Role of Ceramide-activated Protein Phosphatase in Ceramide-mediated Signal Transduction*

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Robert A. Wolff, Rick T. Dobrowsky, Alicja Bielawska, Lina M. Obeid, and Yusuf A. Hannun†
From the Departments of Medicine and Cell Biology, Duke University Medical Center, Durham, North Carolina 27710

Extracellular agonists such as tumor necrosis factor-α (TNF-α) activate the sphingomyelin cycle leading to the generation of ceramide. Ceramide has been suggested as an important mediator of the effects of TNF-α on growth inhibition, c-myc down-regulation, apoptosis, and the activation of the nuclear factor κB. Although there is no clearly defined intracellular target for ceramide activity, previous studies have demonstrated the existence of a ceramide-activated protein phosphatase (CAPP) in vitro. Since c-myc is an early downstream cellular target for TNF-α, we examined the role of ceramide and CAPP in c-myc down-regulation. In intact HL-60 cells ceramide induced down-regulation of c-myc RNA levels. C-derivatives were active at 1–10 μM and caused 40–80% inhibition of c-myc RNA levels at 30–120 min of treatment. In nuclear run-on studies, C,-ceramide induced a block to transcription elongation of the c-myc transcript without affecting transcription through the first exon. Therefore, ceramide appeared to inhibit c-myc expression via a mechanism identical with that of TNF-α. HL-60 cells contained CAPP which was inhibited by okadaic acid (0.1–10 μM). CAPP in HL-60 cells was activated by o-erythro-ceramide but not ω-erythro-dihydroceramide. The specificity of activation of CAPP by ceramide in vitro was matched by a similar specificity of c-myc down-regulation in cells. Moreover, okadaic acid inhibited the effects of ceramide and TNF-α on c-myc down-regulation. On the other hand, okadaic acid did not inhibit the ability of phorbol 12-myristate 13-acetate to down-regulate c-myc, demonstrating the existence of at least two distinct pathways in the regulation of c-myc expression. These results demonstrate that CAPP is important for ceramide-induced down-regulation of c-myc in myeloid leukemia cells. The implications of these findings in further delineating a sphingomyelin signaling pathway with important anti-proliferative effects are discussed.

Recently, a sphingomyelin cycle has emerged as a candidate signal transduction pathway with important biologic consequences. Tumor necrosis factor-α (TNF-α)1 and other extracellular agents, such as 1,25-dihydroxyvitamin D₃, γ-interferon, and interleukin-1, activate a neutral sphingomyelinase resulting in hydrolysis of membrane sphingomyelin (1–4). Ceramide, the product of sphingomyelin cleavage, inhibits cell growth (1) and induces apoptosis (5). In addition, cell-permeable analogs of ceramide have been shown to regulate a number of cellular activities. Ceramides have been shown to increase cyclooxygenase (4), activate mitogen-activated protein kinase (6), and possibly regulate the expression of nuclear factor κB (7–9). These results are beginning to delineate a signal transduction pathway mediated by ceramide.

Despite the growing number of reports demonstrating biologic activity for ceramide in vitro, the direct cellular targets which mediate ceramide activity remain to be elucidated. In cells and cell-free systems, ceramide has been shown to stimulate a protein kinase activity that enhances phosphorylation of the epidermal growth factor receptor on threonine 669 (10). This kinase is also activated in response to TNF-α (11). However, it has not been determined whether this kinase is a direct target for ceramide or perhaps a downstream effector. Recently, both naturally occurring ceramide and C,-ceramide but not other related sphingolipids were found to activate a cytosolic protein phosphatase in vitro (12). This ceramide-activated protein phosphatase (CAPP) is potently inhibited by okadaic acid with an IC₅₀ of 1–10 μM (12). Studies have also shown that CAPP belongs to the heterotrimeric subfamily of the P2A group of serine/threonine protein phosphatases (13). These in vitro studies have identified a direct target for the action of ceramide and raise the possibility that CAPP may serve to mediate the cellular action of ceramide. However, to date no clear link has been established between ceramide-induced phosphatase activation and subsequent intracellular events.

Since previous work has shown that TNF-α potently down-regulates c-myc levels in HL-60 cells (14) and preliminary studies in our laboratory have suggested that ceramide may down-regulate c-myc (1), we attempted to investigate the mechanism of c-myc down-regulation induced by ceramide. Specifically, we speculated that CAPP may play a role in ceramide-induced down-regulation of c-myc. The results from this study show that ceramide produces early, specific, and potent down-regulation of c-myc through a mechanism similar to that of TNF-α (i.e. through a block to transcription elongation). In addition, we provide evidence that c-myc down-regulation in response to ceramide involves activation of CAPP. The implications of these results on TNF-α signal transduction and c-myc down-regulation are discussed.

EXPERIMENTAL PROCEDURES

Materials

C,-ceramide was synthesized from sphingosine as described elsewhere (15). The synthesized products were checked for purity using mass spectrometry and TLC. HL-60 cells were purchased from American Type Culture Collection. RPMI medium and fetal bovine serum were obtained from Life Technologies, Inc. Insulin, transferrin, and salmon sperm DNA were from Sigma. Nitrocellulose filters used were from either Costar or Bio-Rad. Radiolabeled precursors were purchased from Dupont-NEN. Okadaic acid was obtained from Kamiya Biomedical Company.

Methods

HL-60 Cell Culture and Treatment—HL-60 cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (passages 35–
Fig. 1. Down-regulation of c-myc mRNA by C2-ceramide in HL-60 cells. Panel A, C2-ceramide down-regulates c-myc in a dose-dependent manner. Northern blots of total cellular RNA harvested from HL-60 cells treated with ethanol vehicle or increasing concentrations of C2-ceramide hybridized to exon III of c-myc or to 28 S RNA probe. Densitometric quantitation is also shown. Panel B, time course of c-myc down-regulation in response to C2-ceramide. HL-60 cells were treated with 10 μM C2-ceramide or ethanol control for varying lengths of time as indicated. Panel C, specificity of ceramide induced c-myc down-regulation. HL60 cells were treated with D-erythro-C2-ceramide, DL-erythro-dihydro-C2-ceramide (each at 2.5 and 5 μM) or with ethanol vehicle for 2 h. Densitometric quantitation is shown. Panel D, C2-ceramide blocks c-myc mRNA elongation as determined by nuclear run-on analysis. The Northern analysis is representative of seven independent experiments.
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A

FIG. 2. Ceramide-activated protein phosphatase in HL-60 cells. Panel A, HL-60 cells contain a ceramide activated protein phosphatase. CAPP activity (●) was determined as the difference in activity in the presence versus absence (○) of ceramide. One unit of activity is equivalent to 1 nmoI of P i released/min. Panel B, CAPP is inhibited by okadaic acid. Assays were performed using 5 µl of fraction 29, 1 µM 32P-myelin basic protein, in the absence (○) or presence (●) of 20 µM C2-ceramide at okadaic concentrations of 0, 0.1, 0.5, 0.75, 1.0, and 10 nM. Assays were incubated for 5 min at 37°C and extracted as described elsewhere (12). Data are expressed as percent activity in absence of okadaic acid and ceramide. Results are mean ± S.D. of triplicate determinations and are representative of two independent experiments.

B

50). Cells were resuspended at densities between 5 x 10^5–1.0 x 10^6 cells/ml in serum-free medium supplemented with insulin, transferrin, and sodium selenite, allowed to re-equilibrate for 2 h, and treated with TNF-α, C2-ceramide, phorbol 12-myristate 13-acetate, or ethanol vehicle as described in the figure legends and text.

RNA Isolation and Northern Analysis—After treatment with various agonists, cells were harvested and total cellular RNA was isolated using a modification of the method of Chirgwin et al. (16) or by the method of Chomczynski and Sacchi (17). Total cellular RNA was fractionated on a 1% agarose denaturing gel, transferred to nitrocellulose, and hybridized to radiolabeled nick-translated c-myc cDNA as previously described (1).

Nuclear Run-on Studies—HL-60 cells were treated with or without 10 µM C2-ceramide and nuclei were isolated by the method of Bitter and Roeder (18). In vitro transcription was carried out with [35S]UTP by a modification of the method of Salehi and Niedel (19). Radiolabeled mRNA was isolated and hybridized to equal amounts of exon I and III of c-myc cDNA immobilized on nitrocellulose. Hybridization and autoradiography were performed as described for Northern analysis.

RESULTS

Ceramide Regulates c-myc Expression—In HL-60 leukemia cells, TNF-α induces sphingomyelin hydrolysis and ceramide

Protein Phosphatase Assays—HL-60 cells (2.0 x 10^6) were resuspended in 20 ml of homogenization buffer (12) and disrupted by nitrogen cavitation. The supernatant was adsorbed to DEAE-Sephacel and CAPP was eluted as previously described by sequential elution with 100, 200, 300, and 400 mM NaCl in buffer A (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1 mM benzamidine, 0.5 mM dithiothreitol) (12). CAPP activity primarily eluted in the 300 mM NaCl wash (10 ml, 32.7 mg) which was diluted 3-fold with buffer A and applied to a Mono-Q HR5/5 column. Protein was eluted with a 35-ml linear gradient from 100 to 400 mM NaCl in buffer A plus 10% glycerol at a flow rate of 1 ml/min. Fractions were collected and were assayed for CAPP activity as described using 32P-myelin basic protein as substrate.

FIG. 3. Specificity of ceramide in vitro correlates with in vivo specificity. Panel A, effects of ceramide analogs on c-myc down-regulation. HL-60 cells were exposed to 10 µM of each of the six ceramide analogs for 2 h, and Northern blot analysis of c-myc message in total cellular mRNA was performed. Densitometric analysis is shown. Panel B, activation of CAPP by ceramide isomers in vitro. Four specific stereoisomers of C2-ceramide and two dihydro-C2-ceramides were synthesized as previously described (23). Cytosol obtained from untreated HL-60 cells was eluted from a DEAE-Sephacel column and a Mono-Q HR5/5 column as described. CAPP activity was measured in the presence or absence of 10 µM of D-erythro-C2-ceramide, D-threo-C2-ceramide, L-erythro-C2-ceramide, L-threo-C2-ceramide, D-erythro-dihydro-C2-ceramide, and D-threo-dihydro-C2-ceramide. CAPP activity is expressed as percent of control.

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generation within 15–30 min (1). Since this precedes the effects of TNF-α on c-myc down-regulation at 1–4 h (14, 20), the effects of ceramide on c-myc mRNA levels were investigated. C2-ceramide, a cell-permeable analog of ceramide (15), was added to HL-60 cells growing in culture. After 2 h of treatment, C2-ceramide significantly down-regulated c-myc mRNA levels. Initial effects occurred at a ceramide concentration of 1 μM with maximal effects (70% decrease) reached at a concentration of 10 μM (Fig. 1A). The effects of C2-ceramide on c-myc down-regulation were also rapid with 10 μM C2-ceramide resulting in a 40% decrease in c-myc mRNA by 30 min and an 80% down-regulation by 120 min (Fig. 1B). These results show that ceramide induces early and potent down-regulation of c-myc expression.

Whereas C2-ceramide was capable of down-regulating c-myc by 65% in a dose-dependent manner, dl-erythro-dihydro-C2-ceramide was found to up-regulate c-myc mRNA 1.6-fold over control (Fig. 1C). C2-ceramide and dihydro-C2-ceramide differ only in the presence of a double bond at the 4,5-position of the C2-ceramide sphingoid backbone specifying the specificity of the effects on c-myc.

To establish whether ceramide caused c-myc down-regulation through a mechanism similar to TNF-α, studies were undertaken to determine if C2-ceramide down-regulates c-myc through a block to message elongation as described for TNF-α (21). HL-60 cells were treated with 10 μM C2-ceramide, and nuclear run-on studies were performed. The results in Fig. 1D demonstrate basal transcription of the c-myc gene with a modest level of elongation block between the first and third exons in control cells. Exposure to C2-ceramide (10 μM) caused significant inhibition of transcription through exon II with little effects on transcription through exon I; demonstrating the ability of ceramide to induce a block to elongation of c-myc transcription.

**CAPP Is Present in HL-60 Cells**—Previous studies describing CAPP utilized rat brain and glioma cells for identification and purification. Therefore experiments were performed to establish whether HL-60 cells possess CAPP activity. Cytosol from HL-60 cells was fractionated sequentially over DEAE-Sephacel followed by Mono-Q chromatography and assayed for CAPP. As shown in Fig. 2A, peak CAPP activity was eluted at 250 mM NaCl from Mono-Q and was stimulated 2.7-fold by 20 μM C2-ceramide. CAPP eluted ahead of two additional peaks of phosphatase activity (280 and 330 mM NaCl) which lacked ceramide responsiveness. Importantly, CAPP activity from HL-60 cells was found to be sensitive to inhibition by okadaic acid with an IC50 of approximately 1 nM (Fig. 2B).

**Evidence for a Role for CAPP Mediating Ceramide-induced Down-regulation of c-myc**—The role of CAPP in ceramide-induced down-regulation of c-myc was determined. This was investigated by two approaches. First, the specificity of activation of CAPP by ceramide in vitro was compared with the specificity of action of ceramide in vivo. Fig. 3A demonstrates that four distinct stereoisomers of C2-ceramide were capable of c-myc down-regulation at 10 μM by 60–70%. These same analogs also activated CAPP by 190–250%. In contrast, the two dihydroceramide analogs tested did not down-regulate c-myc nor activate CAPP (Fig. 3B). Thus, the ability of ceramide analogs to down-regulate c-myc mRNA levels in vivo closely correlated with the ability to activate CAPP in vitro.

In a second approach, the ability of the phosphatase inhibitor, okadaic acid, to block the effects of ceramide and TNF-α on c-myc down-regulation was investigated. C2-ceramide (10 μM) was added to HL-60 cells exposed to either okadaic acid or ethanol vehicle, and c-myc levels were measured after 2 h. Fig. 4A shows that okadaic acid inhibited down-regulation of c-myc induced by C2-ceramide in a dose-dependent manner with concentrations between 10 and 30 nM. Okadaic acid also inhibited c-myc down-regulation induced by TNF-α by over 50% (Fig. 4B).

Since phorbol esters, such as phorbol 12-myristate 13-acetate (PMA) have also been shown to induce down-regulation of c-myc (22), it became important to determine if phosphatase activation defined a separate signaling pathway for the regulation of c-myc. When HL-60 cells were treated for 2 h with PMA (100 nM) alone, c-myc levels decreased by 46%. However, the down-regulation of c-myc induced by PMA could not be...
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These results also carry implications for the mechanisms regulating c-myc expression. The studies with ceramide, okadaic acid, and PMA define two distinct mechanisms for c-myc down-regulation. PMA, acting on protein kinase C, causes potent down-regulation of c-myc which is not inhibited by okadaic acid. On the other hand, ceramide induces c-myc down-regulation through activation of an okadaic acid-sensitive protein phosphatase. These results suggest that c-myc transcription is closely regulated by phosphorylation/dephosphorylation events.

Finally, these results provide further support for a sphingomyelin/ceramide signaling pathway in HL-60 cells (Fig. 5) activated by TNF-α. The action of TNF-α on its 55-kDa receptor results in activation of a sphingomyelinase which cleaves membrane sphingomyelin and induces generation of ceramide (25, 26). Both ceramide and TNF-α induce c-myc down-regulation through a similar mechanism involving a block to transcription. The action of both TNF-α and ceramide involves an okadaic acid-inhibited phosphatase. In the case of ceramide, evidence was provided for a role for CAPP in mediating c-myc down-regulation. Further studies should explore the activation of CAPP by TNF in cells and the role of CAPP in TNF-α signal transduction. Such studies require the development of specific tools to inhibit ceramide generation and/or CAPP (and not other phosphatases).

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