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A Potential Role for Ceramide in the Regulation of Mouse Epidermal Keratinocyte Proliferation and Differentiation

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We have previously determined that sustained phospholipase D (PLD) activation is associated with differentiation induction in primary mouse epidermal keratinocytes. We therefore investigated the effect of two bacterial PLD on keratinocyte proliferation and differentiation. We found that *Streptomyces sp.* PLD was much less potent at inhibiting proliferation than *S. chromofuscus* PLD, with a half-maximal inhibitory concentration of 0.05 *versus* less than 0.001 IU per ml for *S. chromofuscus* PLD. Similarly, *S. chromofuscus* PLD stimulated transglutaminase activity more effectively and potently than *S. sp.* PLD. When we examined the formation of products by the two PLD, we found that the *S. sp.* PLD showed higher activity at all concentrations. Whereas the PLD from *S. sp.* is relatively inactive on sphingomyelin, *S. chromofuscus* PLD is known to hydrolyze both glycerophospholipids and sphingomyelin. Based on recent data indicating a role for ceramide in regulating cell growth and differentiation, we hypothesized that the ability of *S. chromofuscus* PLD to hydrolyze sphingomyelin might underlie its greater potency. Therefore, we examined the effect of exogenous sphingomyelinase and synthetic ceramides on DNA synthesis. We found that sphingomyelinase exhibited a potent concentration-dependent effect on [^{3}H]thymidine incorporation, much like *S. chromofuscus* PLD. Synthetic cell-permeable ceramides (C_6- and C_2-ceramide) also concentration dependently inhibited DNA synthesis, with a half-maximal inhibitory concentration of ≈12 μM. Finally, we obtained evidence suggesting that ceramide is generated in response to a physiologically relevant agent, because tumor necrosis factor-α, a known effector of sphingomyelin turnover in other systems and a cytokine that is produced and released by keratinocytes, increased ceramide levels in primary epidermal keratinocytes. ***Key words: diacylglycerol/phosphatidic acid/phospholipase D/tumor necrosis factor-α. J Invest Dermatol 110:318–323, 1998***

There is accumulating evidence for a role of lipid signaling systems other than phosphoinositide-specific phospholipase C in regulating various cellular processes (reviewed in Exton, 1990; Liscovitch, 1992; Hannun, 1994; Liscovitch and Cantley, 1994; Zhang and Kolesnick, 1995). In particular, both phospholipase D (PLD)-mediated phospholipid hydrolysis and sphingomyelinase-catalyzed sphingomyelin hydrolysis are thought to result in the production of lipid second messengers. PLD activity yields phosphatidic acid (PA), which may itself be a lipid signal or may be dephosphorylated by phosphatidate phosphohydrolase to form diacylglycerol (DAG). Sphingomyelinase, on the other hand, generates ceramide, which, like DAG, activates particular effector enzymes. In the case of DAG, the effector enzyme is protein kinase C, a family of phospholipid-dependent protein kinase isoforms (reviewed in Nishizuka, 1995). It is likely that ceramide also functions through a protein kinase, the recently discovered ceramide-activated protein kinase, but it may also regulate the activity of a protein phosphatase (reviewed in Hannun, 1994; Zhang and Kolesnick, 1995).

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Abbreviations: DAG, *sn*-1,2-diacylglycerol; IC_{50}, half-maximal inhibitory concentration; PA, phosphatidic acid; PBS^-, phosphate-buffered saline lacking calcium and magnesium; PEt, phosphatidylethanol; PLD, phospholipase D; SFKM, serum-free keratinocyte medium.

Both of these pathways have been shown to modulate differentiated functions in several cell systems. For instance, PLD activity has been suggested to underlie chemotactic peptide-stimulated superoxide anion formation and degranulation of neutrophils (Billah *et al*, 1989), phorbol ester-induced prostaglandin production by a kidney cell line (Sciorra and Daniel, 1996), insulin-elicited glucose uptake in a skeletal muscle cell line (Standaert *et al*, 1996), angiotensin II-mediated aldosterone secretion from adrenal glomerulosa cells (Bollag *et al*, 1990), and gonadotropin-releasing hormone-stimulated steroidogenesis in ovarian granulosa cells (Liscovitch and Amsterdam, 1989). The sphingomyelin pathway has also been implicated in regulating differentiation, as agents that induce monocytic differentiation of HL-60 leukemic cells [1,25-dihydroxyvitamin D_3, tumor necrosis factor-α (TNF-α), and interferon-γ] trigger sphingomyelin hydrolysis, and synthetic ceramides and exogenous sphingomyelinase can mimic these effects (Okazaki *et al*, 1989; Okazaki *et al*, 1990; Kim *et al*, 1991). The role of these two signaling systems (PLD activation and/or sphingomyelin hydrolysis) in keratinocytes is unclear; however, PLD has been implicated in the sustained elevation in DAG content and induction of differentiation in response to ganglioside GQ_{1b} (Yada *et al*, 1991; Seishima *et al*, 1995), and synthetic cell-permeant ceramides have been reported to inhibit proliferation and stimulate differentiation (Wakita *et al*, 1994) in a human squamous cell carcinoma cell line.

We have recently shown that bradykinin, which triggers phosphoinositide turnover in keratinocytes (Talwar *et al*, 1990; Johnson *et al*, 1992), also activated PLD in primary mouse epidermal keratinocytes.[1] Interestingly, however, this activation was transient, such that PLD activity returned to basal levels by 15 min in the continued presence of the hormone. In addition, we proposed that the transience of the

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response underlies the minimal effect of bradykinin on keratinocyte growth, in contrast to the phorbol ester 12-O-tetradecanoyl 13-phorbol acetate, which both profoundly inhibited proliferation and elicited sustained PLD activation.[1]

In this study we examine the effects of two exogenous PLD on keratinocyte growth and differentiation. One PLD from *Streptomyces chromofuscus* is known to hydrolyze both sphingomyelin and glycerophospholipids (Imamura and Horiuti, 1979); the other from *Streptomyces sp.* is reported to be relatively inactive towards sphingomyelin (Dr. Greg Wall, Sigma Technical Service, personal communication, and Table I). We found that the glycerophospholipid specific *S. sp.* PLD was less potent than the *S. chromofuscus* PLD in inhibiting DNA synthesis and stimulating transglutaminase activity, a marker of keratinocyte differentiation. Furthermore, bacterial sphingomyelinase and synthetic ceramides were able to produce similar effects, suggesting a role for sphingomyelin turnover and ceramide generation in regulating keratinocyte growth and differentiation. Finally, we provide evidence that this signaling pathway may have a physiologic role in keratinocytes because TNF-α, a cytokine produced by these cells (Wood *et al*, 1992), increased ceramide levels.

MATERIALS AND METHODS

Cell culture Primary epidermal keratinocytes were prepared from 2 to 3 d old neonatal ICR mice (Harlan Sprague Dawley) according to the method of Marcelo *et al* (1978). Briefly, after trypsinization of the skin, the epidermis was mechanically separated from the dermis and epidermal cells were released by scraping. These cells were then purified by centrifugation through Ficoll and plated in 6 well dishes (Corning, Corning, NY; or Falcon, Franklin Lakes, NJ) in a fetal bovine serum-containing RPMI medium. After 4 h, the cells were washed with phosphate-buffered saline lacking calcium and magnesium (PBS^-) and re-fed with serum-free keratinocyte medium (SFKM), containing 25 μM calcium, 90 μg bovine pituitary extract per ml, ITS (5 μg insulin per ml + 5 μg transferrin per ml + 5 ng selenious acid per ml), 5–6 ng epidermal growth factor per ml, 2 mM glutamine, 0.05% bovine serum albumin, 100 U penicillin per ml, 100 μg streptomycin per ml, and 0.25 μg fungizone per ml, as in Bollag *et al* (1993). Cells were re-fed with fresh medium every 1–3 d.

Measurement of DNA synthesis For measurement of [^{3}H]thymidine incorporation, near-confluent cultures (at densities indicated in the figure legends) were re-fed with SFKM containing various concentrations of the indicated agents. After 24 h in the presence of these agents, cells were labeled with 1 μCi [^{3}H]thymidine per ml for 1 h. Cultures were washed twice with PBS^- and reactions terminated using ice-cold 5% trichloroacetic acid. Cells were washed with an additional volume of 5% trichloroacetic acid and with distilled water, and were solubilized in 0.3 M NaOH. An aliquot of this NaOH extract was counted in a liquid scintillation spectrometer (Packard, Sterling, VA).

For density determination, following trypsinization, a single-cell suspension was generated by repeated aspiration of the cells through an 18 gauge needle and the cells enumerated using an electronic particle counter (Coulter Electronics, Hialeah, FL).

Measurement of transglutaminase activity Near-confluent keratinocytes were incubated for 18 h with the indicated amounts of bacterial PLD in SFKM. The cells were then washed with PBS^-, scraped into homogenization buffer [0.1 M Tris-acetate, pH 7.8, 2 μg aprotinin per ml, 2 μM leupeptin, 1 μM pepstatin A, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 0.2 mM phenylmethylsulfonyl fluoride, and 0.1% β-mercaptoethanol], placed into microcentrifuge tubes and pelleted by centrifugation. The supernatants were removed and the cell pellets subjected to one freeze-thaw cycle prior to disruption by sonication in homogenization buffer. Aliquots of the homogenate were incubated for 14 h at 37°C with 2 μCi per sample [^{3}H]putrescine in a reaction mixture containing 1% dimethylated Hammerstein casein, 0.1 M Tris-acetate (pH 8.5), 10 mM $CaCl_2$, 0.1 mM EDTA, 5 mM dithiothreitol, and 0.1% Triton X-100 (final concentrations). Reactions were terminated by adding trichloroacetic acid (to a final concentration of 5%) and the samples filtered through glass fiber filters. After thorough washing with 5% trichloroacetic acid, the filters were counted by liquid scintillation spectrometry. Protein content of the samples was determined using the micro-BCA protein assay (Pierce, Rockford, IL) with bovine serum albumin as standard.

Table I. *Streptomyces chromofuscus* PLD hydrolyzes sphingomyelin

Treatment[a]	[^{3}H]Sphingomyelin (% control)
Control	100
S. chromofuscus PLD	25.9 ± 6.6[b,d]
S. sp. PLD	82.2 ± 0.5[c]
Sphingomyelinase	24.3 ± 0.7[b,d]

[a][^{3}H]Choline-labeled lipid extracts were incubated for 60 min with 1 IU per PLD per ml from *S. chromofuscus* or *S. sp.* or with 0.05 U PLD per ml from *B. cereus* sphingomyelinase. Radiolabeled sphingomyelin remaining following treatment was then determined as described in *Materials and Methods* and expressed as a percentage of the control (untreated) sample. Data represent the means (± SEM) of three experiments performed in duplicate with control values of 23,370 ± 54, 23,490 ±45, and 12,350 ± 100 dpm per sample.
[b]$p<0.001$ *versus* control.
[c]$p<0.05$ *versus* control.
[d]$p<0.001$ *versus S. sp.* PLD.

Measurement of radiolabeled PA and phosphatidylethanol (PEt) production Radiolabeled PA and PEt production was measured in [^{3}H]oleate-prelabeled primary epidermal keratinocytes as in Bollag *et al* (1990). Briefly, near-confluent cells were labeled in SFKM containing 2.5 μCi [^{3}H]oleic acid per ml for 20–24 h. The medium was then aspirated and replaced with SFKM lacking radiolabel. After a 30 min preincubation period, cells were stimulated with the appropriate agonists and incubated for 30 min in the presence of 0.5% ethanol. The cells were then solubilized in 0.2% sodium lauryl sulfate containing 5 mM EDTA and extracted for 1–2 h with ice-cold chloroform/methanol to which acetic acid (1:2:0.04 vol:vol:vol) was added. Additional chloroform and 0.2 M NaCl were added to break phase, and the lower phases were collected and dried under N_2. Samples were resuspended in chloroform/methanol, also containing 25–50 μg PEt (Biomol, Plymouth Meeting, PA) and PA (Sigma, St. Louis, MO) per sample, and spotted onto heat-activated silica gel 60 thin-layer chromatography plates (0.25 mm thickness aluminum-backed with concentrating zone). Plates were developed in a solvent system consisting of the organic phase of a mixture of ethyl acetate/iso-octane/acetic acid/water (13:2:3:10) and were visualized with iodine vapor and with autoradiography using En^{3}Hance (New England Nuclear/Dupont, Boston, MA). Spots corresponding to PA and PEt, identified by comigration with authentic standards, were cut out, placed in liquid scintillation fluid, and quantitated.

Sphingomyelin hydrolysis Primary epidermal keratinocytes were labeled for ≈48 h with 0.1 μCi [^{3}H]choline per ml. After extensive washing with PBS^-, the cells were scraped in ice-cold methanol, transferred to test tubes containing chloroform and water in a final ratio of 2:1:0.8 vol:vol:vol and extracted for 1–2 h on ice. Additional volumes of chloroform and 0.2 M NaCl were added to break phase (for a final ratio of 2:2:1.6 chloroform:methanol:aqueous phase), and the lower phases collected. The lower phases were washed with methanol and 0.2 M NaCl and dried under N_2. The [^{3}H]choline-labeled lipid extracts were resuspended in sphingomyelinase buffer (100 mM Tris, pH 7.4, 6 mM $MgCl_2$, 0.1% Triton X100) by incubation at 37°C and vigorous vortexing. Aliquots were transferred to clean test tubes and *S. chromofuscus* PLD, *S. sp.* PLD, *Bacillus cereus* sphingomyelinase, or sphingomyelinase buffer (control) alone was added. The samples were incubated for 60 min at 37°C and reactions terminated by the addition of methanol. Lipids were extracted as above and the sphingomyelin present in each sample assayed using the method of Linardic and Hannun (1994). Briefly, lipid extracts were resuspended in sphingomyelinase buffer and 1 U *S. sp.* sphingomyelinase per ml was added. After incubation for ≈90 min, reactions were terminated by addition of chloroform and methanol, and [^{3}H]choline released into the aqueous phase (a measure of sphingomyelin present in the original sample) was determined following extraction according to the method of Folch *et al* (1957). We have confirmed the specificity of the assay in our laboratory using [^{3}H]choline-labeled phosphatidylcholine and sphingomyelin (unpublished data).

Ceramide content Ceramide content was determined using the DAG kinase assay. Briefly, cells were incubated in SFKM in the presence and absence of 10 nM TNF-α for 1 h. Reactions were terminated by the addition of ice-cold methanol; after removing the methanol to a clean test tube containing chloroform, cells were solubilized in 0.2% sodium lauryl sulfate and transferred to the appropriate test tubes. Lipids were extracted into the chloroform/methanol phase as described previously and ceramide content was determined using a bacterial DAG kinase to phosphorylate ceramide in the presence of [γ-^{32}P]ATP to yield [^{32}P]ceramide phosphate. [^{32}P]ceramide phosphate was then

[1] Jung EM, Betancourt-Calle S, Mann-Blakeney R, Bollag WB. Sustained phospholipase D activation is associated with keratinocyte differentiation. *Carcinogenesis*, manuscript in revision.

separated by thin-layer chromatography and quantitated using a PhosphorImager (Molecular Dynamics). This method has been previously described for the measurement of cellular DAG content by Preiss *et al* (1986), and was modified by our laboratory (Isales *et al*, 1989). We have confirmed the linearity of the assay for determining ceramide content in the range of 10 pmoles to 10 nmoles, using Type III ceramides (Sigma) as a standard (unpublished data).

Statistical analysis The significance of differences between mean values was determined using analysis of variance, as performed by the program Instat (GraphPad Software, San Diego, CA).

Materials Bovine serum albumin (fatty acid-free), trichloroacetic acid, dimethylated Hammerstein casein, sphingomyelinase (from *S. sp.* and *B. cereus*), phenylmethylsulfonyl fluoride, and PLD from *S. sp.* and *S. chromofuscus* were obtained from Sigma. Note that 1 IU of PLD will hydrolyze 1 μmole of choline from L-α-phosphatidyl-choline (egg yolk) per minute at 30°C at pH 5.6 (for *S. sp.* PLD) or pH 8.0 (for *S. chromofuscus* PLD). [Methyl-^{3}H]thymidine (specific activity, 40–60 Ci per mmol), [^{3}H]oleic acid, and [^{3}H]putrescine were purchased from DuPont/NEN (Wilmington, DE). Trypsin inhibitor and epidermal growth factor were purchased from Gibco (Gaithersburg, MD); calcium-free minimum essential medium was purchased from Specialty Media (Lafayette, NJ) or Biologos (Naperville, IL) and RPMI 1640 and PBS$^-$ from Mediatech (Herndon, VA). DAG kinase and the cell-permeant ceramides, N-acetylsphingosine (C_2 ceramide), N-acetylsphinganine (C_2 dihydroceramide), and N-hexanoylsphingosine (C_6 ceramide) were obtained from Biomol (Plymouth Meeting, PA). Aprotinin, leupeptin, and pepstatin A were purchased from Boehringer (Indianapolis, IN). Bovine pituitary extract was obtained from Upstate Biotechnology (P-Neurext; Lake Placid, NY) or Collaborative Research (Bedford, MA). ITS was purchased from Collaborative Research; all other tissue culture reagents were obtained from Hazleton Biologics (Lenexa, KS). Thin-layer chromatography plates were purchased from EM Science (Gibbstown, NJ). All other materials were of reagent grade.

RESULTS

***Streptomyces chromofuscus* PLD inhibits growth and stimulates differentiation of mouse epidermal keratinocytes more potently than *S. sp.* PLD** As shown in **Fig 1**, PLD from both *S. chromofuscus* and *S. sp.* inhibited [^{3}H]thymidine incorporation into DNA in primary mouse epidermal keratinocytes; however, whereas PLD from *S. chromofuscus* was effective at very low concentrations (below 0.001 IU per ml), the PLD from *S. sp.* exhibited a very different concentration response. *S. sp.* PLD was much less potent than that from *S. chromofuscus*, with a half-maximal inhibitory concentration (IC_{50}) of 0.05 *versus* less than 0.001 IU per ml for *S. chromofuscus* PLD. At higher concentrations, however, *S. sp.* PLD exhibited a much greater inhibitory effect, with a maximal inhibition of ≈90% *versus* about 50%. Boiling the enzymes completely destroyed their inhibitory effect (data not shown).

Similarly, *S. chromofuscus* PLD stimulated transglutaminase activity, a measure of keratinocyte differentiation, more effectively and potently than *S. sp.* PLD (**Fig 2**). Thus, *S. chromofuscus* PLD elicited an approximate 3.5-fold increase at 0.05 IU per ml. *S. sp.* PLD (0.5 IU per ml), on the other hand, induced only about a maximal 2-fold rise in transglutaminase activity, which was not statistically significant.

Formation of products by the two PLD is comparable but *S. chromofuscus* PLD hydrolyzes sphingomyelin in addition to glycerophospholipids Because the two bacterial PLD exhibit different pH optima (8.0 for *S. chromofuscus* and 5.6 for *S. sp.*), we hypothesized that perhaps *S. chromofuscus* PLD was more active at physiologic pH; however, when we examined the radiolabeled PEt, a novel phospholipid that serves as a marker for PLD activity, and PA formed by the two PLD (**Fig 3**), we found that the *S. sp.* PLD showed higher activity at all concentrations, including 0.005 IU per ml, a concentration at which *S. chromofuscus* PLD was much more effective at inhibiting DNA synthesis. We therefore hypothesized that the difference in potency is the result of a more stringent substrate specificity of *S. sp.* PLD; *S. sp.* PLD is reported to be relatively inactive towards sphingomyelin (Dr. Greg Wall, Sigma Technical Service, personal communication), whereas *S. chromofuscus* PLD hydrolyzes both glycerophospholipids and sphingomyelin (Imamura and Horiuti, 1979). We confirmed this specificity on [^{3}H]choline-labeled keratinocyte lipids *in vitro*. **Table I** illustrates that the PLD from *S. sp.* was relatively ineffective at hydrolyzing sphingomyelin, whereas the PLD from *S. chromofuscus* effectively utilized sphingomyelin as a substrate, hydrolyzing about 75% of the total keratinocyte sphingomyelin within 1 h at a pH of 7.4.

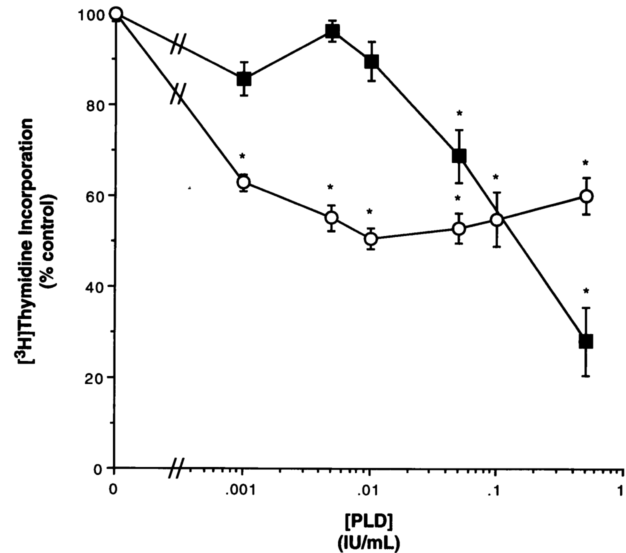

Figure 1. Exogenous PLD from *S. sp.* inhibited DNA synthesis less effectively than the enzyme from *S. chromofuscus*. Sub- to near-confluent keratinocytes (an average cell density of 24,000 ± 3700 cells per cm^2 for *S. chromofuscus* and 25,000 ± 9000 cells per cm^2 for *S. sp.*) were incubated for 24 h with various amounts of *S. sp.* (■) or *S. chromofuscus* (○) PLD, and [^{3}H]thymidine incorporation into DNA was determined as described in *Materials and Methods*. Values are expressed as percentage control (with an average control of 41,000 ± 8000 for *S. chromofuscus* and 55,000 ± 12,000 cpm per well for *S. sp.*) and represent the means (± SEM) of 15–42 samples from at least five separate experiments for *S. chromofuscus* and 8–15 samples from three separate experiments for *S. sp.*; *p < 0.01 *versus* control.

Exogenous sphingomyelinase and cell-permeable ceramides inhibit proliferation Recent data have indicated a role for sphingomyelin turnover, and the resultant production of ceramide, in regulating growth and differentiation status in several cell systems (reviewed in Hannun, 1994; Zhang and Kolesnick, 1995). We hypothesized that the sphingomyelin hydrolysis mediated by *S. chromofuscus* PLD might be involved in its potent inhibition of DNA synthesis and stimulation of differentiation. Therefore, we examined the effects of this sphingomyelin hydrolysis and the product generated, ceramide, on keratinocyte growth and differentiation utilizing exogenous bacterial sphingomyelinase and synthetic ceramides. We found that sphingomyelinase exhibited a very potent but shallow concentration-dependent effect on [^{3}H]thymidine incorporation (**Fig 4*A***), much like *S. chromofuscus* PLD. An IC_{50} of ≈0.005 U per ml was observed, with a maximal inhibition of about 50% at 0.1 U per ml. Synthetic ceramides (C_6- and C_2-ceramide) also showed a concentration-dependent inhibition of DNA synthesis, with an IC_{50} of ≈12 μM, whereas the inactive isomer dihydroC_2-ceramide (dhC_2) inhibited [^{3}H]thymidine incorporation only at the highest concentration of 20 μM (**Fig 4*B***). C_6-ceramide also stimulated transglutaminase activity (unpublished observation). These results concerning the effects of synthetic ceramides on DNA synthesis and growth are in agreement with those reported by other investigators in a human squamous cell carcinoma cell line (Wakita *et al*, 1994) and HaCaT cells (Geilen *et al*, 1996).

TNF-α elevates endogenous ceramide levels and inhibits proliferation Finally, we have obtained evidence that this growth inhibition by sphingomyelin turnover may be physiologically relevant. We have found that TNF-α, a cytokine synthesized by keratinocytes (Wood *et al*, 1992) and a known effector of sphingomyelin turnover in other systems (reviewed in Heller and Krönke, 1994; Zhang and Kolesnick, 1995), increased ceramide, the product of sphingomyelin hydrolysis, in primary epidermal keratinocytes. Treatment of near-confluent keratinocytes for 1 h with 10 nM TNF-α resulted in an increase of ceramide of 1.41 ± 0.16-fold over control (1.00 ± 0.04;

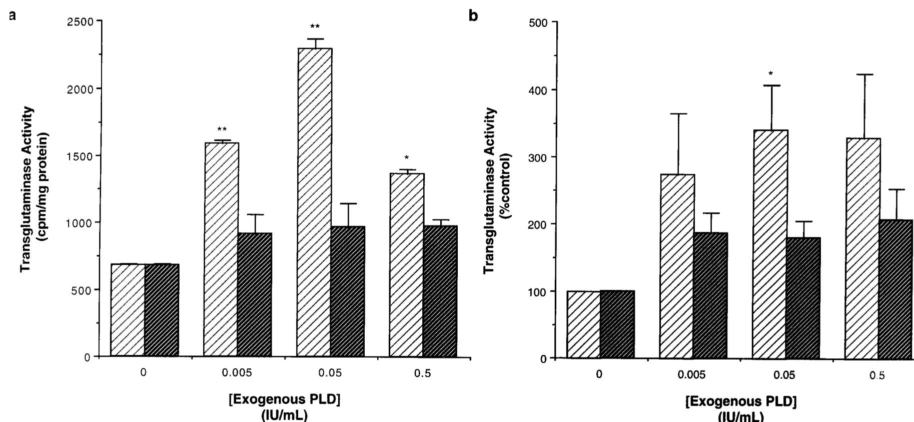

Figure 2. *Streptomyces chromofuscus* PLD stimulated transglutaminase activity more potently than the enzyme from *S. sp.*. Sub- to near-confluent keratinocyte cultures were incubated for 24 h with various amounts of bacterial PLD (from *S. chromofuscus* or *S. sp.*) and transglutaminase activity was measured as [^{3}H]putrescine incorporation into casein, as described in *Materials and Methods*. (*a*) A representative experiment performed in duplicate is expressed as cpm of [^{3}H]putrescine incorporated into casein per mg of protein (± SEM); $*p < 0.05$, $**p < 0.01$ *versus* control. (*b*) Average of the mean values from four additional, separate experiments performed in duplicate are expressed as percentage control ± SEM (controls were 18.3, 21.3, 52.4, and 585.5 cpm per mg protein for the four experiments, with the wide variation due to differences in the lots of the casein substrate used); $*p < 0.05$.

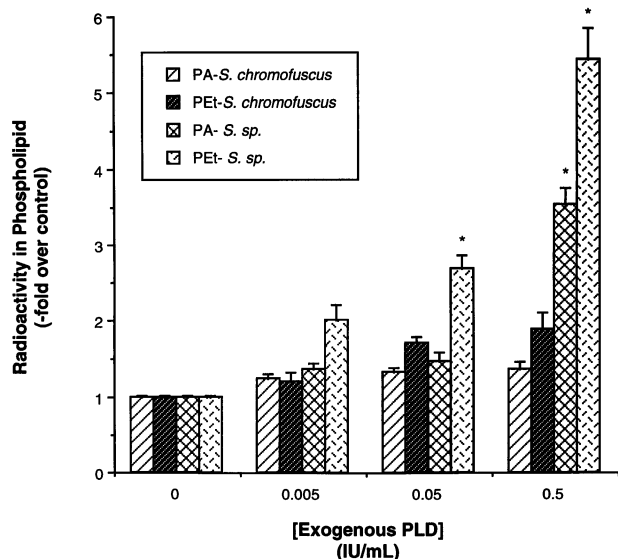

Figure 3. *Streptomyces sp.* PLD increased product formation more potently than the enzyme from *S. chromofuscus*. Near-confluent primary cultures of mouse epidermal keratinocytes were labeled for 20–24 h with [^{3}H]oleic acid and then re-fed with SFKM ± various concentrations of *S. sp.* (*hatched bars*) or *S. chromofuscus* PLD (*striped bars*, as indicated) for 30 min in the presence of 0.5% ethanol. After extracting cellular lipids and separating by thin-layer chromatography, as described in *Materials and Methods*, the radioactivity found in the PLD products, PA and PEt, was quantitated and the results are presented as -fold over the appropriate control value. Radioactivity in control samples averaged 25,000 ± 6100 cpm per 10^6 cells for PA and 11,000 ± 3000 cpm per 10^6 cells for PEt, with an average cell density (± SEM) of 54,000 ± 16,000 cells per cm^2. Values represent the means (± SEM) of six to nine samples from three separate experiments; $*p < 0.01$.

$p < 0.01$). These values represent the mean values ± SEM of 12 samples from six separate experiments.

DISCUSSION

Our finding that *S. chromofuscus* PLD was more potent than that from *S. sp.* in both its growth inhibitory (**Fig 1**) and its differentiation stimulatory effects (**Fig 2**), despite higher activity of the latter enzyme (**Fig 3**), suggested that a *S. chromofuscus* PLD-generated second messenger other than PA, or its metabolite DAG, might be playing a role in these effects. Imamura and Horiuti (1979) demonstrated that PLD from *S. chromofuscus* hydrolyzes sphingomyelin in addition to glycerophospholipids, as we have confirmed (**Table I**), and we hypothesized that this sphingomyelin degradation might provide an additional signaling molecule capable of regulating keratinocyte growth. PLD hydrolysis of sphingomyelin should yield ceramide phosphate, and presumably this compound, or the ceramide produced by the action of phosphatidate phosphohydrolase on ceramide phosphate (Waggoner *et al*, 1996), can modulate keratinocyte proliferation. Indeed, we have demonstrated that synthetic cell-permeant ceramides and exogenous sphingomyelinase both inhibited DNA synthesis (**Fig 4**). Our results are consistent with those of Wakita *et al* (1994), who found that the cell-permeant ceramides inhibited proliferation and increased differentiation (e.g., cornified envelope formation, involucrin expression, and transglutaminase activity) in a human squamous cell carcinoma cell line. Our data extend this finding to normal mouse epidermal keratinocytes and provide additional evidence for a role of ceramides in regulating cellular differentiation status in this epithelial cell system.

Streptomyces sp. PLD also inhibited proliferation, albeit less potently than that from *S. chromofuscus* (**Fig 1**). Nevertheless, the enzyme was extremely efficacious, and our results suggest that the product of PLD activity, i.e., PA, can influence keratinocyte growth, either directly or indirectly, following conversion to DAG. It is possible that the reported ability of exogenous PA and a bacterial PLD to stimulate phosphoinositide turnover (Ryder *et al*, 1993) underlies the growth inhibitory effect of *S. sp.* PLD; however, in the study of Ryder *et al* (1993), only an approximately half-maximal response is elicited by 13 μM PA, with a threshold concentration of 1.3 μM. Our results indicate that 0.05 IU per ml *S. sp.* PLD, a concentration of the enzyme that should generate only ≈50 nmoles of PA per minute in our system, elicited a significant decrease in [^{3}H]thymidine incorporation. Moreover, in a previous study (Ryder *et al*, 1993), the concentration of (*S. chromofuscus*) PLD necessary to generate a response comparable with (although slightly greater than) a concentration of 13 μM PA is equivalent to 1.67 IU per ml (Ryder *et al*, 1993), roughly 30 times the amount of *S. sp.* PLD necessary to generate a half-maximal response in terms of DNA synthesis. For these reasons, we believe that the inhibition of DNA synthesis observed with lower concentrations of *S. sp.* PLD is independent of an effect on phosphoinositide turnover. Instead, we postulate that the PA produced by the action of PLD is converted to DAG and elicits its effects through protein kinase C, which is thought to play a role in inhibiting growth and stimulating differentiation in epidermal keratinocytes (reviewed in Inohara, 1992); however, a more potent stimulatory effect of *S. sp.* PLD on phosphoinositide turnover at higher concentrations may contribute to its greater efficacy in comparison with *S. chromofuscus* PLD.

Recently, Wakita *et al* (1996) have shown that the physiologically relevant cytokine interferon-γ stimulates sphingomyelin hydrolysis in normal human keratinocytes; however, the method used to measure

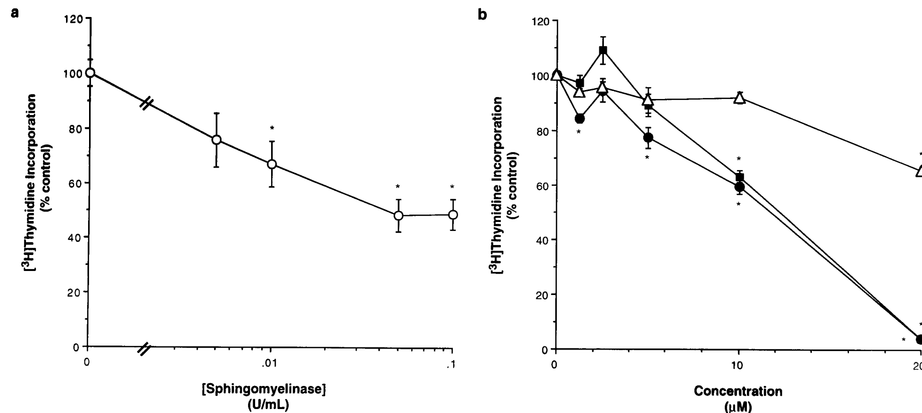

Figure 4. Bacterial sphingomyelinase and synthetic cell-permeant ceramides inhibited DNA synthesis. (*a*) Near-confluent primary epidermal keratinocyte cultures (with an average cell density of 37,100 ± 5000 cells per cm^2) were incubated for 24 h with various concentrations of bacterial sphingomyelinase, and [^{3}H]thymidine incorporation into DNA was determined as described in *Materials and Methods*. Values are expressed as percentage control (with an average control of 38,300 ± 12,000 cpm per well) and represent the means (± SEM) of at least six samples from three separate experiments; *$p < 0.001$ *versus* control. (*b*) Sub- to near-confluent primary epidermal keratinocyte cultures (with an average cell density of 19,000 ± 6100 cells per cm^2) were incubated for 24 h with various concentrations of the active synthetic ceramides N-acetylsphingosine (C_2-ceramide, ■) or N-hexanoylsphingosine (C_6-ceramide, ●), or the inactive N-acetylsphinganine (C_2 dihydroceramide, △), and [^{3}H]thymidine incorporation into DNA was determined as described in *Materials and Methods*. Values are expressed as percentage control (with an average control of 31,000 ± 1100 cpm per well) and represent the means (± SEM) of at least nine samples from three separate experiments; *$p < 0.001$ *versus* control.

sphingomyelin degradation utilized a fluorescent sphingomyelin analog incorporated into the plasma membrane (Wakita *et al*, 1996). Because this exogenous sphingomyelin is likely to become incorporated into the outer leaflet of the lipid bilayer, and should not readily traverse the membrane, it may not be coupled to sphingomyelinase activity and function in signaling in the same way as endogenous sphingomyelin. Indeed, it is not clear why the authors are able to observe interferon-γ-induced exogenous sphingomyelin hydrolysis, because Linardic and Hannun (1994) have demonstrated that the signaling pool in HL-60 cells is contained in the inner leaflet of the plasma membrane. Although it is possible that the 24 h incubation with the fluorescent sphingomyelin could allow "flip-flop" of the lipid into the inner leaflet, Andrieu *et al* (1996) have shown using radiolabeled sphingomyelin that, after a similar 24 h incubation, as much as 60% of the sphingolipid is still accessible to exogenous sphingomyelinase, indicating its location in the outer leaflet of the bilayer. In addition, these authors were unable to demonstrate TNF-α-induced hydrolysis of the radiolabeled sphingomyelin under these conditions (Andrieu *et al*, 1996). Furthermore, and perhaps most importantly, Wakita *et al* (1996) were unable to reproduce the cytokine-induced keratinocyte response with synthetic ceramides or exogenous sphingomyelinase. Nevertheless, the results support the intriguing idea that interferon-γ may function through sphingomyelin hydrolysis.

Our data with TNF-α also give support for a role of the sphingomyelin signaling system in cytokine-induced regulation of keratinocyte growth and differentiation. TNF-α is expressed by keratinocytes in response to perturbations of skin function (Wood *et al*, 1992), and it has been proposed that this cytokine is produced by keratinocytes to regulate immune cells in the skin. The results of Pillai *et al* (1989), who found that TNF-α inhibited the growth and stimulated the differentiation of human keratinocytes, indicates that TNF-α may play an autocrine regulatory role in mouse keratinocytes as well. Similarly, we also found that TNF-α concentration dependently inhibited [^{3}H]thymidine incorporation, with an IC_{50} of 0.3 nM and a maximal inhibition of ≈30% at 10 nM (data not shown). Furthermore, this IC_{50} is nearly identical to the dissociation constant (K_d) calculated by Pillai *et al* for the keratinocyte TNF-α receptors (0.28 nM; Pillai *et al*, 1989). Finally, the ability of TNF-α to elevate cellular ceramide content suggests that the sphingomyelin hydrolysis pathway may be a physiologically relevant signaling pathway involved in the action of this cytokine on keratinocyte growth and differentiation. This interpretation is supported by the recent findings in HaCaT cells that indicate that 1,25-dihydroxyvitamin D_3 induces sphingomyelin hydrolysis (Geilen *et al*, 1996) via release of TNF-α from HaCaT cells (Geilen *et al*, 1997). On the other hand, TNF-α has also been reported to activate a phosphatidylcholine-specific phospholipase C to generate DAG (reviewed in Heller and Krönke, 1994), which could presumably activate protein kinase C and potentially influence growth. Furthermore, because ceramides are constituents of the epidermal lipid barrier (Elias *et al*, 1979; Madison and Howard, 1996), the TNF-α-elicited increase in ceramide content may simply reflect a differentiative effect induced by this cytokine; however, the rapidity of the effect (within a 1 h exposure) argues against such an interpretation. Moreover, our results with the cell-permeant ceramides (**Fig 4**) provide additional evidence for, but do not prove, a role for ceramides in signaling. We are currently investigating the contribution of various pathways for ceramide generation (e.g., from glucosylceramide or sphingomyelin) to the TNF-α-induced increase in cellular ceramide content.

In conclusion, our results suggest a potential role for TNF-α, the sphingomyelin hydrolysis signaling pathway, and the lipid messenger ceramide in the regulation of keratinocyte proliferation and differentiation. In addition, the product of PLD activity, PA, also appears to modulate keratinocyte growth, either directly or indirectly, upon conversion to DAG. Moreover, our results suggest that the PLD and sphingomyelin hydrolysis pathways interact in a synergistic fashion to inhibit growth and stimulate differentiation of epidermal keratinocytes. Further investigation into this interaction and into the ability of other keratinocyte modulators to alter ceramide content and/or PA and DAG levels (via PLD activation), should provide needed information concerning the role of these potentially important lipid messengers in regulating proliferation and differentiation in these cells.

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