Sphingolipids

Lipid Group Colloquium Organized and Edited by S. Pyne (Department of Physiology and Pharmacology, University of Strathclyde). 668th Meeting held at the University of Glasgow, 7–9 April 1999

Identification of intracellular ceramide target proteins by affinity chromatography and TID-ceramide photoaffinity labelling

M. Wickel*, M. Heinrich*, T. Weber†, J. Brunner†, M. Krönke* and S. Schütze*

*Institute of Immunology, University of Kiel, Michaelisstr. 5, D24105 Kiel, Germany and †Department of Biochemistry, Swiss Federal Institute of Technology, Zurich (ETHZ), Univeritasstr. 16, CH D092 Zürich, Switzerland

Introduction

Lipids are not merely structural components of membranes but also have distinct functional roles in cellular signalling. Importantly, they are precursors of second messengers that are generated in cells after stimulation by various exogenous stimuli including cytokines such as tumour necrosis factor (TNF), interferon, interleukin 1 nerve growth factor, or CD28 and CD95 (Fas/APO-1) receptor triggering, ionizing radiation, glucocorticoids, anti-cancer drugs and serum deprivation (reviewed in [1–3]). The biological responses to ceramide range from the induction of proliferation and differentiation to cell-cycle arrest. The most prominent but controversial role of ceramide is the induction of apoptosis in various cell types (reviewed in [4–6]). A current view of the pleiotropic effects of ceramide in p55 TNF receptor (TR55) signal transduction is summarized in Figure 1. The production of ceramide is mediated either by synthesis de novo involving ceramide synthetase located in the Golgi or by hydrolysis of sphingomyelin-engaging sphingomyelinases (SMases). Ligand binding to the TR55, the interleukin 1 receptor 1 and the Fas receptor results in the activation of two SMases, a plasma-membrane-bound N-SMase as well as an endolysosomal A-SMase [7–10]. Each type of SMase generates the second messenger ceramide but with different kinetics and, most importantly, at different intracellular locations. A cytosolic neutral SMase of unknown function has been identified [11]. Specific TR55 domains link to the N-SMase and the A-SMase and to diverse signalling pathways [8]. N-SMase activation is coupled to a neutral SMase activation domain (NSD) via the adaptor protein FAN [12]. The domain of TR55 activating A-SMase corresponds to the death domain that binds the adaptor protein TRADD, which in turn recruits a further protein, FADD, to activate caspase 8 and A-SMase [13]. Fibroblasts derived from FADD-deficient mice are defective in TNF-induced A-SMase activation [14]. Notably, the apoptotic caspase 8 seems not to be involved in the A-SMase activation pathway [13]. The signalling cascade downstream of ceramide might, however, involve caspase 3 (CPP32) [15]. A role for A-SMase in transmitting apoptotic signals in response to Fas/CD95, γ-irradiation, TNF and lipopolysaccharide has been suggested [10,16–20]. However, direct evidence linking ceramide signalling to specific effector elements of the apoptotic response remains to be provided.

To obtain more detailed insights into ceramide action, it is important to identify intracellular target proteins that interact directly with ceramide. As a tool for investigating direct lipid-protein interactions, carbene-generating analogues of lipid second messengers have been used as photoaffinity probes [21,22]. For example, the lysosphatidic acid receptor was identified with the use of 32P-labelled 3-trifluoromethyl-3-(m-iodophenyl)diazirine (TID)-lysosphatidic acid [23], and the binding of ceramide to the protein kinase Raf-1 was demonstrated by employing 125I-TID-ceramide [24]. We are currently using a combination of ceramide affinity chromatography

Abbreviations used: CAPK, ceramide-activated protein kinase; CBP97, 97 kDa ceramide-binding protein; CPP32, caspase 3; CTSD, cathepsin D; MBP, myelin basic protein; TID, 3-trifluoromethyl-3-(m-iodophenyl)diazirine; SMase, sphingomyelinase; THF, tetrahydrofuran; TNF, tumour necrosis factor; TR55; p55 TNF receptor.

1Present address: Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, Box 251, New York NY–10021, U.S.A.
2To whom correspondence should be addressed.
Figure 1

Pleiotropic function of ceramide in TR55 signal transduction

Details are given in the text. Abbreviations: AP-1, activator protein 1; AP24, 24-kDa apoptotic protease; DD, death domain; CAPP, ceramide-activated protein phosphatase 2A; ERK, extracellular signal-regulated protein kinase; MAP, mitogen-activated protein; MEK, MAP kinase/ERK kinase; MEKK1, mitogen-activated protein kinase kinase-1; PKC, protein kinase C; PLA2, phospholipase A2; TAK1, transforming growth factor β-activating kinase.

and photo-cross-linking employing 118I-TID-ceramide (Figure 2) to identify intracellular target proteins that associate directly with ceramide.

Experimental

Ceramide-affinity chromatography

Generation of the affinity matrix
Activated CH-Sepharose 4B (4 g) (Pharmacia Biotech) was resuspended in 1 mM HCl and subsequently transferred to 30%, 70% and 100% tetrahydrofuran (THF). n-erythro-Sphingosine (50 mg) (Sigma) in 6 ml of THF was coupled to this matrix by the addition of 100 µl of N-ethylmorpholine. After incubation for 15 h at 4 °C, the beads were washed in THF and residual active sites were blocked by ethanolamine (2 h at room temperature). The affinity matrix was finally washed three times in THF and three times in water.

Affinity chromatography of cytosolic and membrane proteins
U937 cells (2 x 10^7) were homogenized in 1 ml of buffer H [40 mM Hepes (pH 7.4)/150 mM KCl/5 mM NaF/1 mM PMSF/20 µM pepstatin/20 µM leupeptin/20 µM antipain] by passing the cells through a 28-gauge needle followed by sonication three times (10 s each). Lysates were cleared by centrifugation (5 min, 1000 g); the supernatant was centrifuged for 1 h at 100000 g. The supernant containing cytosolic proteins was saved and the pellet was resuspended in buffer H and adjusted to 0.075% (v/v) Triton X-100. Membrane proteins were solubilized by stirring for 30 min. After centrifugation for 1 h at 100000 g, membrane protein was collected from the supernatants. Cytosolic and membrane proteins were incubated overnight with ceramide-Sepharose at 4 °C. The affinity matrix was washed in buffer H containing 0.075% Triton X-100 to remove un-
bound protein, and the proteins bound to ceramide–Sepharose were eluted with 100 μM ceramide in buffer H containing 0.075% Triton X-100. The eluates were subsequently assayed for TID-ceramide binding as described below.

**125I-TID-ceramide labelling of cytosolic and membrane proteins**

TID-ceramide was synthesized and radiolabelled as described [22]. For labelling of intracellular ceramide-binding proteins, 5 μg of protein was incubated with 1 μCi of 125I-TID-ceramide in 20 μl at 37°C for 5 min. Cross-linking was performed by irradiation with UV for 2 min at 365 nm and 100 W.

After photolysis, the resulting protein/TID-ceramide complexes were analysed by SDS/PAGE or two-dimensional gel electrophoresis followed by autoradiography.

**Kinase assay in vitro**

Lysate protein (10 μg) or affinity-purified protein (2 μg) was incubated with 5 nmol of [γ-32P]ATP (1 μCi; 0.1 mCi) in buffer containing 10 mM MgCl₂, 5 mM NaF and 30 mM Hepes, pH 7.4, for 30 min at 37°C.

**Two-dimensional gel electrophoresis**

Two-dimensional gel electrophoresis was performed by the method of O’Farrell [25] and Hochstrasser et al. [26], with minor modifications.

**Preparation of protein samples**

Proteins precipitated with trichloroacetic acid were redissolved in isoelectric focusing sample buffer [54% (w/v) urea/4% (w/v) 3-[3-(cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulphonic acid (CHAPS)/1% (w/v) dithiothreitol/5% (v/v) Ampholine for the indicated pH range (Pharmacia, Freiburg, Germany)]. After 2 h of gentle shaking at 37°C, the samples were centrifuged at 20000 g for 5 min; the supernatant was loaded on isoelectric focusing gels. The 20 cm isoelectric focusing tube gels contained 5% (w/v) urea, 1.2% (v/v) polyacrylamide (37.5:1), 3% (w/v) CHAPS, 1% (v/v) Nonidet P40 and 5% (v/v) Ampholine for the indicated pH range. For polymerization, 0.1% (v/v) N,N,N',N'-tetramethylethylenediamine and 0.2% (v/v) ammonium persulphate (10%) were added.

**Isoelectric focusing**

The anodolyte was 6 mM H₃PO₄; the cathodolyte was 20 mM NaOH. The polymerized gels were prefocused with isoelectric focusing sample buffer for 15 min at 200 V, 15 min at 300 V and 15 min at 400 V. After prefocusing, the protein samples were loaded and overlaid with 10 μl of 8 M urea,

**Figure 2**

**Detection of ceramide binding proteins by ceramide affinity chromatography and 125I-TID-ceramide photoaffinity labelling**

The structures of natural ceramide, the cross-linking derivative 125I-TID-ceramide and Sepharose-coupled ceramide are shown.
and isoelectric focusing was performed for 18 h at 400 V.

Second dimension

After focusing, the gels were equilibrated for 20 min in an equilibration buffer containing 120 mM Tris/HCl, pH 6.8, 0.1 % SDS and 5 % (v/v) 2-mercaptoethanol. The equilibrated gels were immediately applied to an SDS/polyacrylamide gel and fixed with equilibration buffer containing 1 % (w/v) agarose.

SDS/PAGE was performed by the method of Laemmli. 131I-TID-ceramide-labelled protein was detected by autoradiography of the dried gels.

Results

To screen for intracellular ceramide-binding, we generated a ceramide affinity matrix by coupling sphingosine via an aminohexanoic acid spacer to Sepharose 4B, resulting in an immobilized short-acyl-chain ceramide, leaving its alkyl chain exposed to interact with a hydrophobic cavity of a signalling protein (Figure 2). The alkyl chain in ceramide appears essentially to be involved in binding to proteins ([27], and M. Heinrich and S. Schütze, unpublished work). Incubation of cytosolic or membrane proteins from U937 cells with the ceramide–Sepharose affinity matrix followed by elution of the bound protein by an excess of ceramide resulted in the recovery of approx. 10% of the original protein applied to the matrix. Several proteins seemed to be selectively enriched in the affinity eluates compared with the total proteins before affinity chromatography as revealed by silver staining of the proteins after separation by SDS/PAGE (results not shown). To confirm the ceramide-binding characteristics of the proteins that were eluted from the affinity matrix, we subsequently cross-linked the eluted proteins with 131I-TID-ceramide. As shown in Figure 3(A), cytosolic proteins with molecular masses of 52, and 66 and 97 kDa and membrane proteins with molecular masses of 35, 52 and 74 kDa were predominantly labelled. On the basis of the similarity in molecular mass, the 97 kDa ceramide-binding protein (CBP97) might be identical with the ceramide-activated protein kinase [28]. To characterize CBP97 further, the respective band was pooled from 20 preparations

![Figure 3](image-url)

**Figure 3**

Ceramide-binding proteins in eluates from affinity chromatography

(A) Detection of ceramide-binding proteins in eluates from affinity chromatography. Cytosolic and detergent-soluble membrane proteins were prepared and subjected to ceramide–Sepharose affinity chromatography as described in the Experimental section. Ceramide-binding proteins were eluted from the affinity matrix with 100 μM ceramide and subjected to photolysis with UV in the presence of 1 μCi of 131I-TID-ceramide. Proteins were separated by SDS/PAGE [10% (w/v) gel] and radiolabelled ceramide–protein complexes were detected by autoradiography. The major ceramide-binding proteins are indicated, with their molecular masses in kDa, by arrowheads. (B) Phosphorylation of total lysates or affinity-purified 97 kDa protein in vitro. Total lysates from U937 cells or affinity-purified 97 kDa protein were incubated with [γ-32P]ATP in the absence (left panel: autophosphorylation) or presence (right panel) of MBP. Phosphorylated proteins were separated by SDS/PAGE [10% and 15% (w/v) gel] and analysed by autoradiography. Molecular masses are indicated in kDa alongside arrowheads.
Sphingolipids and re-electrophoresed on two-dimensional gels. The resulting purified 97 kDa protein appeared as a single Coomassie Blue-stained spot with an isoelectric point of 5.2 (results not shown). Tryptic digestion of CBP97 revealed a peptide pattern that is distinct from known proteins as revealed by protein database searches (P. James, personal communication). To obtain functional data on CBP97, kinase assays were performed in vitro. As shown in Figure 3(B), CBP97 was autophosphorylated. In addition, CBP97 is able to phosphorylate myelin basic protein (MBP) added as an exogenous substrate to the purified CBP97. These results suggest that CBP97 might be a protein kinase. The sequencing of purified CBP97 protein will provide us with further information on the CBP97 gene and its function within the ceramide signalling pathway.

To analyse other ceramide-binding proteins detected by TID-ceramide labelling (Figure 3A), we next performed two-dimensional gel electrophoresis of the cytosolic and membrane proteins. As demonstrated in Figure 4, the 35, 52, 66 and 74 kDa and also a higher-molecular-mass complex of 220 kDa are acidic proteins with isoelectric points between 5.7 and 5.1. CBP97 was missing from this experiment but was present in other experiments. The 220 kDa complex in Figure 4(A) might represent a multimer of the 97 kDa protein. Notably, the 35, 52 and 66 kDa proteins are found in both the cytosolic and membrane fractions of U937 cells, whereas the 74 and 97 kDa ceramide-binding proteins are selective for the membrane and cytosolic fractions respectively. It is unclear at present whether under certain conditions a redistribution of the ceramide binding proteins might occur.

We are at present trying to identify and characterize functionally the ceramide-binding proteins detected by combined ceramide affinity chromatography and 125I-TID-ceramide labelling. Because ceramide is a potent inducer of programmed cell death, we investigated whether apoptotic proteases of the caspase family might be a candidate. In particular, we examined whether CPP32, which has been described as being activated in cells treated with exogenous ceramide [19,29,30], could be identical with the 35 kDa ceramide-binding protein shown in Figures 3 and 4. However, a Western blot analysis of the ceramide-affinity eluates employing antibodies against various caspases revealed that the 35 kDa protein is not identical with CPP32. Screening for other known proteins with similar molecular masses to those of the TID-ceramide-binding proteins resulted in the identification of the aspartate protease cathepsin D (CTSD) as a ceramide-binding protein (M. Heinrich, M. Wickel, W. Schneider-Brachert, C. Sandberg, J. Gahr, R. Schwandner, T. Weber, J. Brunner, M. Krönke and S. Schütze, unpublished work). The prepro-CTSD isoform has a molecular mass of 52 kDa, the pro-CTSD 48 kDa and the mature CTSD 32 kDa [31]. All three isoforms could be detected by immunoblotting in the affinity lysates and could be additionally labelled with 125I-TID-ceramide

---

**Figure 4**

Two-dimensional analysis of TID-ceramide-binding proteins

Cytosolic (A) and detergent-soluble (B) membrane proteins from U937 cells were affinity-purified by ceramide-Sepharose, photo-cross-linked with 125I-TID-ceramide and subjected to two-dimensional gel electrophoresis as described in the Experimental section. Radiolabelled ceramide/protein complexes are indicated, with their molecular masses in kDa, by arrowheads. Abbreviation: IEF, isoelectric focusing.
after immunoprecipitation from U937 lysates by using anti-(CTSD) antibodies (results not shown).

**Discussion**

An understanding of the role of ceramide in cell signalling crucially requires the identification of direct targets of ceramide. Several enzymes have been described recently that can be activated by ceramide (summarized in Figure 1), including a ceramide-activated protein kinase (CAPK) [28] that has been suggested to be related to the kinase suppressor of Ras (KSR) [32]. Further ceramide-responsive proteins include: ceramide-activated protein phosphatase 2A (CAPP) [33], protein kinase C [34], protein kinase Raf-1 [35,36], the stress-activated/c-Jun N-terminal protein kinase (JNK) [37–39] and a CPP32-like apoptotic protease [15,19,29,30,40,41]. It should be emphasized that most of the reported ceramide responses are apparently indirect, probably involving unknown intermediates. Evidence for a direct physical interaction of ceramide has been difficult to obtain. In particular, specificity of ceramide binding and activation of the target proteins have not been demonstrated with competitive lipids or ceramide isomers [34,35].

In the present study we sought specific ceramide targets in lysates from U937 cells. Ceramide-affinity chromatography followed by photo-cross-linking of the eluted proteins with the radio-labelled ceramide analogue 115 I-TID-ceramide revealed the predominant labelling of cytosolic proteins with molecular masses of 52, 66 and 97 kDa and of membrane proteins with molecular masses of 35, 52 and 74 kDa. On the basis of the similarity in molecular mass and its characteristics with regard to MBP-directed substrate phosphorylation, CBP97 could be identical with CAPK, which seems to be functionally related to the plasma-membrane-bound neutral SMase [28]. Cloning of the cDNA encoding for CBP97 will provide further information on the functional role of this ceramide-binding protein in intracellular signalling.

As a second ceramide target, the 35, 48 and 52 kDa ceramide-binding proteins could be identical with the different isoforms of the aspartate protease CTSD. Evidence will be provided elsewhere for specific interaction of ceramide with CTSD, leading to enhanced autoprocessing and enzymic activity (M. Heinrich, M. Wickel, W. Schneider-Brachert, C. Sandberg, J. Gahr, R. Schwandner, T. Weber, J. Brunner, M. Krönke and S. Schütze, unpublished work). CTSD is endosomally active and the first ceramide target that co-localized with acidic SMase. CTSD has been implicated in mediating apoptosis in response to TNF, interferon γ, CD95 [42], chemotherapeutic agents such as etoposide and adriamycin [43], and serum deprivation [44,45]. CTSD might link A-SMase to the secretory pathway and to apoptotic signalling events upstream of CPP32.

This study was supported by a grant from the Deutsche Forschungsgemeinschaft (SFB 415)

Abbreviations used: CAP kinase, ceramide-activated protein kinase; PP, protein phosphatase; TNF, tumour necrosis factor.

Ceramide and apoptosis

D. K. Perry

Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, SC 29425, U.S.A.

Introduction

The role of the sphingolipid ceramide in apoptosis has been the subject of considerable, often controversial, interest. Much of the controversy seems to centre on the tendency to place the role of ceramide in one of two diametric positions: being a requirement for apoptosis or being produced as a consequence of apoptosis. Reports have been published arguing for both of these positions. Certainly much remains to be learned of the role of ceramide in apoptosis, and new tools need to be developed. Nevertheless, sufficient data now exist to support the position that ceramide does seem to function in a necessary manner in apoptosis under some conditions. However, it also seems likely that ceramide often has a more complex role in this process. Its specific involvement in apoptosis varies in a tissue-specific manner and is dependent not only on the signal that is inducing the stress but also on the downstream effectors of ceramide action. Here I summarize briefly both the results supporting the role of ceramide in apoptosis and the results that argue strongly against its production as a consequence of apoptosis.

Generation of ceramide during apoptosis

There is widespread agreement over the observation that ceramide is generated in cells undergoing apoptosis. The agents reported to induce ceramide in apoptosis include receptor-mediated transducers such as TNF (tumour necrosis factor) and the Fas ligand, environmental insults such as UV radiation and ionizing radiation, and cytotoxic chemicals such as staurosporine and chemotherapy drugs. The generation of ceramide under these conditions has been documented by several

---

Received 22 March 1999