A critical $\beta 6$ – $\beta 7$ loop in the pleckstrin homology domain of ceramide kinase

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CerK (ceramide kinase) produces ceramide 1-phosphate, a sphingophospholipid with recognized signalling properties. It localizes to the Golgi complex and fractionates essentially between detergent-soluble and -insoluble fractions; however, the determinants are unknown. Here, we made a detailed mutagenesis study of the N-terminal PH domain (pleckstrin homology domain) of CerK, based on modelling, and identified key positively charged amino acid residues within an unusual motif in the loop interconnecting β -strands 6 and 7. These residues are critical for CerK membrane association and polyphosphoinositide binding

and activity. Their mutagenesis results in increased thermolability, sensitivity to proteolysis, reduced apparent molecular mass as well as propensity of the recombinant mutant protein to aggregate, indicating that this loop impacts the overall conformation of the CerK protein. This is in contrast with most PH domains whose function strongly relies on charges located in the $\beta 1$ – $\beta 2$ loop.

Key words: ceramide kinase, ceramide 1-phosphate, Golgi complex, pleckstrin homology domain (PH domain), polyphosphoinositide, subcellular localization.

INTRODUCTION

CerK (ceramide kinase) was identified recently by Sugiura et al. [1]. It is so far the only established enzyme that uses ceramide as a substrate to produce C1P (ceramide 1-phosphate). A CerK-like protein as well as a multi-lipid substrate kinase were also identified as close homologues within the diacylglycerol kinase superfamily, but their activity towards ceramide is either lacking [2,3] or controversial [4,5]. C1P was first described in brain synaptic vesicles, HL60 cells and neutrophils [6–8], and there is now evidence that this lipid is endowed with signalling functions. Chalfant and co-workers [9] have shown that C1P participates in eicosanoid synthesis, where it synergizes with sphingosine 1-phosphate [10]. Their work identified the first molecular target of C1P, namely the cytosolic PLA₂ (phospholipase A₂) α (PLA2GIVA) [11]. Other studies have pointed to a role of CerK/C1P in mast cell degranulation [12], regulation of acidic sphingomyelinase and control of apoptosis and survival in bone-marrow-derived macrophages [13,14], calcium mobilization in GH4C1 pituitary cells and Jurkat T-cells [15,16], and dopamine release in PC12 cells [17]. A role of C1P in fibroblast proliferation was proposed a decade ago [18,19]. However, this may not be general as such an effect was not observed in FRTL-5 thyroid cells [20].

In contrast with C1P signalling, characterization of CerK at the molecular level has been poorly addressed. In particular, there is no detailed information regarding what determines the ability of CerK to associate with the Golgi complex and plasma membrane [21]. We therefore performed a modelling and comprehensive mutagenesis analysis of the N-terminal PH domain (pleckstrin homology domain) of CerK. We identified an unusual loop interconnecting the $\beta 6$ and $\beta 7$ strands of this domain, which provides key positively charged residues required for localization and function of this enzyme.

EXPERIMENTAL

Materials

C₈-ceramide was obtained from Cayman, cardiolipin and the Immunoprecipitation FLAG®-Immunoprecipitation kit were from Sigma, THP [tris(hydroxypropyl)phosphine] was from Novagen, the HALT™ phosphatase inhibitor cocktail was from Pierce, octyl-D- β -glucopyranoside was from Fluka. [γ -³²P]ATP (10 mCi/ml, 3000 Ci/mmol), [³²P]P_i (10 mCi/ml), [³⁵S]methionine and glutathione–Sepharose® 4B were from Amersham Biosciences. Trypsin sequencing-grade, Complete™ protease inhibitor tablets and GSH were from Roche Molecular Biochemicals. BODIPY® (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene) TR-C₈-ceramide was purchased from Molecular Probes. Mutagenesis was performed with the QuikChange® II site-directed mutagenesis kit (Stratagene). SDS/PAGE was performed under reducing conditions on NuPAGE® polyacrylamide gels (Invitrogen). OneShot® TOP10 competent *Escherichia coli* cells were from Invitrogen. Oligonucleotide synthesis and DNA sequencing were performed at VBC Biotech Services. Purified insect cell-expressed GST (glutathione S-transferase)–CerK was obtained from Dr Michael Forstner (Novartis Institutes for BioMedical Research, Basle, Switzerland).

Threading and homology modelling

We used the BMI_db-generation.py script provided with the ProHIT package (© ProCeryon; [22]) to prepare a structure template database for fold recognition. This script essentially downloaded the PDB (Protein Data Bank) [23] and selected 9638 chains that share no more than 95 % sequence identity among each other. According to the SCOP database [24], these 9638 chains contain 25 PH domains. We then ran the ProHIT fold recognition procedure on the putative human CerK PH domain and on similar

Abbreviations used: BODIPY®, 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene; C1P, ceramide 1-phosphate; CerK, ceramide kinase; DIC, differential interference contrast; DTT, dithiothreitol; ESP, electrostatic potential; GFP, green fluorescent protein; GST, glutathione S-transferase; HBSS, Hanks balanced salt solution; HRP, horseradish peroxidase; Ig, immunoglobulin; PA, phosphatidic acid; PB, Poisson–Boltzmann; PH domain, pleckstrin homology domain; PLA₂, phospholipase A₂; PLC, phospholipase C; PPI, polyphosphoinositides; SASA, solvent-accessible surfaces; TAPP2, tandem PH-domain-containing protein-2; THP, tris(hydroxypropyl)phosphine; WT, wild-type.

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but sufficiently different homologous sequences: CerK isoform A, *Homo sapiens* (NP.073603; NCBI); CerK, *Mus musculus* (Q8K4Q7; NCBI); 'similar to CerK isoform a', *Gallus gallus* (NP_001026511; NCBI); 'similar to CerKs', *Rattus norvegicus* (XP_217019; NCBI) and 'mgc84197 protein', *Xenopus laevis* (AAH74110; NCBI). AAH74110 was previously not reported to share a PH domain, but the alignment was nevertheless significant (E -value $8.5e^{-15}$). Each ProHIT run resulted in a list of 9638 sequence–structure alignments (models), represented by various scores. After ranking each result list by the 'threading index', we calculated the median rank of each template. The top seven ranking domains were PH domains, if considering the median rank for each domain over all five sequences (including the proposed human CerK PH domain). Even if we calculated the median rank for the most diverse sequences only, human, chicken and frog, the program indicated PH domain folds for seven ranks out of ten: 1–3, 5–7 and 9. Furthermore, PH domain superfolds that are associated with functions such as the Ran-binding domain [25], the Enabled/VASP (vasodilator-stimulated phosphoprotein) homology domain-1 [26] and the phosphotyrosine-binding domain [27] ranked very low (average rank <40). To find the best fitting structural template for the human CerK PH domain, we inspected the sequence–structure alignments manually. The final model was created with MODELER (Accelrys) [28], based on the sequence–structure alignment.

ESP (electrostatic potential) calculations

ESP calculations based on solutions to the PB (Poisson–Boltzmann) equation were performed with the help of program POLCH [29]. Assuming aqueous solution the dielectric constants were set to 80.0 (bulk) and 4.0 (protein interior). The obtained model structure was stored in pdb format and processed with the program XLEAP from the AMBER suite of programs [30]. Default AMBER RESP charges as well as default AMBER van der Waals radii have been assigned (version 6.0). The WT model structure showed a net charge of +7 prior to PB calculation. For generation of an appropriate molecular surface the program MSROLL was used (version 3.8) employing a probe sphere radius of $2.0 \text{ \AA}$ ($1 \text{ \AA} = 0.1 \text{ nm}$) and resolution/fineness parameters of $-f 1.0$ and $-l 0.3$ [31]. Resulting SASA (solvent-accessible surfaces) were composed of approx. 15 000 surface elements (of flat triangular shape). Upon termination of the PB calculation the structures were embedded in cubic grid boxes of dimension $70 \text{ \AA} \times 70 \text{ \AA} \times 70 \text{ \AA}$ with an edge length of roughly $130 \text{ \AA}$. The ESP was computed at all the grid points including the PB-obtained set of apparent surface charges distributed over the SASA. Grid points approaching any protein atom closer than $3 \text{ \AA}$ were excluded from ESP calculation. Grid maps were superimposed on the protein structure and visualized with VMD (VMD is Copyright © 1995–2005 Board of Trustees of the University of Illinois and others). Representation of the ESP was done with a colour-coded isosurface mode. Selected iso-levels were $\pm 144 \text{ mV}$ (blue/red).

Human CerK constructs and transfection

CerK cDNA, corresponding to GenBank® accession number AB079066, was obtained and subcloned in Gateway™ compatible entry vectors as described previously [33,21]. Site-directed mutagenesis was performed using the following primers (only the forward primers are indicated and changed bases are underlined). Combinations of mutants were performed using the same primers on already established mutants. K17A: cctgtctgtgggtgcacagcagcgctgcgcc; R20A: gtgggtgaagcagcaggcctgcgccgtgagcctg; K17A, R20A: gtgggtggcacagcaggcctgcgcc; K33A: gcgggct-

ctgtgcgctgtggcgagccc; R36A: ctgtgcgctgtggcgagccccgggccc; R29A/R33A/R36A: gcggcgctctgtgcgctgtggcgagccccgggccc; K74A: catcaaggcagtgagcagcatggcagaaaatggaaaagc; K68V, K74A: gaaacagacgttcacgggtgcatcaaggcagtgaaaatg; K77A: cag-tggaaaatggcagcgaatggaaaagccttacg; K80V: ggcagaaaatggaagtgccttacgcttttacg; K74A/K77A: cagtggagcatggcagcgaatggaaaagc; K77A/K80A: ggcaggcaatggaagtgccttacgcttttacg; K90V: gcttttacgttctactgtgtagtgcacgacggcac; R91A: cagttcactgtgtaaggcagcagcagggcaccg; K90V, R91A: gcttttacgttctactgtgtagtggcagcagcagggcaccg; R93A/R94A: ctgtgtaagagagcagcagtgaccgctggaagt; R96A: gagagcagcagggcagcctggaagtggcg; K98V: gacggcaccgctgggtgtggcgagcagtgac; R96A, K98V: gagagcagcagggcagcctgggtgtggcgagcagtgac; K90V, R91A, R96A, K98V: cagcagggcagcctgggtgtggcgagcagtgac.

All constructs were transferred to pcDNA6.2DEST in order to express untagged proteins or pcDNA-DEST53 in order to express N-terminal GFP (green fluorescent protein)-fusion constructs. For transfection, COS-1 cells [obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) and cultured in DMEM (Dulbecco's modified Eagle's medium)/10 % fetal calf serum at $37^\circ\text{C}/5\% \text{ CO}_2$ in a humidified atmosphere] were seeded at 10^5 cells/well in 6-well plates. After 24 h, cells were transfected with $3 \mu\text{g}$ vectors containing expression constructs for various CerK alleles, using FuGENE™ 6 (Roche Molecular Biochemicals). Cells were incubated for 24–48 h following transfection before harvesting.

Fluorescence microscopy

COS-1 cells were seeded into four-chambered coverslips (LabTEK™ II; Nalge Nunc) at 2.2×10^4 cells per chamber. Cells were transfected, 24 h later, with $0.6 \mu\text{g}$ of plasmid per chamber using FuGENE™ 6. Live-cell fluorescence microscopy was performed 24 h after transfection on an inverted microscope Axiovert 200 M equipped with a high-resolution microscopy camera AxioCam MRc (Zeiss) and objectives Plan-Neofluar $40\times/1.30$ oil DIC (differential interference contrast) and Plan-Apochromat $63\times/1.40$ oil DIC, using the following filter set: $\lambda_{\text{ex}} = 470 \text{ nm}/\lambda_{\text{em}} = 525 \text{ nm}$ for GFP fluorescence and $546 \text{ nm}/590 \text{ nm}$ for red fluorescence. For quantification of regions of interest (Figure 4A), pictures were analysed using the AxioVision 4.0 V 4.3.0.101 (Zeiss) software. The first region of interest (x) was defined at the Golgi. A second region of interest was defined on the opposite side of the nucleus (y). Background fluorescence was measured in the extracellular space (z). Golgi/cytoplasm fluorescence ratio values were calculated using the following equation: $(x - z)/(y - z)$.

Cellular fractionation

For Figure 1(C), a one-step fractionation protocol was used. Cells were harvested at 4°C for 2 h on a plate shaker set at maximal speed, in 20 mM Tris (pH 7.5), 1 mM DTT (dithiothreitol), with protease inhibitors, and 2 % Triton X-114, 2 mM EGTA and 500 mM KCl, the latter three either alone or in combination. Cell lysates were spun down at $100\,000 g$ for 30 min at 4°C in a TLA120.2 rotor using a TL-100 ultracentrifuge (Beckman). We did not make use of clouding properties of Triton X-114 (Triton X-100 can also be used, giving rise to similar results).

For Figure 4(B), fractionation was performed in three steps. Cells were first scraped and homogenized by 20 strokes in a Potter–Elvehjem homogenizer, with $500 \mu\text{l}$ of buffer 1 (20 mM Tris, pH 7.5, 1 mM DTT and protease inhibitors). Lysates were centrifuged at $55\,000 \text{ rev./min}$ in a TLA120.2 rotor (Beckman),

Figure 1 Subcellular localization and solubilization of WT CerK

(A) COS-1 cells overexpressing GFP-tagged WT-CerK were incubated for 30 min with 5 μ M BODIPY[®]-TR-ceramide. After medium exchange, cells were analysed by fluorescence microscopy. Left-hand panel, GFP fluorescence; middle panel, BODIPY[®] fluorescence; right-hand panel, merge. (B) COS-1 cells overexpressing GFP-tagged WT-CerK were treated or not with 33 μ M nocodazole for 3 h. Subsequently, cells were osmotically challenged by diluting the medium 1:4 in water and incubated for another 30 min before fluorescence microscopy. White arrows indicate plasma membrane localization. (C) COS-1 cells overexpressing C-terminally FLAG-tagged CerK were fractionated as described in the Experimental section, in the absence or presence of 2% Triton X-114, 2 mM EGTA or 500 mM KCl, as indicated. At the end of the harvesting period, cells were high-speed centrifuged and the resulting supernatants (S) and pellets (P) were analysed with SDS/PAGE and anti-FLAG Western blotting.

yielding a cytosolic fraction. Pellets were dissolved in 500 μ l of buffer 2 (20 mM Tris, pH 7.5, 1 mM DTT, 250 mM NaCl and protease inhibitors) and samples were spun as above. Perimembrane fractions were stored and pellets were dissolved in 500 μ l of buffer 3 (20 mM Tris, pH 7.5, 1 mM DTT, 1% Triton X-100 and protease inhibitors). Samples were spun again and supernatants were collected (Triton extract). Final unextracted pellets were resuspended in 500 μ l of buffer 1 and stored.

Protein expression and purification

CerK-FLAG proteins were obtained following immunoprecipitation. At 48 h after transfection, cells were washed once with PBS and then lysed with 1 ml of lysis buffer (provided with the Sigma FLAG[®]-Immunoprecipitation kit), complete with pro-

tease inhibitors. Samples were incubated for 2 h at 4°C with vigorous shaking then spun down at 4°C for 30 min at 15000 *g* in an Eppendorf 5402 thermo centrifuge. Then, 40 μ l of anti-FLAG M2 affinity gel (beads) was washed four times with the provided wash buffer, and added to each obtained supernatant. Samples were tumbled at 4°C on a wheel overnight. Subsequently, the beads were collected after a short spin and washed three times with wash buffer. Elution was performed by tumbling at 4°C for 2 h in 300 μ l of wash buffer containing 300 ng/ μ l of a provided 3 $\times$ FLAG peptide solution. Expression plasmids for GST-tagged proteins used as controls for the protein-lipid overlay experiments were as follows: murine GRP1 (general receptor for 3-phosphoinositides)-PH, rat PLC δ 1 (phospholipase C δ 1)-PH, human Hrs1 (hepatocyte growth factor-regulated tyrosine kinase substrate 1)-FYVE (Fab1p, YOTB, Vac1p and EEA1)

domain, all in pGEX-2TK and human DAPP1 (dual adaptor for phosphotyrosine and 3-phosphoinositides)-PH, human dynamin-1-PH in pGStag. Plasmids were transformed into TOP10 chemically competent *E. coli* cells. A single colony was grown overnight in liquid culture. The culture was then diluted to reach a D_{600} (attenuance) of 0.06 and grown further until a D_{600} of 0.6. Protein expression was induced with isopropyl- β -D-thiogalactopyranoside (500 μ M) and the suspension was shaken (200 rev./min) for 2 h at 30°C. Finally, cells were spun down and pellets resuspended in 1 ml of lysis buffer (50 mM Tris/HCl, pH 7.5, 270 mM sucrose, 1 mM EDTA, 1 mM EGTA, 5 mM THP, 1 $\times$ phosphatase inhibitor cocktail, 1 $\times$ protease inhibitor cocktail and 1% Triton X-100). Afterwards, samples were snap frozen in liquid nitrogen and thawed. Lysozyme (100 ng/ μ l) was added followed by a 10 min incubation on ice. Samples were sonicated for 30 s on ice using a BANDELIN Sonopuls HD2070 (set at 20%). Lysates were spun down for 30 min at 15000 g and 4°C, in an Eppendorf 5402 thermo centrifuge. Glutathione-Sepharose® 4B (80 μ l per sample) was washed three times in 0.5 ml of lysis buffer. Supernatants were applied to Sepharose beads and tumbled for 1 h at 4°C on a wheel. After a short centrifugation, supernatants were discarded and the Sepharose beads were washed three times with lysis buffer supplemented with 500 mM NaCl. This was followed by ten washing steps with 300 μ l of wash buffer (lysis buffer without EDTA and without Triton X-100). Elution was performed on a wheel in 500 μ l of elution buffer (wash buffer + 20 mM GSH) for 15 min at room temperature (22°C). Eluates were made to 30% (v/v) glycerol and snap frozen until use. *In vitro* translation of untagged CerK was performed using the TNT coupled reticulocyte lysate system (Promega) as described previously [21]. Briefly, 1 μ g of pcDNA 6.2 containing a CerK WT (wild-type) or mutagenized cDNA sequence was mixed with 25 μ l of reticulocyte lysate, 0.5 μ l of methionine and 2 μ l of the reaction buffer. Then, 3 μ l of [³⁵S]methionine (1 mCi/mmol) was added. Reaction was started by adding 1 μ l of T7 RNA polymerase and allowed to proceed for 90 min at 30°C. Subsequently, complete samples were filtered through a micro Bio-Spin 6 chromatography column (Bio-Rad). The flow through was analysed by SDS/PAGE and used in protein–lipid overlay assays.

Protein quantification

Whole cell lysate (10 μ l) was diluted in 1 ml of ethyl alcohol and incubated on dry ice for 15 min. After centrifugation (15 min at maximum speed in a benchtop centrifuge), the supernatant was discarded and 10 μ l of 0.1 M NaOH and 200 μ l of micro BCA (bicinchoninic acid) (Pierce) working solution were added. Following vigorous vortex-mixing and incubation at 37°C for 45 min, 100 μ l was transferred to a 96-well plate and read on a Molecular Devices SpectraMax 340 PC 384 plate reader set at 562 nm. BSA was used as the standard. Normalization for CerK protein content, as used for Figure 5(A) and Table 1, was obtained by measuring GFP fluorescence of the lysates with a Molecular Devices SpectraMax Gemini XS (λ_{ex} = 395 nm/ λ_{em} = 507 nm; cut-off: 495 nm).

CerK assays

For *in vitro* kinase assays, cells were scraped into lysis buffer (10 mM Mops, pH 7.2, 2 mM EGTA, 150 mM KCl, 2% Triton X-100, 1 mM DTT and protease inhibitors). The suspension was homogenized by 20 strokes in a Potter–Elvehjem homogenizer and used immediately. Kinase activity assays were performed in total volumes of 100 μ l composed of: 880 μ M C₈-ceramide, 1 mM cardiolipin, 1.5% β -octylglucoside, 0.2 mM

Table 1 Summary of CerK mutant proteins and associated phenotypes

The CerK mutants are categorized according to the mutagenized region. Localization is depicted using a three-black-dotted scale: (●●●) WT, (●●○) partially compromised, (●○○) severely compromised, (○○○) Δ PH CerK-type localization. Activity is expressed as a percentage of that of WT-CerK, after normalization of CerK protein amounts (compare with Supplementary Figure 1 at <http://www.BiochemJ.org/bj/400/bj4000255add.htm>). Data represent the means $\pm$ S.D. for at least two experiments, performed in triplicate. Deletion of the first seven amino acid residues of CerK (Δ 2–7 CerK) has no effect, consistent with the PH domain starting at residue number 8 (Figure 3) [21]. Further deletion (Δ 2–13 CerK) removes most of the first β -strand and completely abrogates WT localization and activity. An L10A mutation described previously [50] was partially effective in our assays. The results obtained with mutagenesis of the loops interconnecting the β -strands, either alone or in combination, are summarized. Finally, deletion of the C-terminal α -helix, which abrogates PH domain function, is also indicated. —, Not measured.

PH domain region	CerK construct	Localization	Activity	$\pm$ S.D.
	WT	●●●	100	—
	Δ PH	○○○	<1	—
	Δ 2–7	●●●	60	18
	Δ 2–13	○○○	<1	—
β 1– β 2	K17A	●●●	—	—
	R20A	●●●	—	—
	K17A, R20A	●●○	77	8
β 2– β 3	R29A, R33A	●●●	—	—
	K33A	●●●	—	—
β 3– β 4	R36A	●●●	—	—
	R29A/R33A/R36A	●●●	117	13
β 5– β 6	K74A	●●●	—	—
	K68V, K74A	●●●	—	—
	K77A	●●●	—	—
	K80V	●●●	—	—
	K74A/K77A	●●●	—	—
	K77A/K80A	●●●	102	35
	K68V/K74V, K80V	●●●	114	24
β 6– β 7	K90V	●●●	92	16
	R91A	●●○	34	5
	K90V, R91A	●○○	19	4
	R93A/R94A	●●●	—	—
	R96A	●●●	103	26
	K98V	●●●	120	14
	R96A, K98V	●○○	61	9
	K90V, R91A, R96A, K98V	○○○	<1	—
β 1– β 2 + β 6– β 7	K17A, R20A, K90V, R91A	○○○	6	3
	K17A, R20A, K90V, R91A; R96A, K98V	○○○	<1	—
β 3– β 4 + β 6– β 7	R29A, R33A, R36A, K90V, R91A	○○○	2	1
β 5– β 6 + β 6– β 7	K77A, K80A, K90V, R91A	●○○	18	8
	Δ 118–124	○○○	<1	—

diethylenetriaminepenta-acetic acid, 20 mM Mops, pH 7.2, 50 mM NaCl, 10 mM DTT, 2 mM EGTA, 3 mM CaCl₂ and 500 μ M [γ -³²P]ATP (40–100 mCi/mmol). Assays were started by the addition of 20 μ l of protein sample and vortex-mixing, and allowed to proceed for 15 min at 30°C. Reactions were stopped by adding 1 ml of freshly prepared chloroform/methanol (1:1, v/v) and 420 μ l of 1 M KCl in 20 mM Mops (pH 7.2). After vortex-mixing 400 μ l of organic phase was transferred into a new tube and 300 μ l of 1 M KCl in 20 mM Mops (pH 7.2) was added. Samples were vortex-mixed again and spun down. Then, 200 μ l of the final organic phase was collected and counted directly for radioactivity.

For cell-based CerK assays, 48 h after transfection, 10⁶ CerK-transfected COS-1 cells, in a 10 cm diameter dish, were incubated at 37°C for 1 h with 10 ml of fresh DMEM (without serum) and 250 μ l of [³²P]P_i (10 mCi/ml). Cells were then scraped and spun down, the supernatants were discarded, and the pellets were washed with 500 μ l of HBSS (Hanks balanced salt solution) supplemented with 10 mM EDTA. After spinning again and

discarding supernatants, pellets were resuspended in 0.3 ml of HBSS/EDTA. Chloroform/methanol/HCl (100:100:1, by vol.; 1 ml) was added and, following vigorous vortex-mixing and subsequent centrifugation, the organic phase was collected. Saponification was performed by the addition of 1 sample volume of chloroform/methanol (2:1, v/v), 0.25 sample volume of water and a 0.25 sample volume of 1 M NaOH in methanol. Samples were vortex-mixed and incubated for 1 h at 37°C. Finally, the samples were spun down and the organic phase was recovered and dried for 20 min at room temperature in a Thermo Electron SPD Speed Vac®. Dried lipid films were dissolved in chloroform and analysed on an HPTLC silica plate (Merck) using chloroform/methanol/acetic acid (13:3:1, by vol.) as mobile phase. Plates were dried, directly exposed to Kodak X-Omat Blue XB-1 films and processed for autoradiography using an Agfa Curix 60 developer.

Protein–lipid overlays

Protein–lipid overlay assays were performed according to published procedures [34] using PIP (phosphatidylinositol phosphate) strips and PIP arrays (Echelon Biosciences). Briefly, membranes were blocked for 1 h at room temperature in blocking buffer [Tris-buffered saline containing 0.1 % Tween 20 (TBST), with 2 mg/ml fatty-acid-free BSA]. Afterwards, membranes were incubated at 4°C overnight with proteins of interest in hybridization buffer (blocking buffer supplemented with protease inhibitor cocktail). Membranes were then washed ten times with TBST and subsequently incubated for 1 h at room temperature, in blocking buffer, with primary antibody [1:2000 dilution for the FLAG M2 antibody (Sigma) and 1:5000 dilution for the anti-GST–HRP (horseradish peroxidase)-coupled antibody (Upstate Biotechnology)]. After ten washing steps in TBST, hybridization with the secondary antibody, when required (i.e. after FLAG M2), was performed in blocking buffer with a 1:5000 dilution of anti-mouse Ig (immunoglobulin) antibody linked to HRP (no. NA9310V; Amersham Biosciences). Finally, membranes were washed as above and processed with LumiGLO (Cell Signaling Technology) for detection.

Western blot analysis

All fractions were diluted 3:1 in 4 × NuPAGE® SDS sample buffer supplemented with 200 mM DTT, and incubated at 75°C for 10 min before snap freezing and storage at –80°C. PAGE analysis was carried out with NuPAGE Bis-Tris 4–12 % run in Mops buffer at 115 V for 2 h. Gels were transferred to Hybond-ECL® (enhanced chemiluminescence) nitrocellulose membranes (Amersham Biosciences) in a Bio-Rad Trans-Blot SD Semi-Dry Transfer cell (25 V and 100 mA per membrane for 2 h) and probed with an anti-GFP antibody (Abcam ab290 rabbit polyclonal) followed by an anti-rabbit Ig antibody linked to HRP (no. NA9340V; Amersham Biosciences), or with an anti-FLAG antibody (Sigma; M2) followed by an anti-mouse Ig antibody linked to HRP (no. NA9310V; Amersham Biosciences). Detection was carried out with ECL® (Amersham Biosciences) or LumiGLO (Cell Signaling Technology) using either Hyperfilms ECL® (Amersham Biosciences) or a Fujifilm Intelligent Dark Box LAS 3000 imager. Band intensities were measured using ImageJ 1.33u (Wayne Rasband, National Institutes of Health; <http://rsb.info.nih.gov/ij>).

Tryptic proteolysis

Trypsin was dissolved in 1 mM HCl to 10 µg/ml. COS-1 cell lysates were mixed 3:1 with trypsin dilutions, to achieve final trypsin concentrations of 0.001, 0.01 and 1 µg/ml. Samples were

incubated for 30 min at 30°C, and then processed for Western blotting as described above.

RESULTS

CerK subcellular localization and extractability

Using a GFP-tagged protein, we previously reported that CerK mostly localizes within perinuclear regions and cytoplasmic vesicles upon expression in COS-1 cells as well as HUVEC (human umbilical-vein endothelial cells) [21]. Since then we extended this observation to a number of other cell types such as A549, SW1353, CHO (Chinese-hamster ovary) and HEK-293 (human embryonic kidney) cells (C. Graf and F. Bornancin, unpublished work). Such a localization has also been reported for endogenous CerK [35]. In Figure 1(A), we used BODIPY®-labelled ceramide in order to label the Golgi complex and this showed that the perinuclear CerK molecules indeed co-localize with this organelle. Moreover, many vesicles where CerK was detected also displayed BODIPY® fluorescence, an indication that they may originate from the Golgi. Some vesicles instead did not, and thus might be associated with distinct subcellular compartments which remain to be determined. Treatment with nocodazole, a substance known to disrupt microtubules, led to redistribution of CerK within the cell (Figure 1B). Furthermore, the directed motility of CerK-loaded vesicles was lost in cells treated with nocodazole; vesicles showed instead random displacement (see Supplementary material, Movies 1 and 2, at <http://www.BiochemJ.org/bj/400/bj4000255add.htm>). Nocodazole also hindered the plasma membrane accumulation of CerK that takes place upon hypo-osmotic challenge (Figure 1B, right panel). In contrast, treatment with cytochalasin D, which blocks actin polymerization, resulting in microfilament breakdown, did not induce relocation of CerK despite clear morphological changes (results not shown). Also, CerK-displaying vesicles were still capable of directed movement following this treatment (see Supplementary material, Movie 3 at <http://www.BiochemJ.org/bj/400/bj4000255add.htm>). Therefore microtubules rather than actin filaments are required for CerK localization and trafficking.

Before CerK was cloned, it was reported that solubilization of a CerK activity from brain membranes required prolonged treatment with a combination of detergent, salt and chelator [36]. Since then, work with recombinant WT CerK has shown that CerK mostly associates with the particulate fraction [1,21]. Furthermore, the complex Ca²⁺–calmodulin was recently reported to be important for regulation of CerK activity [37]. Therefore we tested the effects of a neutral detergent, Triton X-100 (or X-114), a univalent salt, KCl, and a bivalent ion chelator such as EGTA, either alone or in combination, on the extractability of CerK. As shown in Figure 1(C), only upon combination of these reagents could CerK be efficiently released into the supernatant.

Constitutive Golgi/vesicle targeting, requirement for microtubules as well as strong association with the particulate fraction, prompted us to ask for the responsible determinants within the CerK protein.

CerK PH domain: threading, homology modelling and ESP calculations

The 100–120-amino-acid-long PH domain belongs to one of the most abundant domain classes in the human genome [38,39]. Today, more than 60 structures from PH domains of various proteins and from different species are known, which are all characterized through a structurally conserved superfold: a seven-stranded β -sandwich, capped with a C-terminal α -helix [40]. The PH domain of CerK was originally annotated based on a

Figure 2 Model and ESP calculations for human CerK PH domain

(A) Model of CerK PH domain based on TAPP2 (1V5P.A). (B) ESP calculations. The representation is given in colour-coded isosurfaces at a contour level of ± 144 mV. The red surface encompasses all co-ordinates in the vicinity of the protein that exhibit a negatively signed ESP of -144 mV. The blue surface is similar but represents a layer of positive ESPs of $+144$ mV. The protein structure was colour-coded from red (N-terminus) to blue (C-terminus).

not significant database search hit (*E*-value 5.4) to the SMART (simple modular architecture research tool) PH domain HMM (Hidden Markov Model) SM00233 [41]. In order to select a suitable template for homology modelling, we performed a fold recognition (threading) analysis with the putative CerK PH domain against the PDB. Upon visual inspection of the sequence-structure alignments of the highest ranking models, we selected the PH domain structure of TAPP2 [tandem PH-domain-containing protein-2; 1V5P.A (PDB)] to serve as a template for a low-resolution model of the CerK PH domain. This template is the A chain and the best representative conformer of the 1V5P NMR ensemble of 20 structures.

The modelled PH domain of CerK displays the typical PH domain superfold (Figure 2A). Opposite to the helix are three loops interconnecting $\beta 1$ – $\beta 2$, $\beta 5$ – $\beta 6$ and $\beta 6$ – $\beta 7$. Compared with other PH domains these loops are unusual: $\beta 5$ – $\beta 6$ and $\beta 6$ – $\beta 7$ loops are extended and $\beta 6$ – $\beta 7$ features two additional β -strands, $\beta 6'$ and $\beta 6''$. Instead, $\beta 1$ – $\beta 2$ is very short.

Determination of ESP is a valuable tool to predict properties of PH domains such as their ability to bind membranes [43]. As shown in Figure 2(B), CerK PH domain belongs to the subfamily of PH domains with strong molecular polarization. Notably, the extended hairpin-like $\beta 6$ – $\beta 7$ loop largely contributes to the positive isosurface.

We examined in detail the dependence of the function of CerK on the positively charged amino acid residues of its PH domain (Figure 3). Either alone or in combination we substituted arginine and lysine residues for alanine or valine (Table 1) and studied the intracellular localization, activity and biochemical properties of the resulting mutant proteins.

Golgi localization and membrane association are lost in CerK PH domain $\beta 6$ – $\beta 7$ loop mutants

As shown in Figure 4(A), removal of the PH domain prevents CerK from associating with the Golgi complex. We made use

of this clear readout to characterize CerK PH domain mutant proteins. Compared with WT CerK, localization was almost unchanged upon mutagenesis of positively charged residues of either the $\beta 1$ – $\beta 2$ loop (17/20: K17A and R20A), the $\beta 3$ – $\beta 4$ loop (29/33/36: R29A/ R33A/R36A) or the $\beta 5$ – $\beta 6$ loop (68/74/80: K68A/K74A/K80A) of the PH domain. In contrast, mutagenesis of the $\beta 6$ – $\beta 7$ loop impaired the ability of CerK to localize to the Golgi complex, either partially (90/91: K90A,R91A and 96/98: R96A,K98V) or totally when these two double mutations were combined (90/91/96/98). The latter mutant localized in the cell as would Δ PH-CerK. In comparison, a catalytically defective CerK mutant (Kin[−]) mutagenized in the ATP-binding site (G198D) was correctly localized. These results identify the $\beta 6$ – $\beta 7$ loop as the key provider of positively charged amino acids to allow for localization of the WT protein. A minor role for the $\beta 1$ – $\beta 2$ as well as $\beta 3$ – $\beta 4$ loop becomes visible with mutants displaying a partially compromised $\beta 6$ – $\beta 7$ loop (Table 1). Instead, positive charges within the long $\beta 5$ – $\beta 6$ loop do not appear to be involved, since their substitution does not aggravate the phenotype of such partially compromised mutants (Table 1).

Differential fractionation was performed to compare WT and mutant CerK proteins (Figure 4B). Only minute amounts of WT protein could be solubilized in the absence of detergent, whether at low ionic strength (cytosolic fraction) or at high ionic strength (perimembrane fraction). A significant amount of WT CerK was released with the addition of Triton X-100 to the extraction medium (Triton-extractable fraction; compare also lanes 3 and 4 in Figure 1C), whereas most of the WT CerK protein was still present in the final pellet (Triton-insoluble fraction). The CerK Kin[−] mutant protein, which possesses a functional PH domain and localizes as the WT enzyme when fused to GFP (Figure 4A), fractionated very similarly to WT CerK. In particular, it also displayed membrane extractable material (Triton-extractable fraction). In contrast, neither the CerK Δ PH nor the CerK $\beta 6$ – $\beta 7$ loop (90/91/96/98) mutant proteins were recovered from the membrane extractable fraction.

Figure 3 Structure-based multiple sequence alignment of CerK PH domains

CerK PH domain sequences of multiple species are aligned according to a multiple structure alignment of known PH domains. Highly conserved residues are white on black background, conserved negative residues (DE, conserved marker '-'), positive (HKR, '+'), tiny (AGS, 't'), polar (CDEHKNGRST, 'p') are boldface on white background, conserved aliphatic (ILV, 'l'), hydrophobic (ACFGHILMTVWY, 'h'), aromatic (FHWY, 'a') and big (EFHIKLMQRWY, 'b') residues are set on light grey background (italic font for big residues), conserved charged (DEHKR, 'c') residues on dark grey background, and conserved small (DEHKR, 's') residues are shown in grey font [51]. The asterisks (*) denote mutagenized residues of the present study. The secondary structure elements of the final model are shown in the last row (E = strand and H = helix), following the default Chroma consensus string (80%). The unique and conserved $\beta 6$ - $\beta 7$ loop of CerK PH domains from various species is boxed. The structure identifiers denote the four-letter PDB code, the chain (A or B) and the NMR model number (1), if applicable.

This demonstrates that the PH domain is required for CerK to associate with the membrane, and indicates a critical role played by the $\beta 6$ - $\beta 7$ loop in this process. In the absence of a functional PH domain, the amount of cytosolic protein was somewhat increased. Furthermore and surprisingly, PH domain mutant proteins were still largely present in the particulate fraction.

In order to look for possible regulation of CerK localization by PPI (polyphosphoinositides) binding to its PH domain, we used PPI-spotted nitrocellulose membranes and recombinant CerK proteins. Whether expressed *in vitro* as a untagged protein or produced as an N-terminal GST fusion protein and purified,

CerK was found to bind various PPI: PtdIns(3,5) P_2 , PtdIns(3) P , PtdIns(5) P and PtdIns(4) P (Figure 4C, upper panel, left). To ensure the quality of the overlay procedure, several control proteins, of known and varied PPI binding specificities, were tested in parallel (Figure 4C, upper panel, right) [44-46]. The isolated PH domain of CerK displayed a PPI binding selectivity identical with the full-length protein (Figure 4C, lower panel). In addition, both could bind PA (phosphatidic acid). In the absence of PH domain, a dramatic loss of binding to all PPI (and PA) was observed; accordingly, partial mutagenesis of the $\beta 6$ - $\beta 7$ loop (90/91) only had partial impact (Figure 4C, lower panel). These

Figure 4 Subcellular localization, fractionation and PPI binding of CerK PH domain-mutant proteins

(A) COS-1 cells were transiently transfected with plasmids encoding CerK WT and mutant alleles N-terminally fused to GFP. Cells were analysed using fluorescent microscopy 24 h after transfection. Pictures shown are representative of most of the cellular population, observed in several experiments. Golgi/cytoplasm fluorescence ratios were determined as described in the Experimental section, and are given as the means for measurements obtained with at least eight cells. (B) Cell fractionation of WT CerK compared with a CerK allele lacking the PH domain, to the 90/91/96/98 mutant of this study as well as to the kinase-dead CerK G198D mutant (Kin⁻). All constructs were 6 $\times$ His-FLAG-tagged at the C-terminus. Cell fractionation was performed as described in the Experimental section and obtained fractions were resolved by SDS/PAGE followed by Western blotting using an anti-FLAG antibody. (C) Lipid-protein overlays. Upper panel, left: *in vitro* translated and ³⁵S-labelled CerK, or purified GST-CerK, binding to PPI arrays, were assayed as described in the Experimental section, followed by autoradiography or anti-GST Western blotting respectively. Upper panel, right: control GST protein domains (see the Experimental section for full description), probed with anti-GST Western blotting [34]. Lower panel: phospholipid strip overlay analysis using C-terminally FLAG-tagged immunoprecipitated proteins: full-length CerK, or its PH domain only (CerK-PH), or CerK lacking its PH domain (Δ PH-CerK), or the K90V/R91A CerK mutant protein, followed by anti-FLAG Western blotting. On the right, the PH domain of PLC δ 1 was used as a control.

experiments indicate that an intact $\beta 6$ – $\beta 7$ loop is necessary for the PH domain to mediate CerK binding to PPIs.

CerK PH domain $\beta 6$ – $\beta 7$ loop mutants are functionally compromised

CerK activity was measured *in vitro* (Figure 5A). All mutants displaying WT localization (as seen in Figure 4) also displayed WT catalytic activity (29/33/36 and 68/74/80); those with partial loss of localization had lower activity (e.g. 90/91 and 90/98); finally the mutants with the most aberrant localization showed the weakest activity (90/91/96/98). Therefore it seems that loss of localization parallels loss of activity. Cell-based CerK assays were performed to test compromised mutants more directly. Consistent with the *in vitro* assay, the 90/91 mutant showed partial activity, whereas the 90/91/96/98 was totally devoid of activity (Figure 5B).

In order to characterize how the PH domain of CerK impacts activity, we studied the partially compromised 90/91 mutant with regard to substrate affinities. As can be seen in Figure 6(A), affinities of this mutant CerK protein towards either ceramide or ATP were similar to those of the WT protein (WT-CerK: K_m = 205 and 52 μ M; 90/91 CerK mutant: K_m = 194 and 42 μ M for ceramide and ATP respectively), in line with published results [1]. Hence, the PH domain of CerK influences enzymatic activity through a mechanism that does not involve substrates recognition.

The half-life of CerK enzyme activity was reduced 2-fold when comparing the 90/91 mutant with the WT protein (Figure 6B, left). Indeed, 20 min at 30°C decreased the activity of the WT protein by 50%, whereas a similar reduction occurred in less than 10 min for the mutant. Accordingly, the inclusion of glycerol significantly slowed the inactivation process, allowing the mutant to approach WT activity levels (Figure 6B, right). Therefore positively charged residues in the $\beta 6$ – $\beta 7$ loop of the PH domain allow for conformational stabilization of CerK. This

Figure 5 Activity of CerK PH domain mutant proteins

(A) Different mutant alleles of CerK were expressed in COS cells and assayed *in vitro* as crude cell lysates, in comparison with WT CerK. Results shown represent the means for at least three experiments (– S.D.), performed in triplicates. *** indicates statistical significance with $P < 0.001$ (t test). Expression of mutant proteins with a heavily compromised PH domain reached on average approx. 50% of WT CerK levels (see Supplementary Figure 1 at <http://www.BiochemJ.org/bj/400/bj4000255add.htm>). (B) In-cell kinase assays were performed for N-terminally GFP-tagged WT CerK and key mutants, using ^{32}P labelling followed by lipid extraction (densitometric measurements as percentage relative to WT-CerK are indicated underneath).

was also exemplified when looking at the trypsin-sensitivity of the 90/91/96/98 mutant compared with the WT protein. As shown in Figure 6(C), trypsin concentrations as low as 0.001 $\mu\text{g}/\text{ml}$ result in almost complete disappearance of full-length 90/91/96/98 mutant protein, whereas WT CerK remains undegraded when incubated with 1 $\mu\text{g}/\text{ml}$ trypsin for the same period of time.

As seen in Figure 6(D) (left), mutation of amino acids 90 and 91 induces a small downwards mobility shift after SDS/PAGE, not seen with any other mutations including those at residues 96 and 98. However, mutations at residues 96 and 98 synergize with those at residues 90 and 91, giving rise to a large downward shift. This synergy was not observed when combining mutations at residues 90 and 91 with mutations on other loops (results not shown). Therefore a fast running behaviour typifies mutants with a compromised $\beta 6$ – $\beta 7$ loop. *In vitro* translated $\beta 6$ – $\beta 7$ loop mutants showed a smeary pattern, displaying fast running protein material as well as nearly WT-like running material in variable amounts (Figure 6D, right). This indicates that the mobility shifts observed with $\beta 6$ – $\beta 7$ loop mutants are not a mere consequence of the net loss of positive charges due to mutagenesis. Furthermore, the increased mobility could not be accounted for by proteolysis, as an N-terminally GFP-tagged and C-terminally FLAG-tagged 90/91/96/98 mutant protein could be detected by antibodies raised against the two fusion tags (results not shown). We also examined if glycosylation could have been counteracted in the $\beta 6$ – $\beta 7$ mutant proteins, thus leading to the observed fast running species. However, we did not detect any glycosylation in either WT or 90/91/96/98 CerK mutant proteins (results not shown). Therefore these experiments rather show that the $\beta 6$ – $\beta 7$ loop displays important structural features. Mutagenesis of this loop has resulted in changes that are still visible after denaturation, i.e. the mutant protein shows enhanced globularity and thus reduced apparent molecular size. As shown in Figure 6(D) (left), the structural impact of such mutations also gave rise to protein multimers.

DISCUSSION

We made a detailed mutagenesis study of the PH domain of CerK, to identify positively charged residues important for membrane association and Golgi targeting (Figure 1). Modelling the PH domain of CerK and ESP calculations (Figures 2 and 3) indicate that charged amino acid residues are displayed on four loops $\beta 1$ – $\beta 2$, $\beta 3$ – $\beta 4$, $\beta 5$ – $\beta 6$ and $\beta 6$ – $\beta 7$, on one face of the PH domain. According to the obtained model, the $\beta 6$ – $\beta 7$ loop would make a critical contribution to the positive ESP and this was indeed evidenced by the mutagenesis study reported herein.

The $\beta 6$ – $\beta 7$ loop in the PH domain of CerK has conformational impact, affecting both localization (Figure 4) and activity (Figure 5 and 6). There appears to be a direct correlation between the ability of CerK to bind membrane and to be active, since all mutants that are compromised in membrane binding ability also show reduced activity. The converse, however, does not hold true, since an inactive ATP-binding-site mutant (Kin⁻) localizes like the WT enzyme. Therefore catalytic ability is not a prerequisite for membrane binding. We showed that in the absence of a functional PH domain, recovery from a Triton extractable membrane compartment (Figure 4B) and ability to interact with PPI_n (Figure 4C) are lost. Of note, these mutant proteins unable to bind to the Golgi complex and rather showing diffuse cytoplasmic localization are, for a large part, not cytosolic but rather associate with a Triton X-100-resistant compartment (Figure 4B). Neither a CAAX sequence added at the C-terminus of ΔPH -CerK nor the fusion of ΔPH -CerK with the N-terminal 20 residues of CerK itself (containing the myristoylation signal) was able to target the resulting ΔPH -CerK to the plasma membrane (results not shown), further evidencing that ablation of the PH domain does not render the protein merely cytosolic. We checked if the accumulation of the ΔPH -CerK protein into detergent-resistant compartment might have resulted from misfolding and sequestration into aggregates. These are nocodazole-dispersible Triton X-100-insoluble specialized structures formed by the condensation of mini-aggregates [47]. Treatment of cells overexpressing ΔPH -CerK with nocodazole did not have an effect (results not shown); therefore we believe that Triton insolubility of CerK proteins is functionally relevant. It is actually possible that other parts of the CerK protein contribute to its localization and this will deserve further studies.

Partial mutagenesis of the $\beta 6$ – $\beta 7$ loop allowed for studying how it impacts enzyme activity. As shown in Figure 6(A), neither affinity towards ceramide nor that towards ATP was modified in the 90/91 mutant protein. Rather, $\beta 6$ – $\beta 7$ loop mutants were found to be destabilized proteins, with reduced catalytic stability (Figure 6B) and enhanced susceptibility to proteolysis (Figure 6C). Increased running behaviour and multimer formation (Figure 6D) were also hallmarks of CerK proteins with mutagenized $\beta 6$ – $\beta 7$ loops, indicative of structural changes, maybe through increased hydrophobic surfaces. Overall, mutants with a compromised $\beta 6$ – $\beta 7$ loop displayed an unstable conformation which was subject to enhanced deactivation, proteolysis, and aggregation.

Whether PPI_n participate in WT CerK localization, trafficking and activity will require further investigation. Although early studies had spotted binding to PPI_n as characteristic of PH domains, more recent ones have indicated that selective PPI_n binding, instead, is not a frequent theme [43,48]. In fact, only in very few cases is PPI_n binding by PH domains of high affinity and specificity. This class of PH domains can function independently as signal-regulated membrane-targeting modules. CerK, for a large part, appears to be constitutively targeted to the Golgi complex. Furthermore, we previously showed that it can

Figure 6 Biochemical characterization of CerK PH domain mutant proteins

(A) Substrate kinetic studies were performed using C_8 -ceramide and ATP as substrates and a CerK construct harbouring a $6 \times$ His-FLAG tag at the C-terminus. WT and the 90/91 CerK mutant were overexpressed in COS-1 cells and lysates were directly used for the *in vitro* kinase assays. For each kinetic a Lineweaver-Burk plot is shown, which was used for evaluation of K_m values. This is one of two similar experiments, displayed as means $\pm$ S.D. for triplicate evaluations. (●) CerK WT. (○) CerK 90/91. (B) Left panel: whole cell lysates of COS-1 cells overexpressing WT CerK (solid line) or the CerK 90/91 mutant (broken line), C-terminally FLAG- $6 \times$ His-tagged proteins, were pre-incubated at 30°C for the indicated times, before assaying for the remaining activity. Right panel: The presence of 20% glycerol (grey bars) prevents the inactivation of the CerK 90/91 mutant protein. This is one of two experiments with similar results. (C) WT and

mutant CerK, both C-terminally $6 \times$ His-FLAG-tagged, were overexpressed in COS-1 cells and harvested in lysis buffer. Lysates were incubated with different amounts of trypsin (0, 0.001, 0.01 and 1 μg/ml). After a 30 min incubation, samples were processed for PAGE analysis. Western blotting was performed using an anti-FLAG antibody. This is one of two experiments with similar results. (D) Left panel: N-terminally GFP-tagged CerK and mutant alleles were overexpressed in COS-1 cells. SDS/PAGE was performed on lysates followed by Western-blot analysis using an antibody against the GFP tag. The presence of protein multimers is indicated by arrows heads. Right panel: *in vitro* translated [35 S]methionine-labelled CerK WT and mutants were analysed by SDS/PAGE followed by autoradiography.

interact with liposomes devoid of PPIIn [21]. However, plasma membrane localization also takes place and can be triggered by treatments such as hypo-osmotic shock (Figure 1B). Therefore, although clearly beyond the scope of the present work, it will be important to study if any of the PPIIn identified here using lipid-CerK overlays (Figure 4C) turns out to be important for CerK biology.

The $\beta 1$ - $\beta 2$ loop in the CerK PH domain is strikingly short (Figures 2 and 3). Our study actually underscored that its mutagenesis (17/20) has little effect on localization and activity as long as the $\beta 6$ - $\beta 7$ region is conserved (Figures 2 and 4 and Table 1). Nevertheless, a minor role of the $\beta 1$ - $\beta 2$ region became evident when the $\beta 6$ - $\beta 7$ region itself is compromised (Table 1). A similar finding was found for the $\beta 3$ - $\beta 4$ loop (compare mutant 29/33/36/90/91 with mutant 90/91 in Table 1). Therefore all variable loops previously described to impact PH domain behaviour, namely VL1 ($\beta 1$ - $\beta 2$), VL2 ($\beta 3$ - $\beta 4$) and VL3 ($\beta 6$ - $\beta 7$), are also shown to be important to CerK. However, whereas the VL1 loop is most critical to other PH domains, our findings reveal that in CerK, VL3 is playing a major role. According to the model presented in Figure 2, the $\beta 6$ - $\beta 7$ loop of CerK PH domain ranks among the longest $\beta 6$ - $\beta 7$ loops when compared with PH domains of known structure. Of note, this loop is highly charged and would display two additional strands, $\beta 6'$ and $\beta 6''$, linked by a stretch of intercalated positively charged and hydrophobic residues: KRARRHRWKW, conserved in CerK from all species (Figure 3). The presence of tryptophan residues in this region is remarkable. Hydrophobic residues in this motif may serve as an anchor towards the core of the protein to help position the charged residues. Alternatively, they may be used as buffers between charged residues and be critical to penetration of the protein into the lipid bilayer. It will be interesting to study if electrostatic interactions, as well as tryptophan insertion contributed by the $\beta 6$ - $\beta 7$ loop of its PH domain, might be required for CerK membrane binding and interfacial activation, a mechanism that has been described for PLA₂ [49]. This shall also be clarified when the crystal structure of CerK is available.

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