Published in final edited form as:

Proteomics. 2012 July ; 12(13): . doi:10.1002/pmic.201100527.

Identification of C₆-ceramide-interacting proteins in D6P2T Schwannoma cells

Venkatesh Kota, Zdzislaw M. Szulc, and Hiroko Hama

Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, SC 29425, USA

Abstract

Ceramide is a bioactive molecule involved in numerous cell signaling pathways that are associated with cell cycle control, differentiation, senescence and apoptosis. Although substantial knowledge about ceramide-regulated pathways has accumulated in the past decade, molecular mechanisms of ceramide action remain poorly understood, primarily due to limited information about ceramide-binding proteins. In the present study, we used affinity purification with a synthetic biotin-conjugated C₆-ceramide analogue and LC-MS/MS to identify potential ceramide-interacting proteins in D6P2T Schwannoma cells. The purification resulted in identification of 97 unique proteins. The identified proteins are involved in various cellular processes, including apoptosis, cellular stress, cell cycle, cell differentiation, signaling, transcription, translation, protein biogenesis, metabolism and transport.

Ceramide belongs to a family of biologically active lipid molecule and is comprised of a long-chain sphingoid base and an *N*-acyl chain. Ceramide is involved in signal transduction mechanisms that control the cell cycle, as well as differentiation and apoptosis [1–3]. The signaling and biological responses of ceramide depend upon their subcellular localization, intracellular lipid traffic, and the rate of flux through the network of sphingoid metabolic pathways [3]. Cellular ceramide can change in response to various stimuli, including physiological elements such as growth factors, hormones, and cytokines, as well as xenobiotics such as chemotherapeutic agents. Ceramide is a common intermediary metabolite in several metabolic pathways, and its concentration is controlled by many enzymes involved in *de novo* synthesis or hydrolysis of ceramide, or conversion of ceramide to complex sphingolipids [4–9].

Numerous studies showed that an increase in ceramide was associated with programmed cell death [1, 3, 10]. Similarly, treatment with proapoptotic agents induced accumulation of ceramide, which implicated this lipid in cellular responses to these agents [7]. For instance, several anticancer agents such as daunorubicin, camptothecin, fludarabine, and etoposide stimulate ceramide synthesis in cancerous cells [11]. Because of its apoptosis-inducing effects in cancer cells, ceramide has been termed the “tumor suppressor lipid” [12].

In the last decade ceramide has been extensively studied as potential chemopreventive molecules because they are intimately involved in the regulation of cancer cell growth, differentiation, senescence, and cell death [1, 7, 13]. In many studies, cell-permeable ceramide analogues (C₂- or C₆-ceramide) were shown to have activity against a variety of cancer cell lines. In addition, blocking ceramide clearance by inhibiting specific enzymes can elevate endogenous ceramide, leading to increased cytotoxic effects of chemotherapeutic agents in various cancer cells [1]. Ceramide treatment can also limit tumor growth *in vivo* as shown in a mouse model of breast adenocarcinoma [13]. In many of these studies, however, the direct targets of ceramide and downstream pathways have yet to be established. Currently, a limited number of proteins are known to directly interact with

ceramide, including c-Raf [14], kinase suppressor of RAS (KSR) [15], cathepsin D [16, 17], protein kinase C (PKC- α) [18], ceramide transfer protein CERT [19] and inhibitor 2 of protein phosphatase 2A (I2PP2A) [20]. It is imperative to identify all ceramide-interacting proteins to fully delineate ceramide-mediated signaling pathways and to design ceramide-based therapeutics in cancer treatment. To this end, we have utilized affinity purification with a synthetic biotin-conjugated ceramide analogue and LC-MS/MS to identify potential ceramide-interacting proteins in D6P2T Schwannoma cells. The proteins we identified are involved in apoptosis, cellular stress, cell cycle, cell differentiation, signaling, transcription, translation, protein biogenesis, metabolism and transport.

Rat Schwannoma-derived D6P2T cells were purchased from ATCC (Manassas, VA) and grown in DMEM containing 10% FBS. Biotin-conjugated *D-erythro*-C₆-ceramide analogue (B-C₆-Cer) was prepared by the method of Ong and Brady [21]. Briefly, *D-erythro*-sphingosine (Avanti Polar Lipids) was condensed with *N*-hydroxysuccinimide ester of (+)-biotin (Sigma-Aldrich) in anhydrous dimethylformamide at room temperature for 24 hr. The pure (+)-2-*N*-biotinyl-*D-erythro*-sphingosine was obtained at a yield of 72% as a white microcrystalline powder after silica gel column purification following recrystallization from acetone. The following characteristics were obtained: TLC R_f (CHCl₃-CH₃OH, 50:8, v/v) = 0.45; [α]²³_D = +41° (c = 0.25, CHCl₃-CH₃OH, 9:1, v/v); [α]²²₃₆₅ = +124° (c = 0.25, CHCl₃-CH₃OH, 9:1, v/v).

For affinity purification, cells were harvested at ~60–70% confluency. B-C₆-Cer-interacting proteins were isolated using the Dynabeads® Streptavidin Kit (Invitrogen). Approximately 2 × 10⁶ cells were harvested by trypsin-EDTA treatment, washed with PBS, and disrupted in a lysis buffer containing 10 mM Tris-HCl (pH 8.0); 150 mM NaCl; 1 mM EDTA; 0.1% SDS; 1% sodium deoxycholate; 1% Triton X-100; and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) for 1 hr at room temperature. To exclude non-specific interaction with biotin or the beads, cell lysates were first incubated with biotin-bound beads on an orbital shaker for 1 hr at RT, and bound proteins were discarded. Subsequently, samples were incubated with B-C₆-Cer beads for 1 hr at RT on an orbital shaker. The beads were washed with lysis buffer 5 times and boiled in Laemmli buffer. Proteins bound to B-C₆-Cer beads were resolved on 12% SDS PAGE and stained with Imperial Protein Stain (Thermo Fisher, Rockford, IL). Control experiments were performed in parallel with biotin-bound beads. The proteins were resolved in molecular weight range of 20–150 kDa (Fig. 1). Each lane was cut into pieces and processed for LC-MS/MS analysis.

Gel pieces were placed in Eppendorf tubes and washed with 50 mM ammonium bicarbonate for 10 min, and destained twice with 25 mM ammonium bicarbonate in 50% acetonitrile for 15 min. The gel pieces were dehydrated by immersion in 100% acetonitrile for 15 min and vacuum-dried in a SpeedVac. Dried gel pieces were treated with proteomics grade trypsin (Sigma-Aldrich) at 37 °C overnight. The samples were briefly spun, and supernatants were transferred to clean dry Eppendorf tubes. Peptides were further extracted from the gel once with 25 mM ammonium bicarbonate for 20 min, followed by three washes with 5% formic acid in 50% acetonitrile for 20 min. Pooled supernatants were dried in a SpeedVac to ~2 μL. Prior to LC-MS/MS analysis the samples were reconstituted with 10 μL of 0.2% formic acid in 2% acetonitrile.

Trypsin-digested samples were analyzed by LC-MS/MS. A linear ion trap mass spectrometer (LTQ, Thermo Finnigan) was interfaced with an LC Packings Nano LC system with a 75-μm i.d. C-18 reversed-phase LC column (Microtech Scientific). Peptides were fractionated by a gradient of 2–60% acetonitrile in 0.2% formic acid. Data Dependant Analysis was utilized on the LTQ to perform MS/MS on all ions above an ion count of

1000. Dynamic Exclusion was set to exclude ions from MS/MS selection for 3 min after being selected 2 times in a 30-sec window.

The MS/MS data were searched against the NCBI Rat Genome Database using the Thermo Finnigan Bioworks 3.3.1 SP1 software. Variable modifications of methionine oxidation were considered. Protein identification must meet the minimum criteria of a Protein Probability of 1.0 E-3 or better and have an Xcorr vs charge state >1.5, 2.0, 2.5 for +1, +2, and +3 ions, respectively, with at least 3 unique peptides matching the protein, and a good match for at least 4 consecutive y or b ion series from the MS/MS spectra. Non-specific proteins identified in control samples were excluded from the list of identified proteins.

Overall, 97 unique proteins were identified in this study (Table 1, Supplemental Table S1). The detailed peptide data for identified proteins are provided as Supplemental Table S2. All of the available proteomic data are deposited in the PRIDE proteomics identification database (accession numbers 19308–19311). The identified proteins were annotated using the Gene Ontology Slimmer tool on the AmiGO Gene Ontology database (<http://amigo.geneontology.org>). The detailed distribution of proteins based on biological process, cellular component and molecular function are depicted in Fig. 2. The complete lists of proteins under each classification are provided in Supplemental Table S1.

The cellular locations and functions of the 97 proteins are diverse. As expected, there are proteins involved in apoptosis and cell cycle control. The largest cluster of proteins, however, is involved in gene expression and protein synthesis, including ribosomal proteins, tRNA synthetases, and eukaryotic translation initiation factors. For example, two subunits each of eukaryotic initiation factor 3 and eukaryotic initiation factor 4 were identified in this study. These proteins may participate in the previously reported inhibition of protein synthesis by ceramide [22]. Another large cluster of proteins is involved in metabolic processes, suggesting a possibility that ceramide regulates metabolic rates during cell proliferation, apoptosis, and stress response. There are several proteins involved in protein folding, suggesting a novel role for ceramide in this process. It should be noted that not all 97 proteins would directly bind B-C6-Cer. Some of them would indirectly interact with B-C6-Cer through complexation with other proteins. Further studies are warranted to refine this list to identify direct targets of ceramide.

Interestingly, known ceramide-binding proteins (c-Raf, KSR, cathepsin D, PKC- δ , CERT, I2PP2A) were not identified in this study, indicating that these proteins do not interact with B-C6-Cer under our experimental conditions. It is possible that the acyl chain lengths and lipid environment were not optimal for these proteins, and/or biotin moiety interferes with the binding. Using alternative conditions and different ceramide analogues would likely identify additional ceramide-interacting proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

All the available proteomic data set are deposited in the PRIDE proteomics identification database (accession numbers 19308–19311). This work was supported by National Institutes of Health grant NS060807 (to HH).

References

1. Ogretmen B, Hannun YA. Biologically active sphingolipids in cancer pathogenesis and treatment. *Nat Rev Cancer*. 2004; 4:604–616. [PubMed: 15286740]

2. Struckhoff AP, Bittman R, Burow ME, Clejan S, et al. Novel ceramide analogs as potential chemotherapeutic agents in breast cancer. *J Pharm Exp Ther.* 2004; 309:523–532.
3. Hannun YA, Obeid LM. Principles of bioactive lipid signalling: lessons from sphingolipids. *Nat Rev Mol Cell Biol.* 2008; 9:139–150. [PubMed: 18216770]
4. Chalfant CE, Rathman K, Pinkerman RL, Wood RE, et al. De novo ceramide regulates the alternative splicing of caspase 9 and Bcl-x in A549 lung adenocarcinoma cells. *J Biol Chem.* 2002; 277:12587–12595. [PubMed: 11801602]
5. Perry DK, Carton J, Shah AK, Meredith F, et al. Serine palmitoyltransferase regulates de novo ceramide generation during etoposide-induced apoptosis. *J Biol Chem.* 2000; 275:9078–9084. [PubMed: 10722759]
6. Lucci A, Han TY, Liu YY, Giuliano AE, Cabot MC. Modification of ceramide metabolism increases cancer cell sensitivity to cytotoxics. *Int J Oncol.* 1999; 15:541–546. [PubMed: 10427137]
7. Radin NS. Killing tumours by ceramide-induced apoptosis: a critique of available drugs. *Biochem J.* 2003; 371:243–256. [PubMed: 12558497]
8. Senchenkov A, Litvak DA, Cabot MC. Targeting ceramide metabolism--a strategy for overcoming drug resistance. *J Natl Cancer Inst.* 2001; 93:347–357. [PubMed: 11238696]
9. Strum JC, Small GW, Pauig SB, Daniel LW. 1-beta-D-Arabinofuranosylcytosine stimulates ceramide and diglyceride formation in HL-60 cells. *J Biol Chem.* 1994; 269:15493–15497. [PubMed: 8195192]
10. Lemonnier LA, Dillehay DL, Vespremi MJ, Abrams J, et al. Sphingomyelin in the suppression of colon tumors: prevention versus intervention. *Arch Biochem Biophys.* 2003; 419:129–138. [PubMed: 14592456]
11. Huang WC, Chen CL, Lin YS, Lin CF. Apoptotic sphingolipid ceramide in cancer therapy. *J Lipids.* 2011; 2011:565316. [PubMed: 21490804]
12. Hannun YA. Sphingolipid second messengers: tumor suppressor lipids. *Adv Exp Med Biol.* 1997; 400A:305–312. [PubMed: 9547572]
13. Stover TC, Sharma A, Robertson GP, Kester M. Systemic delivery of liposomal short-chain ceramide limits solid tumor growth in murine models of breast adenocarcinoma. *Clin Cancer Res.* 2005; 11:3465–3474. [PubMed: 15867249]
14. Huwiler A, Brunner J, Hummel R, Vervoordeldonk M, et al. Ceramide-binding and activation defines protein kinase c-Raf as a ceramide-activated protein kinase. *Proc Natl Acad Sci USA.* 1996; 93:6959–6963. [PubMed: 8692926]
15. Zhang Y, Yao B, Delikat S, Bayoumy S, et al. Kinase suppressor of Ras is ceramide-activated protein kinase. *Cell.* 1997; 89:63–72. [PubMed: 9094715]
16. Abbott DE, Margaryan NV, Jeruss J, Khan S, et al. Reevaluating Cathepsin D as a biomarker for breast cancer: Serum activity levels versus histopathology. *Cancer Biol Ther.* 2010; 9:23–30. [PubMed: 19923884]
17. Deiss LP, Galinka H, Berissi H, Cohen O, Kimchi A. Cathepsin D protease mediates programmed cell death induced by interferon-gamma, Fas/APO-1 and TNF-alpha. *EMBO J.* 1996; 15:3861–3870. [PubMed: 8670891]
18. Bourbon NA, Yun J, Kester M. Ceramide directly activates protein kinase C zeta to regulate a stress-activated protein kinase signaling complex. *J Biol Chem.* 2000; 275:35617–35623. [PubMed: 10962008]
19. Hanada K, Kumagai K, Yasuda S, Miura Y, et al. Molecular machinery for non-vesicular trafficking of ceramide. *Nature.* 2003; 426:803–809. [PubMed: 14685229]
20. Mukhopadhyay A, Saddoughi SA, Song P, Sultan I, et al. Direct interaction between the inhibitor 2 and ceramide via sphingolipid-protein binding is involved in the regulation of protein phosphatase 2A activity and signaling. *FASEB J.* 2009; 23:751–763. [PubMed: 19028839]
21. Ong DE, Brady RN. Synthesis of ceramides using N -hydroxysuccinimide esters. *J Lipid Res.* 1972; 13:819–822. [PubMed: 4641422]
22. Ruvolo PP, Gao F, Blalock WL, Deng X, May WS. Ceramide regulates protein synthesis by a novel mechanism involving the cellular PKR activator RAX. *J Biol Chem.* 2001; 276:11754–11758. [PubMed: 11148216]

Fig. 1. Separation of biotin C₆-ceramide-interacting proteins in D6P2T cells by SDS PAGE. The gel was stained with Imperial Protein Stain and bands were excised and subjected to LC-MS/MS analyses. The control sample was obtained with biotin-bound beads.

Fig. 2. Gene Ontology annotations of identified biotin C₆-ceramide interacting proteins in D6P2T cells. Gene Ontology analysis was carried out using the GO Slimmer tool in the AmiGO Gene Ontology database. The 97 proteins were categorized based on biological processes (A), molecular function (B), or subcellular localization (C).

TABLE 1

B-C₆-Cer interacting protein in D6P2T Schwann cells identified by LC-MS/MS

S. No	Protein	Accession No. ^{a)}
1	Adaptor protein complex AP-2, alpha 2 subunit	162138932
2	ADP-ribosyltransferase 1	6978455
3	Arginyl-tRNA synthetase	158631214
4	ATP synthase, H ⁺ transporting, mitochondrial F1 complex, gamma subunit	39930503
5	ATP-binding cassette, sub-family F (GCN20), member 2	157821181
6	Brix domain containing 1	157822643
7	Casein kinase II, alpha 2, polypeptide	157817807
8	Chaperonin containing TCP1, subunit 3 (gamma)	40018616
9	Chaperonin subunit 4 (delta)	33414505
10	Chaperonin subunit 6a (zeta)	76253725
11	Chaperonin subunit 7 (eta)	157819651
12	Coatamer protein complex, subunit beta 1	18158449
13	Coatamer protein complex, subunit gamma	73532768
14	Cullin associated and neddylation disassociated 1	16758920
15	Cytochrome c oxidase subunit II	110189718
16	Cytoplasmic FMR1 interacting protein 1	157822937
17	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 3, X-linked	157823027
18	DEK oncogene (DNA binding)	51948482
19	Developmentally regulated GTP binding protein 1	57528300
20	Dynein cytoplasmic 1 heavy chain 1	148491097
21	Eukaryotic translation initiation factor 3, subunit 6	58865556
22	Fatty acid synthase	8394158
23	Glycosyltransferase 25 domain containing 1	157823499
24	Guanylate nucleotide binding protein 2	19424350
25	High density lipoprotein binding protein	162287194
26	Hydroxyacyl-Coenzyme A dehydrogenase type II	13994225
27	Hydroxysteroid (17-beta) dehydrogenase 4	162287198
28	Hypothetical protein LOC294718	166158346
29	Hypothetical protein LOC497813	66730357
30	Hypothetical protein LOC498736	157819845
31	Kinesin family member 5B	83776543
32	Ly1 antibody reactive clone	58865392
33	Metadherin	19173758
34	Myosin IC	124107592
35	Myosin IE	27465533
36	Nascent-polypeptide-associated complex alpha polypeptide	157786942

S. No	Protein	Accession No. ^{a)}
37	Neural precursor cell expressed, developmentally down-regulated gene 4	158186672
38	NMDA receptor-regulated gene 1	157818303
39	Nucleolar protein 5A	71143106
40	Nucleolar protein family 6 (RNA-associated)	157817474
41	Phosphatidylinositol-4-phosphate 5-kinase, type II, gamma	158534075
42	Pleckstrin homology domain containing, family C (with FERM domain) member 1	58865400
43	Polypyrimidine tract binding protein 1 isoform b	13487910
44	PREDICTED: similar to 40S ribosomal protein S2	62655115
45	PREDICTED: similar to 40S ribosomal protein S9	27665858
46	PREDICTED: similar to 60S ribosomal protein L9	109477682
47	PREDICTED: similar to ARP3 actin-related protein 3 homolog B	109472893
48	PREDICTED: similar to DNA replication licensing factor MCM4 (CDC21 homolog) (P1-CDC21)	34870013
49	PREDICTED: similar to eukaryotic translation initiation factor 3, subunit 8	109462864
50	PREDICTED: similar to eukaryotic translation initiation factor 4, gamma 1 isoform a	109494661
51	PREDICTED: similar to eukaryotic translation initiation factor 4A, isoform 1	109479422
52	PREDICTED: similar to FRG1 protein (FSHD region gene 1 protein)	109504227
53	PREDICTED: similar to GCN1 general control of amino-acid synthesis 1-like 1 isoform 2	109496005
54	PREDICTED: similar to glutamyl-tRNA synthetase	109483957
55	PREDICTED: similar to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	109481109
56	PREDICTED: similar to Histone deacetylase 2 (HD2)	62666128
57	PREDICTED: similar to isoleucine-tRNA synthetase	109505545
58	PREDICTED: similar to Laminin alpha-2 chain precursor (Laminin M chain) (Merosin heavy chain)	109460394
59	PREDICTED: similar to proteasome (prosome, macropain) 26S subunit, non-ATPase, 14	109469981
60	PREDICTED: similar to Putative pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15 (DEAH box protein 15)	109500663
61	PREDICTED: similar to RAB6A, member RAS oncogene family	109462512
62	PREDICTED: similar to ribosomal protein S8	109506240
63	PREDICTED: similar to solute carrier family 25, member 5	62652442
64	PREDICTED: similar to SWI/SNF-related matrix-associated actin-dependent regulator of chromatin a4	109484265
65	PREDICTED: similar to T-complex protein 1 subunit alpha (TCP-1-alpha) (CCT-alpha)	109473894
66	Programmed cell death protein 11	158186708
67	Prohibitin 2	61556754
68	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 3	56605666
69	Proteasome 26S non-ATPase subunit 12	54400716
70	Protein disulfide isomerase-associated 4	16758712
71	Pyrroline-5-carboxylate synthetase (glutamate gamma-semialdehyde synthetase)	157823607
72	RAB33B, member of RAS oncogene family	157822117
73	Radixin	56799432
74	Ribophorin I	6981486
75	Ribosomal protein L13	13592055

S. No	Protein	Accession No. ^{a)}
76	Ribosomal protein L13A	77404207
77	Ribosomal protein L18	13592057
78	Ribosomal protein L5	13592051
79	Ribosomal protein L7a	167466288
80	Ribosomal protein L8	78214309
81	RNA binding motif protein 25	157823201
82	SEC23A (<i>S. cerevisiae</i>) (predicted)	157786714
83	Serine/threonine kinase 2	132626321
84	Small inducible cytokine subfamily E, member 1	75832035
85	Solute carrier family 25, member 4	32189355
86	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5	157817975
87	T-complex protein 1	6981642
88	Topoisomerase (DNA) 2 alpha	38259192
89	Transportin 3	157819279
90	Tropomyosin 1, alpha isoform h	78000201
91	Tropomyosin 3, gamma isoform 2	29336093
92	Tubulin, alpha 1A	11560133
93	Tubulin, beta 6	71043680
94	Tumor rejection antigen gp96 (predicted)	58865966
95	UDP-glucose dehydrogenase	13786146
96	Valyl-tRNA synthetase 2	158186770
97	Zuotin related factor 2	158138509

^{a)} Accession numbers are from NCBI database