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Ceramide-dependent release of ceramide kinase from cultured cells

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Abstract

Ceramide kinase (CERK) and its product, ceramide-1-phosphate (Cer-1-P), are implicated in signaling processes, but the action mechanisms are not fully elucidated. When checking for intracellular effects of Cer-1-P by exposing CERK-expressing CHO cells to truncated ceramides, an unexpected decrease in CERK activity and protein level was observed. This decrease appeared dose-dependent and specific for the D-*erythro*-ceramide configuration and the presence of the double bond. At early time points, CERK clustered near the plasma membrane, followed later by its appearance in the culture medium. In cells expressing CERK lacking the pleckstrin domain or an inactive CERK mutant, this ceramide effect was not observed, indicating that clustering and release of CERK may be mediated by Cer-1-P. Presumably, high local Cer-1-P concentrations will increase the plasma membrane curvature and lead to release of CERK by vesicle shedding. This could be a potential regulatory mechanism in CERK/Cer-1-P signaling so far not investigated.
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Keywords: Apoptosis; Ceramide; Ceramide-1-phosphate; Exocytosis; Membrane fission; Sphingosine; Vesicle shedding

Ceramide, the initial product of the sphingomyelin cycle, functions as a key component in the regulation of various cellular functions like differentiation, proliferation, apoptosis, and inflammation. More recently, the ceramide metabolite ceramide-1-phosphate (Cer-1-P) also gained attention and turned out to be a bioactive sphingolipid. Ceramide kinase (CERK), initially described as a Ca^{2+}-dependent lipid kinase that co-purified with brain synaptic vesicles [1], can phosphorylate ceramides with a different *N*-acyl chain length or variation in the sphingoid base, but it displays a highly stereoselectivity for the D-*erythro* configuration [2–4]. The cellular localization of CERK is a matter of debate: membrane-associated in tissues [3], predominantly cytosolic in RBL-2H3 cells [5], largely Golgi-associated in Cos7 and HUVEC cells [6], mainly plasma membrane bound in CHO cells [3]. Membrane association of CERK is mediated by its pleckstrin domain [3,6,7] which interacts with phosphatidylinositol-4,5-bisphosphate [6,7]. Apparently, CERK can respond to stimuli by changing its localization, e.g. from Golgi to plasma membrane upon osmotic swelling [6], explaining perhaps the conflicting subcellular findings.

Cer-1-P has been implicated as a regulator of different cellular processes, like mitosis, apoptosis, phagocytosis, and inflammation. Cer-1-P stimulates DNA synthesis, but the mechanism is currently unknown. Inhibition of apoptosis would occur by inhibiting acid sphingomyelinase [8] and by stimulating the phosphatidylinositol 3-kinase (PI3-K)/protein kinase B (PKB) pathway [9]. Stimulation of phagocytosis was suggested to be caused by an increase in the plasma membrane Cer-1-P level, causing a change in the structural order of lipid rafts, which might promote phagosome formation [10]. Cer-1-P was also found to be a

Abbreviations: C_n, chain with n carbon atoms; Cer, ceramide; Cer-1-P, ceramide-1-phosphate; CERK, ceramide kinase; SPHK, sphingosine kinase.

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potent and specific inducer of arachidonic acid release and prostaglandin synthesis in cells by directly activating cytosolic phospholipase A_2 (cPLA$_2$) [11,12]. Recently, the extracellular Cer-1-P effects have been questioned and some have been shown to be related to their delivery in dodecane/ethanol [13].

The mechanisms by which CERK and its product, Cer-1-P, would regulate these important cellular processes, are only partly elucidated and defining the involved signaling pathways remains a major challenge. During an attempt to investigate Cer-1-P induced protein phosphorylation, we noticed a peculiar effect of ceramide on CERK in CERK-overexpressing cells, we like to report here. Apparently, ceramide triggers the release (or exocytosis) of CERK, and this response is mediated by intracellular Cer-1-P.

Materials and methods

Materials. C_6-ceramide (*N*-hexanoyl-sphingenine) was obtained from Acros Organics, sphingenine from Avanti Polar Lipids, anandamide from Larodan and MG132 from Sigma–Aldrich. The C_6-ceramide isomers and C_6-dihydroceramide (*N*-hexanoyl-sphinganine) were synthesized as described in [3].

Cloning. Vectors pHVO001, pHVO002, pHVO010, and pPVV072, coding for FLAG-*Hs*CERK, GFP-*Hs*CERK, FLAG-*Hs*CERK$_{116\text{-}537}$, and His$_6$-*Hs*CERK, respectively, were made as described [3]. A plasmid coding for an inactive FLAG-CERK (pHVO0023) was made as follows. Using pPVV072 as template, two amplicons were made by *Pfx* DNA polymerase (Gibco-BRL); primers for first PCR, fw 5′-ATGGGG ATCCGAGCTC and rv 5′-GAACATA**TC**ATCTCCGCC; for second PCR, fw 5′-GGCGGAGAT**GA**TATGTTC and rv 5′-CTAAAGCTTC AGTTTGTCAGCAGGACGCC (in bold the mutated codon to change the glycine at position 198 into aspartate). After fusion PCR, the product was digested with BamHI and HindIII and inserted into BamHI/HindIII restricted pCMV-Tag-2B (Stratagene).

Cellular studies. Chinese hamster ovary (CHO) cells, grown in serum-supplemented medium [3], were (co)transfected with appropriate vectors by using the Lipofectamine Plus Reagent (Invitrogen). The day after transfection, the medium was replaced by α-Minimal Essential Medium, containing 0.2% (v/v) Ultroser (Invitrogen), 1% (v/v) Glutamax, and antibiotics/antimycotics and cells were exposed to ceramide or a related lipid (prepared from an ethanolic stock, diluted with filter-sterilized defatted BSA solution to obtain a lipid/BSA molar ratio of 2; final ethanol concentration $\leqslant$0.2 % (v/v)). After the indicated incubation period, the adherent cells were harvested, resuspended in phosphate-buffered saline (PBS) containing a mix of protease inhibitors and sonicated on ice (Branson Sonifier B115, microtip).

When extracellular release was studied, the culture medium was also collected and centrifuged successively at $800g \times 10$ min, $10{,}000g \times 20$ min, and $100{,}000g \times 60$ min. The consecutive pellets and final supernatant were kept for analysis.

For subcellular localization studies by immunofluorescence microscopy, CHO cells were grown on coverslips, incubated as described above, and immunostained as described [14]. Antibodies used were diluted in 1 % (w/v) BSA in PBS (primary antibody anti-FLAG M2 (Stratagene); secondary antibody anti-mouse IgG FITC (Sigma)). Fluorescence was observed under an inverted IX81 Olympus microscope equipped with U-MNUA2/U-MNIBA3 filters.

Enzyme measurements and immunoblotting. CERK activity was measured using C_6-ceramide bound to BSA as substrate and ^{32}P-γ-ATP [3]. Sphingosine kinase (SPHK) activity was analyzed as described before [15], except for the use of CHAPS (1.25 (w/v)% final concentration) instead of ethanol/BSA to dissolve (4,5-^{3}H)sphinganine. β-galactosidase was measured by following the hydrolysis of 1 mM chlorophenol red β-D-galactoside (Boehringer Mannheim) at 575 nm at 37 °C in the presence of 10 mM DTT–1 mM $MgCl_2$–200 mM Hepes pH 8.0–0.1 % (w/v) Triton X-100 (final assay volume 500 μl) (modified from [16]).

Western blots (from 10% acrylamide gels; 50 μg protein/lane) were transiently visualized by Ponceau S staining, followed by blocking with 5% (w/v) dried milk (Protifar®, Nutricia) in Tris-buffered saline containing 0.05% Tween 20 (TBST). After overnight incubation with primary antibody [anti-FLAG M2; anti-caspase-3 (8G10; rabbit mAb, Cell Signalling Technology)], diluted in 1 % (w/v) dried milk in TBST, the membrane was washed and incubated with the appropriate secondary antibody coupled to peroxidase. The protein-antibody complex was visualized by chemiluminescent detection (ECL Plus Western blotting detection reagents, GE-Healthcare).

Results and discussion

Previously, we succeeded in expressing human CERK in eukaryotic cells in an active form as an N-FLAG-decorated fusion protein [3]. In order to study the possible effect of Cer-1-P on protein phosphorylation, without relying on exogenous addition of Cer-1-P, we attempted to increase the intracellular levels by exposing such CERK-expressing CHO cells to C_6-ceramide. Unexpectedly, when measuring CERK, a drastic decrease was observed. The specific CERK activity in cells exposed for 16–18 h to 5 μM C_6-ceramide dropped to $18.7 \pm 4.2\%$ (mean $\pm$ SE; eight separate transfection experiments) compared to that of non-treated cells. This consistent decrease was not due to an inactivation, as it was also seen at the protein level based on immunoblotting (data not shown). Further investigations revealed that the effect of C_6-ceramide was dose-dependent (Fig. 1A) and also observed with C_2-ceramide or with a different active fusion (GFP-*Hs*CERK) (data not shown). However, when the CERK-overexpressing cells were incubated with sphingenine (as a precursor of ceramide) or anandamide (as an inversed C_2-ceramide analogue), no effect on FLAG-CERK (Fig. 1A) or GFP-CERK (data not shown) was seen. Incubations with the different isomers of C_6-ceramide revealed a strong stereospecificity: CERK activity and protein level were decreased by the D-*erythro*-isomer, but weakly or not by the D-*threo*-, L-*erythro*-, and L-*threo*-isomers of C_6-ceramide, the saturated analogue, D-*erythro*-C_6-dihydroceramide, or an analogue with a reduced amide bond, *N*-octyl-sphingenine (C_8-ceramine) (Fig. 1B; data not shown).

As shown in Fig. 1C and D, the decrease of CERK levels and activity was time-dependent, and the cellular response to ceramide exposure appeared quite fast, already very obvious after 3 h. Once again, this decrease in activity was accompanied by the disappearance of CERK, visualized either by indirect immunofluorescence microscopy of the cultured cells (Fig. 1C) or by immunoblotting (data not shown). Interestingly, the microscopic analysis revealed a pronounced clustering of CERK at discrete sites near the plasma membrane few hours after the addition of ceramide. When expressing GFP-*Hs*CERK, this clustering could be followed *in vivo* in non-fixed cells (data not shown).

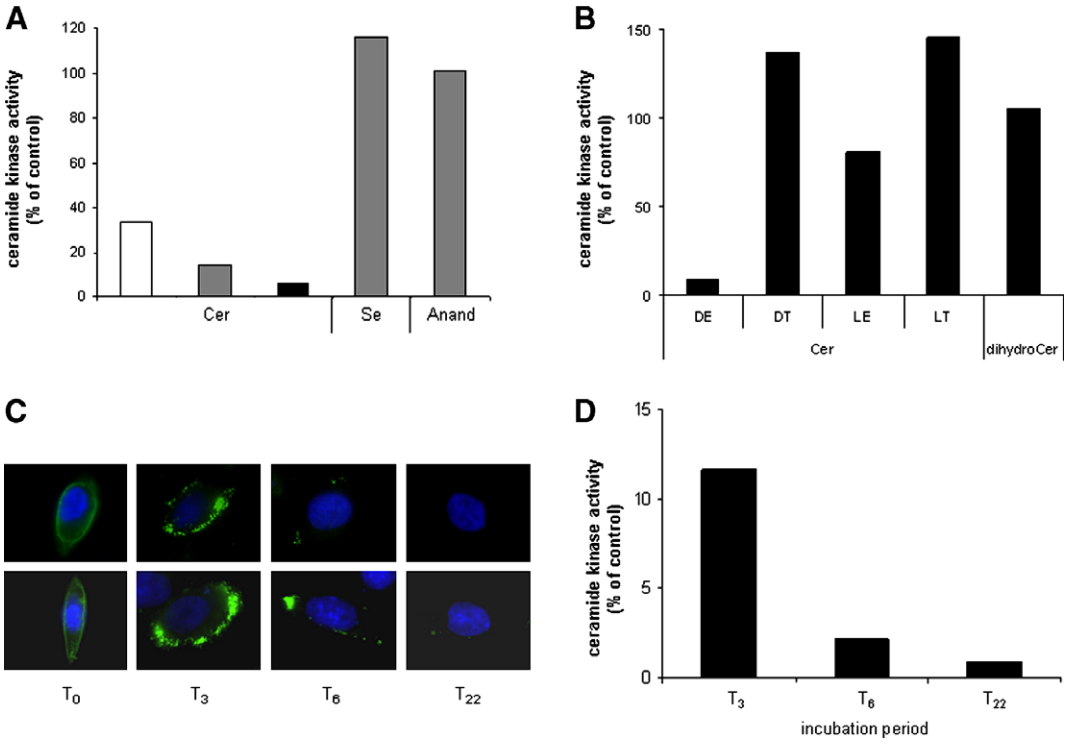

Fig. 1. Dose- and time-dependent modulation and isomer-specific regulation of CERK by ceramide in CERK-overexpressing cells. CHO cells were transfected with pHVO001 coding for FLAG-*Hs*CERK, and the next day incubated further overnight (A,B) or for shorter periods (C,D) in the presence of ceramide or other lipids, followed by collection of the cells, preparation of lysates, and CERK activity measurements. Data shown in (A,B) and (C,D) were obtained in separate representative experiments. (A) Cultures were exposed for 17 h to 5 μM D-*erythro*-C_6-ceramide (Cer), sphingenine (Se) or anandamide (Anand) (grey bars). For C_6-ceramide, incubations with 2.5 μM (white bar) and 10 μM (black bar) are also shown. Activities are expressed as percentage of control, receiving only ethanol/BSA, and are average of two experiments (CERK activity 6.4 and 16.8 nmol/min mg protein). (B) Cells were incubated overnight with 5 μM D-*erythro* (DE), D-*threo* (DT), L-*erythro* (LE) or L-*threo* (LT) C_6-ceramide (Cer) or C_6-dihydroceramide (dihydroCer). Activities are expressed as percentage of control, receiving only ethanol/BSA (CERK activity 4.6 nmol/min mg protein). (C,D) The day after transfection, cells were incubated with 5 μM C_2-ceramide for 0 (T_0), 3 (T_3), 6 (T_6) and 22 (T_{22}) hours, followed by immunohistochemistry (C) or CERK activity measurement (D). In (C) cells were stained with DAPI, incubated with monoclonal mouse anti-FLAG M2 (Stratagene)/anti-mouse FITC (green) and analyzed by immunofluorescence microscopy (for each time point, two representative pictures). In (D) activities are expressed as percentage of control, receiving ethanol/BSA for the same incubation period (CERK activity 6.0, 7.3, and 3.8 nmol/min.mg protein at T_3, T_6, and T_{22}, respectively; the lower value at T_{22} is due to the transient plasmid-driven expression of CERK, being maximal between 20 and 30 h after transfection).

Given the fact that both C_6-ceramide [3] and C_2-ceramide [17] are phosphorylated when given to CERK-transfected cells, the D-*erythro*-stereospecificity of CERK [3,4] and the lower *in vitro* activity of CERK on dihydroceramide compared to ceramide [3], the ceramide effect seems to be correlated with the substrate specificity of CERK [3,4]. Hence, we examined the possibility that it was mediated by Cer-1-P produced by CERK. Therefore, based on the known catalytic site of CERK and the example of an inactive sphingosine kinase mutant whereby conserved residues G in this site were mutated [18], a CERK mutant was generated by introducing a mutation in the ATP-binding site (changing glycine198 to aspartate), designated as $CERK_{G198D}$. As expected, this mutant kinase was catalytically defective but the mutation did not result in an altered subcellular localization (Fig. 2A and C), which is mediated by the pleckstrin domain [6]. A similar mutant has recently been reported by Rovina et al. [19]. Cells expressing inactive $CERK_{G198D}$ did not respond to C_6-ceramide (data not shown) or C_2-ceramide: the protein level did not decrease (Fig. 2B) and the enzyme remained associated with the plasma membrane (Fig. 2A). Similarly, no ceramide effect was seen in cells expressing FLAG-CERK lacking the pleckstrin domain (data not shown). As reported before, this truncated CERK does not interact with the membrane and is inactive [3,6]. These findings strongly implicate that the ceramide effect is mediated by intracellularly formed Cer-1-P.

To reveal the mechanism of the ceramide/Cer-1-P effect on the kinase, a few possibilities were further explored including proteolytic degradation and cell death. Proteasome-mediated proteolytic degradation seems unlikely given the lack of any protective effect of the proteasome-inhibitor MG132 [20] at 20 μM (data not shown). Although ceramide is a considered as a pro-apoptotic agents, the fact that it has no effect in cells expressing the mutated or truncated CERK variants, exclude a ceramide-mediated apoptosis under our conditions. This is consistent with immunoblotting with a caspase-3 antibody. Generally in transfected cells, a small amount of 17 kDa cleavage product was seen, but the signals were not enhanced by C_6-ceramide (data not shown). Also the DAPI

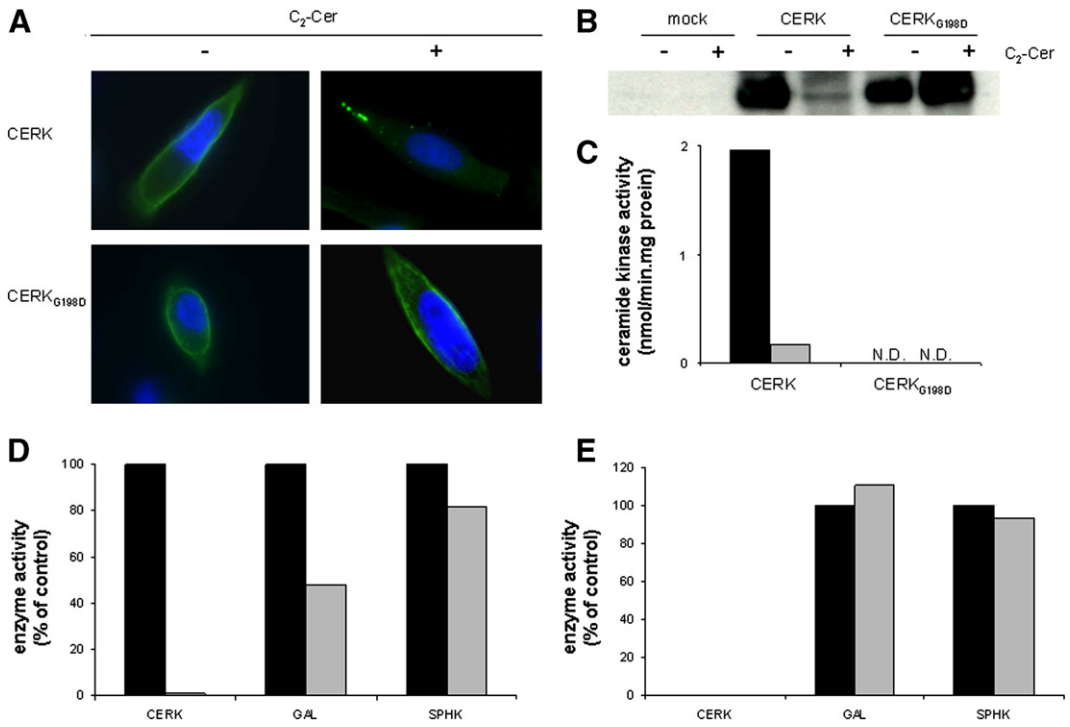

Fig. 2. Modulation of CERK by ceramide is dependent on the formation of Cer-1-P. CHO cells were transfected with plasmids encoding FLAG-*Hs*CERK (pHVO001) or FLAG-*Hs*CERK$_{G198\ D}$ (pHVO023) (A–C) or cotransfected with a β-galactosidase expression vector pCMVβ (Clontech) and these vectors (D,E). The day after transfection, cells were incubated overnight with (+) or without (−) 5 μM C_2-ceramide (C_2-Cer), followed by immunohistochemistry, immunoblotting or CERK activity measurement in lysates. (A) FLAG fusions of *Hs*CERK and *Hs*CERK$_{G198D}$ were visualized with mouse anti-FLAG-M2/anti-mouse FITC and analyzed by immunofluorescence microscopy. (B) Immunoblot analysis of cell lysates using anti-FLAG-M2 antibody decorating a band of the expected size of the fusion protein (62 kDa) (mock : empty vector pCMV-Tag 2B). (C) CERK activity in lysates of cells incubated overnight with (grey bars) or without (black bars) C_2-ceramide (ND, not detectable at the specific radioactivity of ATP used in these experiments). (D,E) Ceramide kinase (CERK), β-galactosidase (GAL) and sphingosine kinase (SPHK) activity in lysates of CHO cells co-transfected with pCMVβ and pHVO001 expressing FLAG-*Hs*CERK (D) or pHVO023 expressing FLAG-*Hs*CERK$_{G198\ D}$ (E) and incubated overnight with (grey bars) or without (black bars) 5 μM C_2-ceramide. Activities are expressed as percentage of enzyme activity in absence of C_2-ceramide (control) (D: CERK 8.4 nmol/min mg protein, GAL 1.8 μmol/min mg protein, SPHK 56 pmol/min mg protein; E: CERK not detectable (ND), GAL 3.1 μmol/min mg protein, SPHK 56 pmol/min mg protein).

staining did not reveal more nuclear fragmentation/condensation in the presence of ceramide in the CERK-expressing cells.

As CERK activity measured in these experiments is mainly plasmid-derived and under control of the CMV-promoter, an effect on transcription seems remote but cannot be excluded. Since expression of CERK$_{G198D}$, under similar control, was not influenced, an effect of ceramide, but not of Cer-1 P, on transcription can be ruled out. Finally, in order to correct for any cytotoxic effect, due to transfection agents, expression of CERK itself, formation of Cer-1P or addition of ceramide/solvent, cells were co-transfected with CERK and β-galactosidase expression vectors, and the CERK/β-galactosidase balance was evaluated. As shown in Fig. 2D, incubation with ceramide lowered the β-galactosidase activity, although substantially less than the CERK activity (48 versus 1% compared to control incubation). A drop of β-galactosidase activity was not observed when inactive CERK$_{G198D}$ was expressed (Fig. 2E). As an additional control, we measured in the transfected cells an endogenous, not plasmid derived, enzyme, in casu sphingosine kinase (SPHK), being closely related to CERK [2]. SPHK activity decreased slightly by incubation with ceramide (82% for CERK and 94% for CERK$_{G198D}$ compared to control incubations) (Fig. 2D and E). The selective decrease of β-galactosidase activity, but not of a related kinase, in CERK-transfected cells incubated with ceramide, might point to an effect of Cer-1-P on the protein synthesis.

Hence, it seemed that disappearance of CERK from the cells cannot exclusively be explained by proteolysis or a cytotoxic effect. Consequently, we analyzed the culture medium. The cell debris (and detached cells, collected by centrifugation at 800*g*) from cells exposed to ceramide contained relatively more CERK (Fig. 3). This is in agreement with a higher degree of cell detachment, seen sometimes in ceramide treated CERK-expressing cells (at 5 μM C_6-ceramide for 16–18 hours, 18.1 ± 10.3 % less protein in the washed monolayers compared to cultures exposed to ethanol/BSA; $n = 6$). A small amount of CERK was recovered upon pelleting the 800*g* supernatant at 10,000*g*, independent of the exposure to ceramide. Finally, upon analyzing the material sedimenting at 100,000*g*, the presence of CERK was revealed exclusively in the pellet from CERK-overexpressing cells incubated with ceramide (Fig. 3). The most straightforward explanation is that cer-

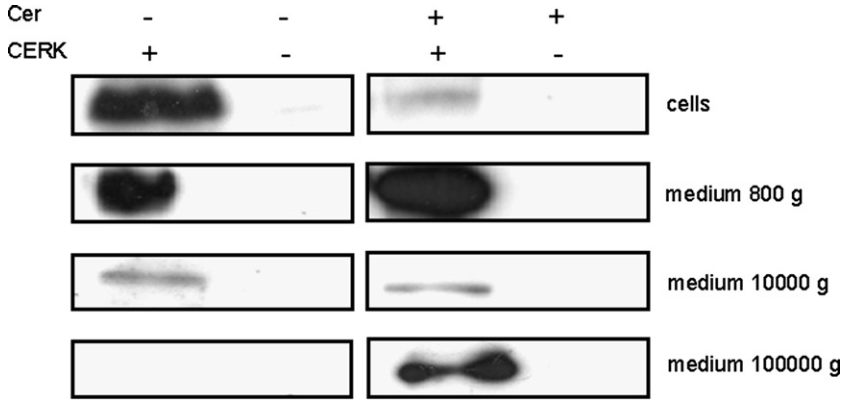

Fig. 3. Ceramide/Cer-1-P mediated release of CERK.CHO cells were transfected with a FLAG-*Hs*CERK expression vector (pHVO001; +CERK) or the empty vector (pCMV-Tag2B; -CERK). The day after transfection, cells were incubated overnight with or without 5 μM C_6-ceramide (+/− C_6-cer). Subsequently, the medium was removed and centrifuged sucessively at 800*g*, 10,000*g* and 100,000*g* and cells were collected. Proteins of the cell lysate (1/30 of total) and the pellets (1/5 of total) were separated by SDS–PAGE and analyzed by immunoblotting with anti-FLAG-M2.

amide/Cer-1-P somehow triggers the release or exocytosis of CERK into the medium. Being pelleted at 100,000*g*, CERK is not freely soluble but likely associated with a small vesicle, and this would rule out a conventional exocytotic release by membrane fusion. The clustering of CERK near the plasma membrane, shortly after ceramide exposure (Fig. 1C), is likely preceding/triggering the vesicular release.

In order to explain the sedimentable nature of the released CERK, the involved mechanism could either be exosomal release [21] or vesicle shedding [22]. The first one is so far mainly described in hemopoietic cells [21], and there is no evidence that multivesicular bodies are involved in the phenomenon described here. On the other hand, the accumulation of CERK at restricted sites beneath the plasma membrane is in line with vesicle shedding [22] and a similar time frame has been reported for the externalization of fibroblast growth factor-2 in shed vesicles from SK-Hep1 cells [23]. A putative mechanism, based on outward budding of the plasma membrane induced by higher levels of cone-shaped Cer-1-P in the inner leaflet, is shown in the Supplement.

Summarizing, we showed a cellular effect of ceramide on (expressed) CERK resulting in reduced cellular CERK levels and release of CERK in the culture medium, likely mediated by the ceramide metabolite Cer-1-P. The latter conclusion is based on the use of truncated ceramides known to be phosphorylated *in vivo* [3,17], the correlation between kinase recognition and CERK release with regard to stereospecificity and desaturation of the ceramides, and the lack of an effect when an inactive kinase is expressed. Our findings have potential regulatory consequences for the Cer-1-P/CERK mediated response(s) and suggest that Cer-1-P might play a role in vesicle shedding. Because of structural resemblance, Cer-1-P may have indeed effects on membranes comparable to those of phosphatidate for which a key role in intracellular membrane fission/budding events has been described [24,25].

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.09.117.

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