Exogenous C2-ceramide activates *c-fos* serum response element via Rac-dependent signalling pathway

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Ceramide is an important regulatory molecule implicated in a variety of biological processes in response to stress and cytokines. To understand the signal transduction pathway of ceramide to the nucleus, in the present study, we examined whether C2-ceramide, a cell permeable ceramide, activates *c-fos* serum response element (SRE). Treatment of Rat-2 fibroblast cells with C2-ceramide caused the stimulation of *c-fos* SRE-dependent reporter gene activity in a dose- and time-dependent manner by transient transfection analysis. Next, we examined the role of Rho family GTPases in the ceramide-induced signalling to SRE activation. By reporter gene analysis following transient transfections with various plasmids expressing a dominant negative mutant form of Cdc42, Rac1 or RhoA, C2-ceramide-induced SRE activation was shown to be selectively repressed by pEXV-RacN17 encoding a dominant negative mutant of Rac1, suggesting that Rac activity

is essential for the signalling cascade of ceramide to the nucleus. In a further study to analyse the downstream mediator of Rac in the ceramide-signalling pathway, we observed that either pretreatment with mepacrine, a potent and specific inhibitor of phospholipase A₂, or co-transfection with antisense cytosolic phospholipase A₂ (cPLA₂) oligonucleotide repressed the C2-ceramide-induced SRE activation selectively, implying a critical role of cPLA₂ in C2-ceramide-induced signalling to nucleus. Consistent with these results, the translocation of cPLA₂ protein as well as the release of arachidonic acid, a principal product of phospholipase A₂, was rapidly induced by the addition of C2-ceramide in a Rac-dependent manner. Together, our findings suggest the critical role of 'Rac and subsequent activation of phospholipase A₂' in ceramide-signalling to nucleus.

INTRODUCTION

Ceramide, a product of sphingomyelin (SM) hydrolysis by sphingomyelinase (SMase) and a proposed lipid second messenger, has been shown to mediate many cellular processes including cell growth, differentiation or apoptosis [1–5]. Despite these suggested actions being implicated in a variety of biological processes, the signalling mechanism of ceramide in the cell remains unclear. Recent report suggests that ceramide activates the *c-Jun* amino-terminal kinase (JNK) cascade, composed of MEKK (mitogen activated kinase kinase-1), JNKK (JNK kinase) and JNK (*Jun* N-terminal kinase), which is also termed the stress-activated protein kinase (SAPK) pathway [6]. For example, in response to tumour necrosis factor (TNF)- α , Fas ligand, ultraviolet (UV) radiation, or X-rays, ceramide is generated by SM hydrolysis in the cell membrane and stimulates the JNK cascade [6]. Thus, JNK-linked signalling pathway could be one of the major signalling cascades by which ceramide mediates the stress or proinflammatory cytokine signals in the cell.

It has been shown that the JNK cascade activates TCF (ternary complex factor) protein, Elk-1/p62^{TCF} [7–9]. For example, in response to interleukin-1 (IL-1) or UV radiation, JNK signalling cascade is stimulated, thereby activating Elk-1/p62^{TCF} to activate *c-fos* SRE (serum response element) [7–9]. Thus, the signalling connection between ceramide and *c-fos* SRE activation via JNK-Elk-1/p62^{TCF} cascade may potentially exist to mediate the intracellular action of environmental stress or cytokines. To investigate the possible activity of ceramide to stimulate SRE, in this present study, we analysed the effect of C2-ceramide, a cell permeable ceramide, on the activation of *c-fos* SRE by transient

transfection analysis. Our results clearly demonstrate that exogenous C2-ceramide stimulates SRE in a dose- and time-dependent manner in Rat-2 fibroblast cells. In our further study to understand the downstream signalling cascade of ceramide, we demonstrated that Rac, a member of Rho family GTPases [10], and subsequent phospholipase A₂ (PLA₂) activation [11] are essential for the ceramide-induced signalling pathway to *c-fos* SRE. Thus, we propose that 'Rac and Rac-activated PLA₂ cascade' is one of the major nuclear signalling pathways by which ceramide stimulates *c-fos* SRE.

MATERIALS AND METHODS

Chemicals and reagents

C2-ceramide was obtained from BioMol Co. (Plymouth Meeting, PA, U.S.A.). Epidermal growth factor (EGF) was purchased from Boehringer Mannheim GmbH (Mannheim, Germany). Antisense cytosolic phospholipase A₂ (cPLA₂) oligonucleotide (GsTsGCTGGTAAGGATCTsAsT) is directed against codons 4–9 of the human cytosolic, Ca²⁺-dependent PLA₂ and the two linkages are phosphothioated at both the 5' and 3' ends. Antisense and control (GsTsGCTCCAAGTTTCTsAsT) cPLA₂ oligonucleotides were purchased from BioMol Co. (Plymouth Meeting, PA, U.S.A.). Fetal bovine serum (FBS), gentamycin, Dulbecco's modified Eagle's medium (DMEM) were from Gibco-BRL (Gaithersburg, MD, U.S.A.). Polyclonal antibodies to cPLA₂ were purchased from Santa Cruz Biotechnology (CA, U.S.A.). All other chemicals were from standard sources and were molecular biology grade or higher.

Abbreviations used: SM, sphingomyelin; SMase, sphingomyelinase; JNK, *c-Jun* N-terminal kinase; SAPK, stress-activated protein kinase; PLA₂, phospholipase A₂; cPLA₂, cytosolic phospholipase A₂; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate buffered saline; SRE, serum response element; SRF, serum response factor; AA, arachidonic acid; TCF, ternary complex factor; LPA, lysophosphatidic acid; EGF, epidermal growth factor.

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Cell culture, transfections and luciferase assay

Rat-2 fibroblast cells were obtained from the American Type Culture Collection (ATCC, CRL 1764). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, 10% (v/v) fetal bovine serum (FBS) and gentamycin as described before [12]. Transient transfection analysis was performed as described previously [13]. To control for variations in both cell numbers and transfection efficiency, all clones were co-transfected with 1 μ g of pCMV- β GAL, an eucaryotic expression vector in which *E. coli* β -galactosidase (lac Z) structural gene is under the transcriptional control of the CMV promoter [13]. Lysates prepared from the harvested cells were assayed for both luciferase activity and β -galactosidase activity, which was used as an internal standard to normalize the luciferase activity directed by the test plasmid [13]. Luciferase activity was determined as described previously [13]. Transfection experiments were performed in triplicate with two independently isolated sets and the results were averaged. Rat-2 clones stably expressing RacN17 were prepared by co-transfecting pEXV-RacN17 plasmid with pSV-Neo[®] plasmid followed by the selection of clones in the presence of G418 antibiotic (400 μ g/ml) for 2–3 weeks as described previously [14]. Expression of RacN17 protein in the clones was confirmed by Western blot hybridization using anti-myc epitope antibody.

[³H]Arachidonic acid release

Rat-2 or Rat2-RacN17 cells in DMEM medium containing 10% FBS were plated to 1×10^5 cells per well (12 well plate). After 3 h, 0.5 μ Ci/ml of [³H]arachidonic acid (250 μ Ci; Amersham) was supplemented to each well and incubated for 24 h, then washed at least four times with DMEM containing 0.5% FBS. Then, cells were labelled with [³H]AA at 0.5 μ Ci/ml for 48 h in DMEM/0.5% FBS, washed eight times with DMEM/0.5% FBS, and finally added with 2 ml of DMEM/0.5% FBS containing C2-ceramide (10 μ M), EGF (50 ng/ml), LPA (lyso-phosphatidic acid; 10 μ M), or control buffer for 1 h. The released [³H]AA into the medium was quantitated by scintillation counting of 0.5 ml medium each time. At the end of experiments, the cells were solubilized with 0.5 ml of ethyl alcohol (EtOH) for the determination of total intracellular incorporation. Counts were corrected for total incorporation.

Subcellular fractionation of cell lysates

Rat-2 cells were serum-starved for 18 h in serum-free DMEM and appropriate agonists were treated for the indicated times. The medium was removed and the cells were washed twice with ice-cold phosphate buffered saline (PBS), scraped, harvested by micro-centrifugation, and resuspended in 0.2 ml of buffer A (137 mM NaCl, 8.1 mM Na₂HPO₄, 2.7 mM KCl, 1.5 mM KH₂PO₄, 2.5 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenyl-methylsulphonyl fluoride, 10 μ g/ml leupeptin, pH 7.5). The resuspended cells were lysed by 20 passes through a 21.1-gauge needle on ice. Lysates were ultra-centrifuged at 10000 *g* for 1 h to prepare cytosolic and total particulate fractions. The particulate fractions containing membrane fraction were washed twice and resuspended in 50 μ l of buffer B (buffer A plus 1% Triton X-100). The supernatant fractions were precipitated with 5 volume of acetone, incubated on ice for 5 min, centrifuged (14000 r.p.m. for 20 min at 4 °C), and the pellet was resuspended in 50 μ l of buffer A. Protein concentrations were determined using the Bradford procedure with Bio-Rad Dye Reagent and bovine serum albumin as a standard.

SDS/polyacrylamide gel electrophoresis and Western blotting

Sodium dodecyl sulphate (SDS)/polyacrylamide gel electrophoresis was performed on 8% acrylamide gels, and proteins were transferred onto polyvinylidene difluoride membranes for 2 h at 100 V using a Novex wet transfer unit. The membrane were blocked overnight with TBS [PBS containing 0.01% (v/v) Tween 20] with 5% (w/v) nonfat dried milk. Blots were incubated for 2 h with primary antibody (to cPLA₂) in TBS and then for 1 h with a horseradish peroxidase-conjugated secondary antibody, prior to development using an enhanced chemiluminescence kit (Amersham Corp.). Bands corresponding to cPLA₂ were measured by densitometry and the purified recombinant proteins were used as standards to calibrate the densitometer.

RESULTS

c-fos SRE as one of the nuclear targets by C2-ceramide

As a first step towards understanding the signalling pathway of ceramide toward gene regulation, we have examined whether C2-ceramide, a cell permeable ceramide, activates *c-fos* serum response element (SRE) [15]. To do this, Rat-2 cells were transiently transfected with a reporter plasmid, pSRE-Luc containing *c-fos* SRE oligonucleotides inserted upstream of *c-fos*

Figure 1 Dose- and time-dependent *c-fos* SRE stimulation by C2-ceramide

(A) Diagram of pSRE-luciferase reporter gene plasmids that were used. The structure of construct containing SRE oligonucleotide sequences (23 mers) inserted to -53 position of the truncated *c-fos* promoter fused to the luciferase gene is shown. (B) Transient transfection assay with either pSRE-Luc or pO-Luc (vector without SRE insert). After transient transfections, Rat-2 cells were serum-starved in 0.5% FBS/DMEM for 36 h before C2-ceramide treatment (10 μ M). C2-ceramide was treated for 1 h before cell harvest. The relative activation of pSRE-luciferases to pO-luciferase was calculated as described in 'Materials and Methods' and histograms of the results of the luciferase are shown. Values were representative of triplicate transfections. (C) Dose and time responses of C2-ceramide on SRE activation. (upper) Various amounts of C2-ceramide (0, 2.5, 5, 10, 20 μ M) were treated for 1 h to serum-starved transfected cells. (lower) Time-dependent response of C2-ceramide (10 μ M) on SRE activation. Serum-starved Rat-2 cells were treated with C2-ceramide for the lengths of time indicated. Luciferase activities were measured and normalized with co-transfected β -galactosidase activities as described in 'Materials and Methods'.

Table 1 Elk-1/p62^{TCF}-independent signalling pathway for C2-ceramide-induced SRE activation

(A) Sequences of wild type and mutant SRE oligonucleotides (23 mers) which were inserted to —53 position of the truncated *c-fos* promoter fused to luciferase gene. Also, the methylation interference pattern for SRF ternary complex with Elk-1/p62^{TCF} is designated as closed circles. The mutant SRE has two point mutations (AGG to TGT, shown by lower-case letters) in the Elk-1/p62^{TCF}-binding region that abolish the SRF-Elk-1/p62^{TCF} complex-formation. (B) Transient transfection assay with either pSREwt-Luc or pSREmt-Luc. Control buffer (—), PMA (10 ng/ml), C2-ceramide (10 μ M), or LPA (10 μ M) was treated for 1 h before cell harvest, and the relative activation of luciferase activities were measured. The results are means $\pm$ S.D. obtained with three samples.

(A)

SRE-wt AGGATGTCATATTAGGACATCT

●●● ●● ●●

SRE-mt t GtATGT CCATATTAGGACATCT

(B)

Transfected DNA	Relative luciferase activity			
	—	PMA	C2-ceramide	LPA
pSREwt-Luc	1	6.1 ± 0.2	4.6 ± 0.3	7.7 ± 0.3
pSREmt-Luc	1	2.3 ± 0.3	4.3 ± 0.4	7.9 ± 0.5

minimal promoter fused to luciferase coding sequences (Figure 1A) [16]. Following transient transfections with pSRE-Luc (3 μ g) plasmid, Rat-2 cells were serum-starved in DMEM containing 0.5 % FBS for 36 h before treating C2-ceramide. SRE activation was monitored by measuring luciferase activities normalized with co-transfected β -galactosidase activity. As shown in Figure 1C, C2-ceramide stimulated *c-fos* SRE-dependent reporter gene activity in a dose- and time-dependent manner. A five-fold increase in the luciferase activity occurred at 1 h after the addition of exogenous C2-ceramide (10 μ M) and then the *c-fos* SRE luciferase level declined (Figure 1C). Maximal luciferase activity was detected at 10 μ M concentration of C2-ceramide (Figure 1C).

In a control experiment to demonstrate the specific action of C2-ceramide toward SRE, Rat-2 cells were transiently transfected with pO-Luc (vector without SRE) and very little stimulation of luciferase activity was observed in response to C2-ceramide addition. We observed a dramatic stimulation ($\approx$ 40-fold) of the relative luciferase activity of pSRE-Luc compared to pO-Luc, suggesting that the C2-ceramide signal to SRE activation is quite specific (Figure 1B). Together, these results indicate that *c-fos* SRE is one of the nuclear target sequences by C2-ceramide in Rat-2 fibroblast cells.

C2-ceramide activates *c-fos* SRE mostly by Elk-1/p62^{TCF}-independent signalling pathway

As ceramide has been reported to stimulate JNK cascade which had been shown to induce the activation of Elk-1/p62^{TCF}, one of the TCFs (ternary complex factors) [7–9], we examined whether Elk-1/p62^{TCF}-dependent signalling pathway is required for the C2-ceramide-induced SRE activation. To do this, a mutant SRE-luciferase plasmid defective in Elk-1/p62^{TCF} binding (pSREmt-Luc) was transiently transfected, treated with C2-ceramide, and the resulting luciferase activity was compared to that obtained using pSREwt-Luc, a wild type SRE-luciferase. As shown in Table 1, the oligonucleotide in pSREmt-Luc plasmid contains two point mutations (AGA to TGT) which abolish Elk-1/p62^{TCF} binding [16]. C2-ceramide (10 μ M) activated both SREwt-luciferase ($\approx$ 4.6-fold) and SREmt-luciferase ($\approx$ 4.3-fold) about equally as shown in Table 1. In a control test, PMA (phorbol-12-

Table 2 Inhibition of C2-ceramide-induced SRE activation by RacN17

(A) A reporter gene plasmid, pSRE-Luc (3 μ g) was transiently co-transfected with indicated amounts (0, 1, 2.5, 5 μ g) of pEXV, pEXV-Cdc42N17, pEXV-RacN17, or pEXV-RhoN19. (B) Also, the effect of C3-transferase was analysed by co-transfecting with indicated amounts (0, 0.5, 0.75, 1.0 μ g) of pEF or pEF-C3. Total amounts of DNA was kept at 20 μ g with calf thymus carrier DNA. Transfected cells were serum-deprived in DMEM containing 0.5% FBS for 36 h before harvest. Control buffer, C2-ceramide (10 μ M) or LPA (10 μ M) was added 1 h prior to cell harvest, and the relative activation of luciferase activities were measured. The results are means of $\pm$ S.D. obtained with three samples.

Agonist + co-transfected DNA	Relative luciferase activity			
(A)	0	1.0	2.5	5.0 (μ g)
Control buffer				
pEXV	1	1.1 $\pm$ 0.1	1.0 $\pm$ 0.2	1.1 $\pm$ 0.2
pEXV-Cdc42N17	1	1.1 $\pm$ 0.2	0.9 $\pm$ 0.1	1.0 $\pm$ 0.2
pEXV-RacN17	1	1.0 $\pm$ 0.2	0.9 $\pm$ 0.2	0.8 $\pm$ 0.3
pEXV-RhoN19	1	1.0 $\pm$ 0.1	0.9 $\pm$ 0.1	0.9 $\pm$ 0.3
C2-ceramide				
pEXV	4.9 $\pm$ 0.2	4.8 $\pm$ 0.1	4.8 $\pm$ 0.2	4.7 $\pm$ 0.2
pEXV-Cdc42N17	4.9 $\pm$ 0.2	5.6 $\pm$ 0.8	4.7 $\pm$ 0.4	4.6 $\pm$ 0.2
pEXV-RacN17	4.9 $\pm$ 0.2	3.5 $\pm$ 0.2	2.2 $\pm$ 0.4	1.5 $\pm$ 0.3
pEXV-RhoN19	4.9 $\pm$ 0.2	4.7 $\pm$ 0.4	4.6 $\pm$ 0.6	4.5 $\pm$ 0.3
LPA				
pEXV	7.9 $\pm$ 0.2	7.8 $\pm$ 0.2	8.0 $\pm$ 0.1	7.8 $\pm$ 0.2
pEXV-Cdc42N17	7.9 $\pm$ 0.2	7.8 $\pm$ 0.2	7.6 $\pm$ 0.1	7.7 $\pm$ 0.2
pEXV-RacN17	7.9 $\pm$ 0.2	7.8 $\pm$ 0.4	7.6 $\pm$ 0.3	7.5 $\pm$ 0.5
pEXV-RhoN19	7.9 $\pm$ 0.2	5.6 $\pm$ 0.3	3.7 $\pm$ 0.3	2.6 $\pm$ 0.4
(B)	0	0.5	0.75	1.0 (μ g)
Control buffer				
pEF	1	1.1 $\pm$ 0.1	1.0 $\pm$ 0.2	0.9 $\pm$ 0.2
pEF-C3	1	1.0 $\pm$ 0.1	0.9 $\pm$ 0.3	0.8 $\pm$ 0.4
C2-ceramide				
pEF	4.9 $\pm$ 0.3	4.8 $\pm$ 0.1	4.7 $\pm$ 0.2	4.5 $\pm$ 0.3
pEF-C3	4.9 $\pm$ 0.3	4.8 $\pm$ 0.2	4.4 $\pm$ 0.5	4.3 $\pm$ 0.4
LPA				
pEF	7.9 $\pm$ 0.4	7.8 $\pm$ 0.2	7.7 $\pm$ 0.1	7.6 $\pm$ 0.2
pEF-C3	7.9 $\pm$ 0.4	5.6 $\pm$ 0.4	3.5 $\pm$ 0.4	2.3 $\pm$ 0.5

myristate-13-acetate; 10 ng/ml) activated SREmt-luciferase activity much less compared to wild type SRE ($\approx$ 38 % activation of SREwt-luciferase activity). In contrast, LPA (lysophosphatidic acid; 10 μ M), which had been previously shown to activate SRE by Elk-1/p62^{TCF}-independent pathway [17], stimulated both SRE mutant- and SRE wild type-dependent luciferase activity to the same level. Together, these results suggest that Elk-1/p62^{TCF}-dependent pathway is not the major signalling route of C2-ceramide to *c-fos* SRE although C2-ceramide appears to require Elk-1/p62^{TCF}-dependent pathway for the full activation of SRE. Thus, C2-ceramide stimulates mostly Elk-1/p62^{TCF}-independent signalling cascade to activate SRE in Rat-2 fibroblasts.

Rac activity is essential for the C2-ceramide-induced *c-fos* SRE activation

Recently, several Rho family GTPases including Rac and Cdc42 have been reported to be involved in the signalling cascade induced by cytokines and environmental stress [18,19]. To investigate any potential role of Rho family GTPases in ceramide-signalling cascade to nucleus, pSRE-Luc (3 μ g) was transiently co-transfected with various amounts (0, 1, 2.5, 5 μ g) of pEXV (control), pEXV-Cdc42N17 (encoding a dominant negative mutant of Cdc42), pEXV-RacN17 (encoding a dominant negative

Table 3 PLA₂ activity is necessary for C2-ceramide-induced SRE activation

Effect of mepacrine, a potent PLA₂ inhibitor, and antisense cPLA₂ oligonucleotide on C2-ceramide-induced SRE activation was analysed. Rat-2 cells were transiently transfected with a reporter gene plasmid, pSRE-Luc (3 µg). Mepacrine (1 µM) or control buffer (—) was treated at 30 min prior to C2-ceramide (10 µM), LPA (10 µM), or control buffer addition. Also, Rat-2 cells were transiently co-transfected with 0.1 µM of antisense or control cPLA₂ oligonucleotide (for the sequences of oligomers, see 'Materials and Methods') along with a reporter gene plasmid, pSRE-Luc (3 µg). Transfected cells were serum-deprived in DMEM containing 0.5% FBS for 36 h before treatment with C2-ceramide (10 µM), LPA (10 µM), or control buffer. After 1 h of treatment, cells were harvested for the relative luciferase assay. The relative activities were measured as described in 'Materials and Methods'. The results are means ± S.D. obtained with three samples.

Inhibitor	Co-transfected DNA	Relative luciferase activity		
		Buffer	C2-ceramide	LPA
—	—	1	4.8 ± 0.2	7.7 ± 0.3
Mepacrine	—	1.0 ± 0.1	1.9 ± 0.4	7.3 ± 0.5
—	cPLA ₂ control oligomer	1	4.9 ± 0.3	7.8 ± 0.5
—	cPLA ₂ antisense oligomer	1.0 ± 0.1	2.1 ± 0.3	7.4 ± 0.8

mutant of Rac1), or pEXV-RhoN19 (encoding a dominant negative mutant of RhoA). As shown in Table 2, C2-ceramide-induced SRE activation is dramatically inhibited by co-transfection with pEXV-RacN17 (≈ 68% reduction of luciferase activity by 5 µg transfection), suggesting that Rac is critical for the C2-ceramide-induced signalling to *c-fos* SRE. In contrast, no detectable inhibition was observed by co-transfection with either pEXV-Cdc42N17 or pEXV-RhoN19 (Table 2). On the other hand, LPA (lysophosphatidic acid; 10 µM)-induced SRE activation was significantly inhibited only by RhoN19, not by Cdc42N17 or RacN17, indicating that LPA-signalling to *c-fos* SRE is primarily through RhoA-linked pathway [17]. Together, these results indicate an essential role of Rac in C2-ceramide-induced signalling to *c-fos* SRE. To analyse further the role of Rho, we have tested the effect of botulinum C3 transferase expression which has been shown to specifically inhibit Rho via ADP-ribosylation at Asn-41 [17]. Cells were co-transfected with EF (vector) or EF-C3 (C3 transferase) together with SRE-luciferase reporter gene to see whether C3 transferase co-transfection blocks C2-ceramide-induced SRE activation. As shown in Table 2, we observed that EF-C3 blocked dramatically LPA (10 µM)-induced SRE activation (≈ 80% reduction of luciferase activity by 1 µg of EF-C3), whereas C2-ceramide (10 µM)-induced SRE activation was not affected (Table 2B). This result again suggests that C2-ceramide-signalling to *c-fos* SRE is not dependent upon RhoA.

Role of PLA₂ in the C2-ceramide-induced signalling to SRE activation

Recently, arachidonic acid (AA) has been shown to be released via phospholipase A₂ (PLA₂) in response to Rac activation, implying a potential role of 'Rac-activated PLA₂ in Rac-mediated signalling pathway' [11,12,20,21]. In our further study to understand the downstream mediator of Rac GTPase in ceramide-signalling cascade, we examined whether Rac-activated PLA₂ is involved in the C2-ceramide-induced SRE activation. To do this, we first tested whether mepacrine, a potent and specific inhibitor of PLA₂, could inhibit the C2-ceramide-induced SRE activation. As shown in Table 3, mepacrine (1 µM) treatment inhibited the C2-ceramide-induced SRE activation by approx. 60% without affecting LPA-induced SRE activation. Similarly, we observed

Figure 2 C2-ceramide stimulates translocation of cPLA₂ in a time-dependent manner

Cells were stimulated with 10 µM C2-ceramide for the indicated times (1, 0 min; 2, 1 min; 3, 2 min; 4, 5 min), lysed, and the membrane fraction was prepared, as described in 'Materials and Methods'. (A) The increasing level of cPLA₂ (≈ 100 kDa) was detected by Western blotting of equal amounts of membrane fraction proteins with time (0, 1, 2, 5 min). The positions of the protein markers, their molecular masses expressed in kDa, are shown at the left side of the picture. (B) Data are expressed as a relative percentage of the basal value (0 min; 100%) and are the average ± S.E. of duplicate experiments.

that co-transfection with antisense cytosolic phospholipase A₂ (cPLA₂) oligonucleotide, not control cPLA₂ oligonucleotide, could significantly inhibit C2-ceramide-induced SRE activation (≈ 60% inhibition by co-transfection with 0.1 µM of antisense cPLA₂ oligomer) with no effects on LPA-induced SRE activation, suggesting that cPLA₂ is clearly involved in the C2-ceramide-induced signalling to *c-fos* SRE (Table 3).

To examine further the role of cPLA₂ in the C2-ceramide signalling pathway, we determined whether C2-ceramide stimulates the translocation of cPLA₂ to membrane. To do this, serum-starved Rat-2 cells were stimulated with 10 µM of C2-ceramide for the indicated times (0, 1, 2, 5 min). Then, the cells were lysed, and the particulate membrane fractions were analysed for cPLA₂ by Western blotting. As shown in Figure 2, cPLA₂ (≈ 100 kDa) was translocated to the membrane in a time-dependent manner. By 5 min treatment of C2-ceramide (as shown in lane 4), approx. 4.5-fold increase in translocation was observed, demonstrating the apparent stimulation of cPLA₂ by ceramide. We observed an equivalent loss of cPLA₂ levels from the cytosolic fraction with time (data not shown). This result again points to a critical role of cPLA₂ in the ceramide-signalling in the cell. In addition, we determined whether AA, a principal product of PLA₂, is released by treating with C2-ceramide to Rat-2 cells which had been pre-labelled with [³H]AA as described in

Table 4 C2-ceramide induces the release of [³H]AA in a Rac-dependent manner

Rat-2 and Rat2-RacN17 cells were labelled with [³H]AA (0.5 µCi/ml; 48 h) and washed eight times with DMEM containing 0.5% FBS as described in 'Materials and Methods'. Control buffer (—), EGF (50 ng/ml), LPA (10 µM), or C2-ceramide (10 µM) was added 1 h before the harvest for the determination of [³H]AA release. For the inhibitor test, mepacrine (1 µM) was pretreated 30 min before the addition of C2-ceramide or LPA. The released [³H]AA into the medium was quantitated by scintillation counting and normalized by the total counts as described in 'Materials and Methods'. The results are means ± S.D. obtained with triple samples (N.D., not determined).

Inhibitor	Cell type	[³ H]AA release (c.p.m.)			
		—	EGF	C2-ceramide	LPA
—	Rat-2	1950 ± 150	2980 ± 120	8250 ± 420	3410 ± 120
—	Rat2-RacN17	1890 ± 140	1930 ± 150	3150 ± 360	3350 ± 330
Mepacrine	Rat-2	1860 ± 130	N.D.	2950 ± 220	3320 ± 250

'Materials and Methods'. As shown in Table 4, C2-ceramide induced a significant liberation of AA (≈ 4.2-fold stimulation of AA release over control), but this over-induced liberation of AA was significantly diminished by 30 min pretreatment with mepacrine (1 µM), suggesting that PLA₂ plays a major role in mediating C2-ceramide-induced AA release. Next, we determined whether this AA liberation requires Rac activity or not. By comparing the level of AA liberation between control cells and cells stably expressing RacN17 (Rat2-RacN17), it was shown that in Rat2-RacN17 cells the release of AA was reduced significantly (≈ 38% level compared to that in Rat-2 cells) in response to C2-ceramide, suggesting that C2-ceramide induces AA liberation via Rac-dependent manner (Table 4). In contrast, LPA-induced AA release was not inhibited in Rat2-RacN17 cells. Together, these results point to a critical role of Rac-activated PLA₂ in the C2-ceramide-induced signalling cascade.

DISCUSSION

The present study demonstrated that Rac activity is essential for the signalling pathway of C2-ceramide to *c-fos* SRE. By reporter gene analysis following transient transfections with pEXV-RacN17 encoding a dominant negative Rac mutant, RacN17, C2-ceramide-induced SRE activation was shown to be selectively repressed, revealing that Rac activity is critical for the signalling cascade of ceramide to nucleus. Although Rac appears to be essential for C2-ceramide signalling pathway to SRE activation, the downstream kinase cascade that mediates this effect is yet to be identified. Recently, Rac has been shown to function in the regulation of kinase cascades. For example, Cdc42 and Rac regulate PAKs (p21-activated kinases), a family of highly related serine/threonine kinases, JNK, and also p38/HOG kinase, MAP kinase homologues [18,19,22,23]. Another kinase, 70 kDa ribosomal S6 kinase (pp70^{S6K}), is recently reported to be activated by Cdc42 and Rac1 in response to growth factors such as EGF and platelet-derived growth factor [24]. In response to proinflammatory cytokines and environmental stress, Rac has been reported to activate JNK signalling cascade [18,19]. As JNK pathway was shown to stimulate Elk-1/p62^{TCF} to activate SRE, the signalling cascade of Rac-JNK-Elk-1/p62^{TCF} may potentially exist. However, our result (Table 1) suggests that JNK-linked cascade is unlikely to be the major signalling pathway by which C2-ceramide mediates *c-fos* SRE activation, as C2-ceramide still activates *c-fos* SRE mutant defective in Elk-1/p62^{TCF} binding. Thus, Rac may also mediate Elk-1/p62^{TCF}-independent signalling

pathway to SRE activation, depending on the type of agonists or stress. Consistent with this idea, Hill et al. demonstrated that Rho family GTPases including Rac activate *c-fos* SRE through Elk-1/p62^{TCF}-independent pathway [17].

Our results demonstrate that 'Rac-activated PLA₂' is clearly involved in the ceramide-signalling cascade to *c-fos* SRE, suggesting that PLA₂ may be one of the target molecules of Rac in the ceramide-induced nuclear signalling pathway. Besides activating PLA₂ activity, C2-ceramide was also shown to stimulate the transcription of cytosolic PLA₂ mRNA [25]. Thus, it is plausible that one of the key roles of ceramide is to amplify the magnitude of signals generated through the PLA₂ pathway. Consistent with the role of PLA₂ as one of the downstream target molecules of ceramide signal, PLA₂ was shown to participate in the cell death programme in several cell types [26–28]. Also, there is an increasing amount of evidence suggesting signalling-link between the sphingomyelin cycle and PLA₂-mediated arachidonic acid metabolism [25,29–31].

In summary, in the present study, we have shown that C2-ceramide activates *c-fos* SRE via Rac-dependent signalling pathway in Rat-2 fibroblast cells. Although the exact mechanism of Rac action in C2-ceramide-signalling remains unclear yet, we suspect that the role of Rac in the C2-ceramide-induced SRE activation is, at least partly, exerted through the activation of phospholipase A₂, especially cytosolic PLA₂.

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