shown).

DMS inhibits CerK

We found that DMS, a commonly used inhibitor of cellular sphingosine kinase activity, inhibited CerK in our assay (Figure 5A). Whole reaction mixes resolved on TLC illustrate the good correlation between the amount of product formed and the fluorescent data derived from our plate reader assay (Figure 5B). DMS was a less effective inhibitor of C6-NBD ceramide phosphorylation when detergent micelles, rather than BSA, were used to deliver substrate (Figure 5A). These results suggest that the TritonX-100 interferes with the association between DMS and CerK. The inhibition was greater than that reported by Sugiura *et al* [5], who observed less than 50% inhibition of CerK activity with 100 μ M DMS, using detergent/cardiolipin micelles to deliver substrate.

For the purpose of testing inhibitory compounds such as DMS, the assay described above yields a Z-factor [23] of 0.75, using DMS at 100 μ M to obtain a minimum value and vehicle (methanol) control for the maximum value. The coefficient of variation for the vehicle condition was 0.07 (n=3). These values did not change significantly when the number of replicates was increased to 6. In contrast, we obtain coefficients of variation of no less than 0.15 (n=4) when running conventional [32 P]-ATP/TLC assays, with quantitation of radioactivity by Phosphorimager.

Discussion

In this paper we have presented a simplified assay for CerK, based on a single chloroform/methanol extraction of the reaction mix, after which the product (C6-NBD Cer1P) partitions into the aqueous phase, whilst the substrate (C6-NBD ceramide) remains in the organic phase. Although the signal over background improves when the extraction is carried out at pH 8.5 – 9, adjusting the pH of the reaction mix during extraction is not necessary to obtain a good, quantitative signal. Product formation, as determined by TLC, correlates well with the aqueous fluorescence after extraction at pH 7.4 (Figure 5A and B). An important aspect of our assay format was that we could detect lipid/CerK interactions with greater sensitivity than is achieved with the mixed micelle assay, which uses detergents. When substrate was delivered in BSA, DMS was inhibitory at sub-micromolar concentrations, and 80% inhibition was observed at 10 μ M DMS. By contrast, DMS incorporated into the mixed micelle assay was not inhibitory until it reached 30 μ M, which is equivalent to 9% of the micelle lipids if we assume full incorporation of DMS into the micelles. We have also observed a direct inhibitory interaction between CerK and the endogenous glycolipid sulfatide. The inhibitory interaction is observed in the presence of TritonX-100 at 1 $\times$ critical micelle concentration, but is lost when the TritonX-100 concentration is increased to 10 $\times$ critical micelle concentration (unpublished observations). It is up to the reader's interpretation as to whether the mixed micelle format is

a closer approximation of the lipid environment of the cell, and in this report our intent is only to point out the greater sensitivity of the BSA delivery method in identifying lipid/enzyme interactions. We note, however, that the activity of CerK on its endogenous C16–C24 ceramide substrates can not be determined with BSA as the vehicle [4].

The $V_{\max}$ for our assay (11–22 nmoles/min/mg; Figure 2A and B) is similar to the 35 nmoles/min/mg reported for phosphorylation of C6 ceramide by bacterially-expressed human CerK using the [32 P]-ATP assay [4]. Our K_m of 1.0 μ M is much lower than the 30 μ M reported by van Overloop *et al* [4], but low micromolar K_m values have previously been reported by Bajjalieh *et al* [24] for synaptic vesicle CerK activity, and Wijesinghe *et al* [3] for phosphorylation of C14–C24 ceramides by human CerK expressed in insect cells. We note that the kinetics of this assay are sensitive to the substrate delivery mode, and that the K_m will vary depending on the concentration of BSA or detergent used. In terms of sensitivity, we are able to detect specific activities that range down to 5 pmoles/min/mg cell lysate protein, in untransfected HEK293 cell lysate. Below this, the signal/noise becomes problematic. In our experience, the fluorescent plate assay is more sensitive than conventional radioactive/TLC assays that use 5 μ Ci [32 P]-ATP per reaction. However, specific activities as low as 2 pmole/min/mg protein have been measured in lysates of HL60 cells, using 60 μ Ci [32 P]-ATP per reaction [6]. When measuring endogenous CerK activity in cell lysates, we recommend running TLC to confirm that extracted material represents C6-NBD Cer1P and not another metabolite of the reaction. In our experience Cer1P is the only metabolite that is effectively extracted into aqueous solvent.

Our assay offers several advantages over the conventional [32 P]-ATP based assay: firstly, our assay is much faster (it can be completed in 1 hour), since it requires only a single extraction step followed by detection of aqueous phase fluorescence. By contrast the [32 P]-ATP assay involves multiple extractions to remove radiolabelled ATP, followed by TLC, then detection of radioactivity either by phosphorimager or scintillation counting. Secondly, as this is a plate reader assay with fewer steps, one can more easily run a large number of assay replicates at one time. Thirdly, our assay is non-radioactive, and therefore circumvents the combination of radioactive materials and organic solvents which create safety and disposal difficulties. Our assay could potentially be adapted to a high-throughput format using microtitre plates with hydrophobic filters to adsorb the substrate, although we have not yet investigated this possibility. This assay format would eliminate the need for chloroform and methanol, further reducing the adverse environmental impact of the assay.

Acknowledgements

This work was supported in part by the National Institutes of Health (AI055509 and NIMH-074404 for H.R.) and a grant from Kyorin Pharmaceutical Company.

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Figure 1. Development of fluorescent Cerk assay

(A) TLC showing formation of product after 0, 20, 40, or 60 min incubation of C6-NBD ceramide or C12-NBD ceramide, delivered in TritonX-100/cardiolipin micelles, with MBP-Cerk. Reaction mix was spotted directly on to the TLC plates. (B and C) CerK assay was carried out as described in experimental procedures, using BSA to deliver the C6-NBD ceramide substrate. Separation of product from substrate following chloroform/methanol extraction of the reaction mix was gauged by TLC. (B) For this experiment, the organic phase (lane 3) was extracted a second time with aqueous reaction buffer. Lane 1, whole reaction mix, no enzyme control; lane 2, whole reaction mix (including enzyme); lane 3, organic phase; lane 4, first aqueous extract; lane 5, second aqueous extract. (C) Reaction mix was extracted in the presence of 10 mM (lanes 1 and 2) or 800 mM (lanes 3 and 4) KCl. Lanes 1 and 3, organic phase; lanes 2 and 4, aqueous phase. All images are scans of the TLC plate for NBD fluorescence (emission 488 nM; absorption, 530 nM).

Figure 2. Characterisation of fluorescent CerK assay

(A) CerK reaction rate as a function of substrate concentration, with C6-NBD Cer1P fluorescence quantitated after extraction. (B) CerK reaction rate as a function of ATP concentration. Results for (A) and (B) are mean of duplicate reactions. Curves were fitted and K_m and V_{max} values determined using GraphPad PRISM software. (C) Effect of BSA on CerK reaction rate. Reaction buffer was as described in experimental procedures, with the BSA concentration varied as indicated on the x-axis. (D) Effect of TritonX-100 on CerK reaction rate. BSA was omitted from the reaction buffer for this assay. Results for (C) and (D) are mean and standard error from three reactions, and enzyme used for all reactions was MBP-CerK.

Figure 3. Influence of pH on reaction rate and Cer1P extraction

(A) Formation of C6-NBD Cer1P (product) with MBP-Cerk was tested at different pH values as described in experimental procedures. Reaction mix was resolved by TLC, and numbers below TLC panel indicate pH of reaction. Results are representative of two assays. (B) Extraction of C6-NBD Cer1P into the aqueous phase was tested at different pH values, as indicated on x-axis. Aqueous phase fluorescence (y-axis) was measured in the presence (■) or absence (○) of enzyme (MBP-Cerk). Data points are the mean and standard error derived from three assays.

Figure 4. Assaying CerK activity in cell lysates and cultured cells

(A) TLC scanned for NBD fluorescence, showing reaction of C6-NBD ceramide with J774 lysate. Lane 1, Cer1P standard; lane 2, no lysate control reaction; lane 3, reaction mix after incubation with J774 lysate; lane 4, aqueous phase after chloroform/methanol extraction of the reaction mix from lane 3. (B) CerK activity of J774 lysates, as a function of time. Results are mean and standard error of three assays. (C) CerK activity of HEK293 cells transfected with CerK in pIRES-EGFP (■), or vector only (○). Activity was assayed by incubating cells for indicated times with 5 μ M C6-NBD ceramide, then extracting cells as described in experimental procedures. Results are mean and standard error of three treatments.

Figure 5. DMS inhibits CerK

(A) Recombinant human CerK activity as a function of DMS concentration, using 0.2 mg/mL fatty acid free BSA (■) or TritonX-100/cardiolipin micelles (○) to deliver the substrate. DMS was delivered in 2 μL methanol/100 μL reaction. Data points are mean and standard error from three assays. (B) 1 μL of each reaction mix (prior to chloroform/methanol extraction), from reactions in which BSA was used as vehicle (Part A), was resolved on TLC and scanned for NBD fluorescence. Numbers below the TLC scan indicate the concentration of DMS; Ctrl: no enzyme control.