Ceramide Regulates Protein Synthesis by a Novel Mechanism Involving the Cellular PKR Activator RAX*

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The sphingolipid ceramide is an important second signal molecule and potent apoptotic agent. The production of ceramide is associated with virtually every known stress stimulus, and thus, generation of this sphingolipid has been suggested as a universal feature of apoptosis. Recent studies suggest that an important component of cell death following diverse stress stimuli (e.g. interleukin-3 withdrawal, sodium arsenite treatment, and peroxide treatment) is the activation of the double-stranded RNA-activable protein kinase, PKR, resulting in the inhibition of protein synthesis (Ito, T., Jagus, R., and May, W. S. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7455–7459). The recently discovered cellular PKR activator, RAX, is phosphorylated in association with PKR activation (Ito, T., Yang, M., and May, W. S. (1999) J. Biol. Chem. 274, 15427–15432). Since RAX is phosphorylated by an as yet undetermined SAPK and ceramide is a potent activator of SAPKs such as JNK, a role for ceramide in the activation of RAX might be possible. Results indicate that overexpression of exogenous RAX potentiates ceramide-induced killing. Furthermore, ceramide can potently inhibit protein synthesis. Since ceramide potently promotes RAX and eukaryotic initiation factor-2α phosphorylation, a possible role for ceramide in this process may involve the activation of PKR by RAX. Since 2-aminopurine, a serine/threonine kinase inhibitor, blocks both the potentiation of ceramide killing by RAX and ceramide-induced inhibition of protein synthesis, ceramide appears to promote PKR activation, at least indirectly. Collectively, these findings suggest a novel role for ceramide in the regulation of protein synthesis and apoptosis.

In addition to their role in membrane structure, sphingolipids play a critical role in cellular signal transduction pathways, particularly in response to stress stimuli (1–6). Diverse stress stimuli, including chemotherapeutic drug treatment, irradiation, and growth factor withdrawal, promote the generation of ceramide, which more often than not results in cell death or cell growth arrest (1, 2, 6). Clearly, ceramide regulates stress-signaling pathways by multiple actions (e.g. activation of stress kinases, inhibition of protein kinase C, activation of protein phosphatases) (1–6). However, a role for ceramide has yet to be determined in the stress-mediated arrest of protein synthesis that has recently been found to precede apoptosis (7).

The most well characterized mechanism for regulating protein synthesis involves the reversible phosphorylation of the α-subunit of eukaryotic initiation factor-2 (eIF2α) (8–10). The phosphorylation of eIF2α at serine 51 prevents protein translation initiation events (11–13). A physiologic eIF2α kinase and key regulator of this process is the double-stranded RNA-activable protein kinase, PKR (14–16). However, until recently, much of what is known about PKR has been derived from its role in inhibiting host cell protein synthesis in response to viral infection, thereby activating the host antiviral defense mechanism (17, 18). Recent data, however, suggest that PKR may play a more expanded role in regulating protein synthesis in response to cellular stresses such as IL-3 growth factor withdrawal (7), serum deprivation (19), tumor necrosis factor-α (20, 21), or lipopolysaccharide treatment (21). Recently, RAX (PKR activator X) (22) and its human homolog, PACT (23), were independently discovered as the first known cellular activators of PKR (22, 23). Consistent with this role, RAX/PACT has been shown to activate PKR in response to stress applications such as IL-3 withdrawal, sodium arsenite treatment, and peroxide treatment (22, 24, 25). In response to stress stimuli, RAX is phosphorylated by an unknown stress-activated protein kinase (SAPK) (22). Phosphorylated RAX associates with PKR, resulting in PKR activation and inhibition of protein synthesis (22). Thus, RAX appears to be directly involved in the regulation of PKR during diverse cellular stress events.

Ceramide is a naturally occurring sphingolipid that has emerged as an important second messenger molecule in apoptosis signaling (26, 27). Ceramide is produced during diverse stress stimuli, including chemotherapeutic drug treatment (28), ischemia/reperfusion injury (29), FAS antigen activation (30), corticosteroid treatment (31), and irradiation (31). Indeed, the generation of ceramide is so common during apoptosis that it has been considered a universal feature of this process (1, 3). Ceramide has been demonstrated to activate a number of stress-activated kinases, including the mitogen-activated protein kinases JNK1 and JNK2 (4, 32, 34). Considering that ceramide is produced during diverse apoptotic stress applications and has been demonstrated to activate stress-activated kinases, it is possible that ceramide may regulate RAX. The findings presented indicate a novel signal pathway linking ceramide to the regulation of protein translation, via a mechanism involving RAX. These data point to a unique level of cellular homeostatic control involving this important second messenger molecule.

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1 The abbreviations used are: eIF2α, eukaryotic initiation factor-2α; IL-3, interleukin-3; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinase; HA, hemagglutinin; 2-AP, 2-aminopurine.
Materials—All reagents used were purchased from commercial sources unless otherwise stated.

Cell Lines—Mature 13 N1 cells and N1H7 cells stably transfected with HA-RAX or vector control plasmid were maintained as previously described (22).

Analysis of Cell Viability and Apoptosis—Cells were treated with increasing doses of C2-ceramide (Calbiochem) or 50 μM C2-dihydroceramide (Calbiochem) for 3, 6, or 24 h. Where appropriate, cells were treated with increasing doses of 2-amino purine (Sigma) for 24 h. Cell viability was measured by trypan blue dye exclusion, and apoptosis was determined by detecting DNA laddering in apoptotic cells as previously described (7, 22).

Analysis of RAX Phosphorylation—Cells (1×10^6 cell eq) were lysed, and HA-RAX was immunoprecipitated with an anti-HA antibody as previously described (22). Where indicated, cells were treated with 1, 10, or 50 μM C2-ceramide for 30 min. The immunoprecipitated protein was resuspended in sample buffer, and protein was subjected to vertical slab isoelectric focusing and immunoblot analysis with an anti-RAX polyclonal antibody as previously described (22). The same blot was used for Western blotting with anti-eIF2 α antibodies as described (22). Where indicated, cells were treated with 25 μM C2-ceramide for 30 min. Samples were electrophoresed on a 0.1% SDS-12% acrylamide gel, transferred to nitrocellulose, and exposed to Kodak X-OMat film at −80 °C. The nitrocellulose was probed with anti-eIF2 α antiserum and developed using an ECL kit (Amersham Pharmacia Biotech) as previously described (22).

Co-immunoprecipitation Analysis—Cells were treated with increasing doses of C2-ceramide for 30 min and lysed, and PKR was immunoprecipitated with an anti-PKR antibody as previously described (22). The immunoprecipitated protein was electrophoresed on a 0.1% SDS-12% acrylamide gel and transferred to nitrocellulose, and Western blot analysis was performed with an anti-RAX polyclonal antibody as described (22).

Measurement of Cellular Protein Synthesis—Cells were treated with C2-ceramide (0, 1, 10, or 50 μM) or C2-dihydroceramide (50 μM) for 30 min. Where indicated, cells were treated with 1 mM 2-AP for 30 min. Measurement of protein synthesis was performed as previously described (7). After treatment, ~1× 10^6 cell eq were incubated with L-[35S]methionine (Amersham Pharmacia Biotech) at 2 μCi/ml for 10 min at 37 °C. The reaction was terminated by the addition of 20% (w/v) trichloroacetic acid, and the radioactivity in the acid-precipitable fraction was measured in a scintillation counter.

RESULTS

RAX Overexpression Potentiates Ceramide-induced Cell Killing via a Mechanism Involving PKR—Protein synthesis, which is required for cell growth, is inhibited by activated PKR (7). Ceramide is a potent apoptotic agent and inhibitor of cell growth (3–5), yet the effect of ceramide on protein translation is unknown. To determine whether RAX, the first cellular activator of PKR to be discovered, can participate in a ceramide-mediated pathway, the effect of ceramide treatment was compared in cells stably expressing exogenous HA-RAX versus vector-only or parental control cells. Overexpression of RAX has previously been shown to accelerate the cell death of murine IL-3-dependent myeloid cell lines following IL-3 withdrawal or after treatment with apoptotic agents such as peroxide and sodium arsenite in the presence of IL-3 (22). Since ceramide is generated during virtually all known stress stimuli (2, 3), we tested whether stress activation of RAX may be mediated by ceramide. If ceramide can participate in RAX-mediated stress, it is predicted that RAX may sensitize cells to ceramide-induced cell death in a mechanism similar to that by which RAX sensitizes factor-dependent cells to killing following factor deprivation (22). The results revealed that cells expressing exogenous HA-RAX were at least 2.5-fold more sensitive to active C2-ceramide killing compared with vector-transfected or untransfected cells (Fig. 1A). The IC50 for the untransfected or vector-transfected cells treated with ceramide for 24 h was >50 μM, whereas the IC50 for cells overexpressing RAX was dramatically reduced to 18 μM. Cell killing resulted from apoptosis as determined by the classic pattern of DNA fragmentation observed (Fig. 1B) (35, 36). As a further control, cells overexpressing HA-RAX were also treated with an inactive C2-ceramide analog, C2-dihydroceramide (2), at 50 μM. The inactive analog had no effect on cell viability (Fig. 2). Although a role for RAX in ceramide-induced cell killing is suggested but not proven by these findings, it was initially unclear whether PKR is involved. Therefore, 2-AP, a serine/threonine kinase inhibitor that has previously been shown to inhibit PKR (37, 38), was used. A high concentration of 2-AP (10 mM) was found to have no effect on cell viability. However, in cells overexpressing HA-RAX, 2-AP was found to protect cells from C2-ceramide-induced cell killing in a dose-dependent manner (Fig. 2). Using a concentration of C2-ceramide that approximates the IC50 at 24 h (i.e. 18 μM), cells treated with >1 mM 2-AP were protected from ceramide-induced cell killing. These data strongly suggest a novel mechanism for ceramide-mediated apoptosis involving both RAX and PKR.

Ceramide Promotes RAX Phosphorylation in a Dose-dependent Manner—Ceramide is known to activate serine/threonine stress-activated kinases, including JNK (3–5, 26, 27). Since RAX is serine-phosphorylated by an as yet unidentified stress-activated kinase (22), it is possible that a mechanism by which ceramide can induce PKR activation and inhibit protein synthesis is by promoting RAX phosphorylation and activation. Therefore, whether C2-ceramide could induce RAX phosphorylation was assessed in vivo. Previously, isoelectric focusing analysis revealed that exponentially growing NPS/N1H7 cells contain predominantly a single unphosphorylated species of RAX with an approximate pl of 8.6, whereas stress applications to cells, including IL-3 deprivation or treatment of cells with sodium arsenite or thapsigargin, induces a prominent acidification (i.e. via phosphorylation) of RAX with a pl 8.3 (22). Thus, cells not exposed to stress stimuli contain primarily the unphosphorylated form of RAX, whereas stress treatment induces
phosphorylation of RAX. The results demonstrated that ceramide could also induce the phosphorylation of RAX in a dose-dependent manner. Although little, if any, of the phosphorylated form of RAX (observed as the slower migrating band following vertical slab isoelectric focusing electrophoresis) could be detected by Western blot analysis of protein lysates from untreated cells or cells treated with a low concentration of C₂-ceramide (i.e. 1 μM), cells treated with >10 μM C₂-ceramide expressed virtually all RAX as a phosphoprotein (Fig. 3). Thus, ceramide appears to activate a RAX kinase.

Ceramide Promotes PKR Activation—Since RAX phosphorylation promotes the activation of PKR by RAX (22), it is likely that ceramide activates PKR. Co-immunoprecipitation studies have previously demonstrated that cellular activation of PKR strongly correlates with the association of PKR with RAX (22). To determine whether ceramide can promote RAX/PKR association, protein extracts from cells treated with increasing doses of C₂-ceramide were immunoprecipitated with anti-PKR antisera as previously described (22). RAX was detected by Western analysis using an anti-RAX polyclonal antibody that was produced as previously described (22). The results indicated that ceramide promoted RAX/PKR association in a dose-dependent manner (Fig. 4). However, whether ceramide induced PKR activation was not clear. Previously, it has been demonstrated that stress application induces RAX phosphorylation and promotes RAX/PKR association concomitant with PKR activation and eIF2α phosphorylation (22, 24). Since the biogenesis of ceramide can result from many diverse stress stimuli, we tested whether ceramide may trigger this cascade by inducing PKR activation. To determine whether ceramide can promote the activation of PKR in a RAX-dependent manner, the effect of C₂-ceramide treatment on eIF2α phosphorylation was assessed. Cells were metabolically radiolabeled with [³²P]orthophosphate and treated with C₂-ceramide for 30 min, followed by incubation with a ¹⁴C-radiolabeled amino acid mixture for 10 min at 37 °C and precipitation of protein by the addition of trichloroacetic acid as previously described (7). Protein synthesis rates were determined by measuring the amount of radioactivity incorporated in the acid-precipitable fraction. The results indicated that ceramide inhibited protein synthesis in a dose-dependent manner (Fig. 5). Cells that were treated with >10 μM C₂-ceramide exhibited a markedly reduced level (i.e. ~60%) of nascent protein synthesis compared with untreated cells. Collectively, these findings strongly support a mechanism whereby ceramide can promote RAX activation and inhibit protein synthesis.

Next, a role for PKR in ceramide-mediated inhibition of protein synthesis was examined using 2-AP. The results revealed that the inactive ceramide analog (C₂-dihydroceramide) failed to inhibit protein synthesis, whereas 50 μM C₂-ceramide resulted in potent inhibition of protein synthesis (Fig. 7). Importantly, ceramide-induced inhibition of protein synthesis was prevented when cells were co-treated with 2-AP (Fig. 7). These results indicate a role for PKR in this process and suggest a novel role for ceramide in the regulation of protein synthesis involving both RAX and PKR.

DISCUSSION

The role of PKR in viral host defense is well known (17, 18). In response to viral double-stranded RNA, host PKR is activated, resulting in the phosphorylation of eIF2α with a shutdown of protein synthesis to prevent viral replication (13–16). Yet it is only recently that cellular activation of PKR has been established (7). The identification of RAX/PACT as a cellular PKR activator and its participation in programmed cell death suggest an important link between the regulatory mechanisms
that mediate cell survival and protein synthesis (22–25). But how these pathways may be linked remains unclear. One mechanism for linkage would be via a second signal molecule that is rapidly generated such as ceramide (1–5). Considering that ceramide production is associated with most, if not all, stress stimuli (1, 3), we examined whether ceramide may provide the link between these pathways. Our findings clearly indicate that ceramide can regulate cell survival by multiple mechanisms involving diverse signal pathways. For example, ceramide can affect transcriptional mechanisms (e.g. c-Jun) (4, 34). In addition, ceramide was found to regulate stress-signaling pathways involved in apoptosis by stimulating dephosphorylation of Bcl2 and Bad (39, 40). Now we have discovered a novel mechanism whereby ceramide can regulate protein synthesis by a mechanism(s) involving both PKR and RAX. PKR is likely involved since 2-AP can reverse ceramide-induced killing (Fig. 2) and ceramide-induced inhibition of protein synthesis (Fig. 7). In addition, ceramide promotes RAX/PKR association, which strongly correlates with PKR activation and inhibition of protein synthesis (Fig. 4) (22). Although it was not formally tested whether RAX may also activate another cellular eIF2α kinase such as HRI (heme-regulated inhibitor of translation) (9) or PERK (41), an association of RAX with any other eIF2α kinase has yet to be reported. Thus, the findings presented here strongly suggest that the upstream event triggering the inhibition of protein synthesis involves RAX activation of PKR. The precise mechanism by which phosphorylated RAX may activate PKR remains to be elucidated. PKR is a ribosomal protein that is apparently activated when it is released from the ribosome (42, 43). Recently, PKR has been shown to interact with the L18 ribosomal protein (44). Interestingly, L18 inhibits PKR autophosphorylation, and phosphorylation of eIF2α is blocked in vitro and in vivo (44). Since ribosome-associated PKR is in a monomeric (inactive) form (45), it is possible that activation of PKR coincides with displacement from the ribosome (43). Since RAX can associate with and activate PKR, it is tempting to speculate that at least one mechanism by which RAX may activate PKR is by blocking L18 binding to PKR. A role for ceramide in this potential mechanism remains to be determined.

An elaborate homeostatic regulatory mechanism for apoptosis involving ceramide is beginning to emerge. On the one hand, during stress that can lead to cell death, ceramide activates protein phosphatases such as protein phosphatase 2A that can promote the functional dephosphorylation and inactivation of anti-apoptotic signal molecules, including Bcl2 (38), Akt (46), and protein kinase C (33). On the other hand, ceramide can directly activate SAPKs such as JNK (Fig. 4) (4, 32). Although it remains to be determined which SAPK(s) may be responsible for RAX phosphorylation, a role for ceramide in inhibiting protein synthesis may involve activation of a ceramide-activated SAPK to phosphorylate RAX and lead to PKR activation, eIF2α phosphorylation, inhibition of protein synthesis, and apoptosis (Fig. 8). Further studies are necessary to identify a physiologic RAX kinase to fully understand the mechanism(s) by which protein synthesis may be negatively regulated under stress.

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