UV-C Light Induces Raft-associated Acid Sphingomyelinase and JNK Activation and Translocation Independently on a Nuclear Signal*

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The initiation of UV light-induced signaling in mammalian cells is largely considered to be subsequent to DNA damage. Several studies have also described ceramide (CER), a lipid second messenger, as a major contributor in mediating UV light-induced c-Jun N-terminal kinase (JNK) activation and cell death. It is demonstrated here that UV-C light irradiation of U937 cells results in the activation and translocation of a Zn²⁺-independent acid sphingomyelinase, leading to CER accumulation in raft microdomains. These CER-enriched rafts aggregate and play a functional role in JNK activation. The observation that UV-C light also induced CER generation and the externalization of acid sphingomyelinase and JNK in human platelets conclusively rules out the involvement of a nuclear signal generated by DNA damage in the initiation of a UV light response, which is generated at the plasma membrane.

Mammalian cells respond to UV light irradiation by activating a complex signaling network that implies radical oxygen species (ROS) production, activation of transcription factors, and stimulation of kinases (1). Among the latter, the c-Jun N-terminal kinase (JNK), a main regulator of the AP-1 transcription factor, is considered as one of the most critical components of the UV light response. Indeed, the JNK/AP-1 pathway has been implicated in various UV light effects depending on the cellular model, including tumor promotion (2), apoptosis (3), and cell cycle arrest (4). Therefore, it is not surprising that the characterization of the signal transduction pathway leading to JNK activation has attracted a great deal of attention. From these studies, ceramide (CER), a lipid second messenger, has emerged as a major contributor in mediating UV light-induced JNK activation (5). Further studies have confirmed the general function of CER in stress-activated JNK activation and in mediating cell death (6).

The mechanism by which CER is produced upon UV light activation has been investigated. Hitherto, two main metabolic pathways have been identified for CER accumulation, namely hydrolysis from sphingomyelin (SM) through sphingomyelinase (SMase) stimulation and de novo synthesis by CER synthase activation. The latter appears not to be involved in UV light-induced CER production. Indeed, UV-A, -B, and -C light induce SM hydrolysis in most cellular models because of SMase stimulation (7), although in human keratinocytes a third, non-enzymatic mechanism of CER formation has been described (8). Despite some controversies, it appears that both neutral SMase (N-SMase) and acid SMase (A-SMase) have been implicated in UV light-induced CER production and apoptosis, depending on the cellular origin and experimental conditions (9, 10). However, the functional role of A-SMase, but not N-SMase, in UV light-induced JNK activation has been established (11).

Although A-SMase stimulation appears to be a critical upstream event for JNK activation, the enzyme and how it operates following UV light irradiation have not been determined. The A-SMase gene encodes for at least two forms of A-SMase produced by post-translational processing, namely a lysosomal form (L-A-SMase), which is lacking in Niemann-Pick disease, and a so-called secretory form (S-A-SMase) identified by Schissel et al. (12). S-A-SMase targets the plasma membrane, requires exogenous Zn²⁺ for activity, and has been involved in the cellular response to inflammatory cytokines (13). Finally, a third form of Zn²⁺-independent A-SMase has been identified recently that is present on the cell membrane surface of CD95- or CD40-stimulated cells (14). Finally, it remains to be determined what role, if any, does UV-C light-mediated DNA damage and, hence, a potential intranuclear signaling cascade have in initiating A-SMase activation.

Based on these considerations, we hypothesized that UV light irradiation may induce the activation and translocation of a Zn²⁺-independent A-SMase independently of a nuclear signal, resulting in CER formation in raft microdomains. We also hypothesized that CER-enriched membrane platform formation plays a role in JNK activation.

MATERIALS AND METHODS

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Drugs and Reagents—Silica gel 60 thin layer chromatography plates were from Merck (Darmstadt, Germany). Aquasafe 300 scintillation mixture was purchased from EG & G Wallac (Evry, France), SR33557 was kindly provided by Dr. J. M. Herbert (Sanofi-Synthelabo, Toulouse, France), and maltose-binding protein-lyslein was provided by Dr. T. Kobayashi (Lipid Biology Laboratory, RIKEN, Saitama, Japan.) All other drugs and reagents, unless specified, were purchased from Sigma (St. Quentin Fallavier, France) or Alexis Biochemicals (Paris, France).
**Cell Culture**—The human myeloblastic cell line U937, obtained from the American Type Culture Collection (Manassas, VA) was cultured in RPMI 1640 medium at 37 °C in 5% CO₂. The culture medium was supplemented with 10% heat-inactivated fetal calf serum (Amersham Biosciences). Normal human lymphoblast cells or Niemann-Pick disease lymphoblast MS1418 was a generous gift from Prof. T. Levade (INSERM U468, CHU Rangueil, Toulouse, France).

**Cell Irradiation**—U937 cells were irradiated with UV-C light (254 nm) in PBS for 30 s, corresponding to 30 joules/m² at a concentration of 1 million cells/ml.

**Sphingomyelin Hydrolysis**—SM quantitation was performed by labeling cells to isotopic equilibrium with 0.5 μCi/ml [methyl-³H]choline (81 Ci/mmol; Amersham Biosciences, Sascary, France) for 48 h in complete medium. CER was processed by labeling cells to isotopic equilibrium with 1 μCi/ml [³H]choline (33 Ci/mmol; Amersham Biosciences) for 48 h in complete medium as described previously (16). Cells were then washed with PBS and resuspended in serum-free medium for kinetic experiments. Lipids were extracted and resolved by thin layer chromatography and were detected by autoradiography (5:3:1). Lipids were identified by comparing the metabolic products.

**Confocal Microscopy**—Cells were irradiated with 30 J/m² of UV-C light and incubated with 15 μg/ml cholera toxin subunit B conjugated to Cy5 (Molecular Probes) for 20 min. Cells were then fixed with 0.5% paraformaldehyde (w/v) in PBS and washed with PBS containing 3% BSA (w/v) and 2 μM HEPES (BPS-BSA). Cells were then incubated for 20 min with either a rabbit polyclonal anti-ROS (Abcam), an anti-JNK (Santa Cruz Biotechnology) and anti-ROCK (Cell Signaling Technology) and anti-phospho stress-activated protein kinase/JNK (Cell Signaling Technology) and anti-JNK (Santa Cruz Biotechnology) antibodies. Bound proteins were detected by enhanced chemiluminescence (Amersham Biosciences).

**Fluorescence-activated Cell Sorter Analysis**—Cells were irradiated with 30 J/m² of UV-C light, fixed for 10 min in 4% paraformaldehyde (w/v) in PBS and washed with PBS containing 3% BSA (w/v) and 1 μM HEPES (BPS-BSA). Cells were then incubated for 20 min with either a rabbit polyclonal anti-ROS (Abcam), an anti-JNK (Santa Cruz Biotechnology) and anti-phospho stress-activated protein kinase/JNK (Cell Signaling Technology) and anti-JNK (Santa Cruz Biotechnology) antibodies. Bound proteins were detected by enhanced chemiluminescence (Amersham Biosciences).

**Gradient Centrifugation**—Total cellular CER was quantitated using a FACScan cytomter (BD Biosciences). Samples were analyzed in the FACScan mode, using a single-laser (488 nm) and two-photon detectors (FL1, 530–540 nm; FL2, 575–585 nm).

**Western Blotting**—Each aliquot was subjected for 5 min at 95 °C to denaturating buffer (62.5 mM Tris pH 6.8, 10% glycerol, 2% SDS, 0.04% bromophenol blue, and 0.005% SDS) and subjected to electrophoresis in a 10% SDS-polyacrylamide gel. The protein bands were transferred to a nitrocellulose membrane (Hybond-C, Amersham Biosciences) and blocked with 10% nonfat milk in Tris-buffered saline-Tween 20 (0.1%) for 2 h, the filter was incubated overnight at 4 °C with the β-subunit of cholera toxin, which has an affinity for GM1. The filter was then washed, and bound proteins were detected by enhanced chemiluminescence (Amersham Biosciences). A specific signal was detected using a 1:1000 dilution of rabbit polyclonal anti-ROS (Abcam) and anti-JNK (Santa Cruz Biotechnology) antibodies. Bound proteins were detected by enhanced chemiluminescence (Amersham Biosciences).
Experiments performed in triplicate. Non-irradiated control A-SMase values were shown in Fig. 1.

**Table I. Effect of SR33557 on UV-C light-induced A-SMase externalization.**

| µM | Fluorescence (U937 cells) |
|----|--------------------------|
| None | 167 ± 15 |
| 1    | 171 ± 8 |
| 6    | 116 ± 9 |
| 10   | 107 ± 11 |
| 30   | 96 ± 7 |

Note: Results are expressed as the percentage of total cell surface fluorescence compared to non-irradiated controls and the mean of three independent experiments ± S.D.

**Fig. 1.** Effect of UV-C light on intracellular CER levels and SMase activity. A and B, U937 cells were prelabeled with [9,10-3H]palmitic acid (A) or [methyl-3H]choline (B) to equilibrium for 48 h and then treated with 30 J/m² UV-C light. C and D, neutral SMase activity (C) and A-SMase activity (D) were quantitated as described under “Materials and Methods.”

**Fig. 2.** Effect of UV-C light on intracellular CER levels and SMase activity. A and B, U937 cells were prelabeled with [9,10-3H]palmitic acid (A) or [methyl-3H]choline (B) to equilibrium for 48 h and then treated with 30 J/m² UV-C light. C and D, neutral SMase activity (C) and A-SMase activity (D) were quantitated as described under “Materials and Methods.”

To confirm the role of rafts microdomains, we pretreated U937 cells with the cholesterol-sequestering agent MβCD under conditions where it is still possible to isolate rafts but with their content in cholesterol significantly reduced (~50%). UV-C light induced a time-dependent recruitment of A-SMase to the outer leaflet of the cell membrane as revealed by flow cytometry analysis performed with a FITC-coupled anti-A-SMase antibody on non-permeabilized cells (Fig. 3A). Confocal analysis of A-SMase accumulation on the cell surface was detected as early as 10 min and peaked at 12 min, after which the fluorescence signal decreased and returned to basal level after 15 min (data not shown). These results suggested that, upon UV light activation, a fraction of internal A-SMase was rapidly and transiently externalized to the cell surface. Moreover, the temporal association between A-SMase stimulation and externalization suggested that activation was a critical event for enzyme relocation.

**UV-C Light-induced SMase Translocation to the Plasma Membrane Outer Leaflet**—We next investigated the subcellular distribution of A-SMase in UV-C light-treated cells. UV-C light induced a time-dependent recruitment of A-SMase to the outer leaflet of the cell membrane as revealed by flow cytometry analysis performed with a FITC-coupled anti-A-SMase antibody on non-permeabilized cells (Fig. 3A). Confocal analysis of A-SMase accumulation on the cell surface was detected as early as 10 min and peaked at 12 min, after which the fluorescence signal decreased and returned to basal level after 15 min (data not shown). These results suggested that, upon UV light activation, a fraction of internal A-SMase was rapidly and transiently externalized to the cell surface. Moreover, the temporal association between A-SMase stimulation and externalization suggested that activation was a critical event for enzyme relocation.

**UV-C Light-induced A-SMase Translocation to Raft Microdomains**—Based on our biochemical studies, we hypothesized that upon UV-C light activation, A-SMase relocation was not a random process but that A-SMase was redirected toward raft microdomains. We first investigated whether UV-C light activation could interfere with raft distribution by using a Cy3-coupled antibody directed against flotillin (data not shown).
shown) or Cy5-coupled cholera toxin, which specifically binds the raft component ganglioside GM1. As shown in Fig. 4A, UV light irradiation induced a rapid and marked reorganization of rafts into larger platforms, producing a capping effect on the cells at 12 min post-irradiation (10% GM1 clustering in controls (depending on the experiment) compared with >80% in irradiated cells). Moreover, in irradiated cells A-SMase colocalized with cholera toxin, suggesting that the enzyme translocated into raft microdomains (Fig. 4B). Based on these results, we hypothesized that CER production preferentially occurred at the raft level. Indeed, upon UV-C light activation CER co-localized with GM1 as shown in Fig. 4C. These results strongly suggested that plasma membrane microdomains are essential constituents in UV-C light-mediated A-SMase activation and externalization. Because SM is an essential constituent of plasma membrane rafts, we elected to investigate SM distribution in irradiated U937 cells. As shown in Fig. 4D, the SM-specific toxin lysenin (here coupled to maltose-binding protein) bound uniformly to the plasma membrane of control U937 cells, as described previously for normal fibroblasts (41). However, there appeared to be a modest redistribution after irradiation that colocalized with GM1 aggregation.

To further confirm the role of rafts in our study, we pre-treated U937 cells with MβCD. As shown in Table II, UV-C light-induced A-SMase externalization was completely inhibited in MβCD-treated cells. Moreover, we observed, using con-
focal microscopy, that both CER and GM1 redistribution in UV-C light-treated cells were inhibited (as expected) in MβCD-treated cells (data not shown). These results suggested that UV-C light activated an ordered signaling cascade consisting of A-SMase activation and relocalization into raft microdomains, SM consumption, and CER release, resulting in the formation of large CER-enriched platforms. To further confirm this hypothesis, we compared A-SMase externalization under UV-C light treatment in normal and Niemann-Pick disease lymphoblasts. Again, UV-C light treatment induced significant A-SMase externalization in normal lymphoblasts, which was comparable with that observed in U937 cells but not in Niemann-Pick disease cells (Table II).

**Fig. 4. UV-C light-induced A-SMase translocation to raft microdomains.** U937 cells were irradiated or not irradiated with UV-C light at 30 J/m². 12 min post-irradiation, cells were analyzed by confocal microscopy using cholera toxin subunit B conjugates with Cy5 (blue emission) (A–D), rabbit FITC/anti-A-SMase (B), rabbit FITC/anti-CER (C) (green emission), or rabbit Cy3/anti-maltose-binding protein-lysénin (red emission) (D). Turquoise and purple are FITC/Cy5 and Cy3/Cy5 merge, respectively. Results are representative of three independent experiments.

**ROS Regulates A-SMase Activation and Translocation to Raft Microdomains**—In an attempt to characterize the signaling pathways leading to A-SMase activation induced by UV-C light, we first studied the role of ROS in UV-C light signaling. Indeed, treatment of U937 cells with UV-C light resulted within 5 min in a burst of H₂O₂ production (Fig. 5A), stimulation of A-SMase activity (Fig. 5B), and translocation of A-
SMase activity to the outer leaflet of the plasma membrane (Fig. 5C). Moreover, inhibiting UV-C light-induced ROS production by PDTC abolished both A-SMase activation (Fig. 6A) and membrane externalization (Fig. 6B).

**JNK Is Activated and Relocalized into Raft Fractions Following UV-C Light Irradiation—**CER has been described as triggering distinct intracellular signaling pathways, including the stimulation of JNK (5). Therefore, we evaluated whether UV-C light could activate this pathway through CER production. These experiments showed that, in U937 cells, treatment with UV-C light resulted in rapid (as early as 10 min) and prolonged (up to 24 h) JNK phosphorylation (Fig. 7A). Furthermore, the inhibition of A-SMase, by pretreatment with SR33557 (data not shown) or desipramine, another potent A-SMase inhibitor (23), blocked UV-C light-induced JNK kinase activity using the myelin basic protein as substrate (Fig. 7B). Moreover, we observed that under UV-C light treatment, JNK and a fraction of P-JNK was redistributed toward raft microdomains (Fig. 7C) and that this translocation was also inhibited by desipramine and MβCD (data not shown). By confocal microscopy, we clearly observed P-JNK externalization in UV-C light-treated cells, a portion of which colocalized with GM1 (Fig. 7D). These results suggested that SM-derived CER, resulting in CER-enriched rafts, mediated the stimulatory effect of UV-C light on these signaling kinases.

**UV-C Light-induced CER Production and JNK Activation Is Not Dependent on a Nuclear Signal—**It has been suggested that cell signaling induced by UV-damaged DNA in the nucleus is rapidly transferred to the cytosol, leading to downstream events (25, 26). To determine whether a nucleus is necessary for UV-C light-induced CER generation, we attempted to prepare U937 cytoplasts. However, karyophilic staining revealed that enucleation efficiency was <70%, making it impossible to interpret a clear result. Therefore, we elected to irradiate platelets with UV-C light. As shown in Fig. 8, A and B, exposure to UV-C light induced CER generation and A-SMase translocation to the external leaflet of the plasma membrane similar to that in observed in intact U937 cells. Furthermore, because platelets also express JNK (42), we were able through flow cytometry analysis to demonstrate JNK externalization in UV-C light-treated platelets (Fig. 8C). Hence, as described previously by Devary et al. (27), UV-C light-induced JNK activation was observed to be independent of a nucleus. These experiments conclusively rule out the involvement of a nuclear signal generated by DNA damage in the production of CER.
Indeed, not only was UV light-induced A-SMase stimulation found to be Zn\(^{2+}\)/H\(_{11001}\)-independent, but UV-C light irradiation also resulted in an increase in A-SMase localized on the outer leaflet of the cell surface. This finding suggested that, upon UV-C light stimulation, A-SMase probably translocated from the cytoplasm to the external leaflet of the plasma membrane by intracellular vesicles, as this event was found to be brefeldin-sensitive (29). Because we were unable to detect any modification of the total intracellular distribution of A-SMase by confocal microscopy following UV-C light irradiation, only a very small sub-population fraction of the enzyme was implicated.

Little is known about the regulation of A-SMase in the context of stress response. ROS has been described as one of the major signaling components of the UV light response (30, 31). For this reason, we investigated whether ROS could be involved in the stimulation and/or the translocation of the enzyme. Our study shows that the antioxidant PDTC, a ROS scavenger, not only inhibited A-SMase stimulation but also prevented its translocation to the cell surface. Indeed, UV light-induced ROS generation could be localized in raft microdomains (32). Of course, one cannot rule out the possibility that UV-C light induced cholesterol peroxidation; however, UV-C light did not induce ROS generation in Niemann-Pick disease cells (data not shown). Hence, our study not only shows that ROS operated upstream of A-SMase but also suggests that stimulation of the enzyme is required for its translocation to the cell surface. This hypothesis is supported by the fact that SR33557, an inhibitor of A-SMase activity, also abrogated its translocation to the cell surface.

This study also shows that UV-C light induced significant redistribution of GM1, suggesting a major reorganization of raft microdomains. Indeed, we found that non-irradiated cells displayed dispersed GM1 distribution at the surface of the cell,
Light at 30 J/m². Results are representative of three independent experiments. It has been proposed that it is the consumption of SM in cytoskeleton, and sustained cell signaling and activation (33, 34). It has been suggested in which the T cell receptor-asso-

and photocarcinogenesis.

Furthermore, our results are consistent with the hypothesis of Karin and colleagues, as we observed that the initiation of UV cell response (i.e. CER generation, A-SMase and JNK activation, and externalization to the external plasma membrane) was similarly observed in platelets. A recent study also proposed that mitochondrial CER generation was implicated in UV signaling (38), perhaps as a consequence of mitochondrial DNA damage (39). In the field of cell response to UV light, our observations should provide new investigative paths into UV light-induced photocaging, immunosuppression, and photocarcinogenesis.

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Finally, our study questions the involvement of a nuclear signal generated by UV light-induced DNA damage in the induction of A-SMase activation and CER generation. Indeed, it is generally postulated that UV light response occurs by induction of a nuclear signaling cascade by damaged DNA (25). However, Karin and colleagues proposed that UV light response (such as JNK activation) was likely to be initiated at or near the plasma membrane through alterations at the cell surface leading to receptor clustering (27, 37). In our study we also observed UV-C light-induced JNK activation in U937 cells, as well as its translocation to the outer surface of raft microdomains. Furthermore, our results are consistent with the hypothesis of Karin and colleagues, as we observed that the initiation of UV cell response (i.e. CER generation, A-SMase and JNK activation, and externalization to the external plasma membrane) was similarly observed in platelets. A recent study also proposed that mitochondrial CER generation was implicated in UV signaling (38), perhaps as a consequence of mitochondrial DNA damage (39). In the field of cell response to UV light, our observations should provide new investigative paths into UV light-induced photocaging, immunosuppression, and photocarcinogenesis.

whereas upon UV activation GM1 concentrated to one pole of the cell in a majority of cells. One could speculate that UV-C light induces the aggregation of raft components, leading to the formation of a major polarized signaling microdomain. Indeed, a model has been suggested in which the T cell receptor-associated signaling machinery initiates raft aggregation by promoting F-actin reorganization, permitting full activation of the tyrosine phosphorylation cascade, reorganization of the actin cytoskeleton, and sustained cell signaling and activation (33, 34). It has been proposed that it is the consumption of SM in rafts by A-SMase that results in the formation of large CER-enriched membrane domains which, in turn, could facilitate
UV-C Light Signaling

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