Ceramide-binding and activation defines protein kinase c-Raf as a ceramide-activated protein kinase

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ABSTRACT Interleukin 1 is the prototype of an inflammatory cytokine, and evidence suggests that it uses the sphingomyelin pathway and ceramide production to trigger mitogen-activated protein kinase (MAPK) activation and subsequent gene expression required for acute inflammatory processes. To identify downstream signaling targets of ceramide, a radioiodinated photoaffinity labeling analog of ceramide ([125I]3-trifluoromethyl-3-(m-iodophenyl)diazirine-ceramide) was employed. It is observed that ceramide specifically binds to and activates protein kinase c-Raf, leading to a subsequent activation of the MAPK cascade. Ceramide does not bind to any other member of the MAPK module nor does it bind to protein kinase C-ζ. These data identify protein kinase c-Raf as a specific molecular target for interleukin 1β-stimulated ceramide formation and demonstrate that ceramide is a lipid cofactor participating in regulation of c-Raf activity.

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MATERIALS AND METHODS

Cell Culture. Rat renal mesangial cells were cultured as described (12). Single cells were cloned by limited dilution on 96-microwell plates. Clones with apparent mesangial cell morphology were used for further processing. The cells were grown in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum, penicillin (100 units/ml), streptomycin (100 µg/ml), and bovine insulin (0.66 units/ml). For the reported experiments, passages 5–18 of mesangial cells were used.

Lipid Extraction and Separation. Confluent cells in 30-mm diameter dishes were labeled for 24 h with [3H]serine (0.3 µCi/ml; specific activity, 53 mCi/mmol) and stimulated as indicated. The reaction was stopped by extraction of lipids (13), and sphingomyelin and ceramide were resolved by sequential one-dimensional TLC using chloroform/methanol/ammonia (65:35:7.5; vol/vol) followed by chloroform/methanol/acetic acid (9:1:1, vol/vol). Spots corresponding to ceramide and sphingomyelin were analyzed and quantitated using a Berthold (Nashua, NH) LB 2842 automatic TLC scanner.

In Vivo P32-Labeling of Cells and Immunoprecipitation. Confluent mesangial cells in 100-mm diameter dishes were incubated for 2 days in DMEM containing 0.1 mg of fatty acid-free BSA per ml and then washed three times with phosphate-free DMEM to remove all phosphate. Then the cells were metabolically labeled for 4 h at 37°C with [32P]orthophosphate (0.5 mCi/plate) in phosphate-free DMEM. After labeling, cells were stimulated as indicated. To stop the reaction, cells were washed twice with ice-cold buffer containing 50 mM Tris-HCl (pH 7.5) and 150 mM NaCl and scraped into 1 ml of buffer A (50 mM Tris-HCl, pH 7.5/150 mM NaCl/10% glycerol/1% Triton X-100/2 mM EDTA/2 mM EGTA/40 mM β-glycerophosphate/50 mM NaF/10 mM sodium pyrophosphate/25 µg of leupeptin per ml/200 units of aprotinin per ml/1 µM peptatin A/1 mM phenylmethylsulfonyl fluoride) and homogenized by 10 passes through a 26-gauge needle. The homogenate was centrifuged for 10 min at 14,000 x g, and the supernatant was taken for immunoprecipitation.

Abbreviations: IL-1, interleukin 1; MAPK, mitogen-activated protein kinase; TID, 3-trifluoromethyl-3-(m-iodophenyl)diazirine; MEK, MAPK kinase; PKC, protein kinase C.

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Samples (1 ml), containing 150 × 10⁶ cpm of labeled proteins, 5% fetal calf serum, and 1.5 mM iodoacetamide in buffer A were incubated with the indicated antibodies for 3 h at room temperature, or overnight at 4°C. Then 100 μl of a 50% (vol/vol) slurry of protein A-Sepharose 4B-CL in PBS was added, and the mixture was incubated for 1 h under mild shaking. After centrifugation for 5 min at 3000 × g, immunocomplexes were washed three times with 1 ml of a low-salt buffer (50 mM Tris-HCl, pH 7.5/150 mM NaCl/0.2% Triton X-100/2 mM EDTA/2 mM EGTA/0.1% SDS) and three times with a high-salt buffer (50 mM Tris-HCl, pH 7.5/500 mM NaCl/0.2% Triton X-100/2 mM EDTA/2 mM EGTA/0.1% SDS). Pellets were boiled for 5 min in 50 μl of Laemmli dissociation buffer and subjected to SDS/PAGE.

3-Trifluoromethyl-3-(m-iodophenyl)diazirine (TID) Labeling Studies. Confluent mesangial cells were incubated for 2 days in DMEM containing 0.1 mg of fatty acid-free BSA per ml. Thereafter cells were stimulated for 5 min with 50 nM N-[3-[2-(125I)Iodo-4-[3-(trifluoromethyl)-3H-diazirin-3-yl]-benzyl]oxy]carbonylpropanoyl]-d-erythro-sphingosine (125I)TID-ceramide; 0.2 mCi/ml) and subjected to photolysis for 30 s using a high-pressure mercury lamp (Osram HBO, 350 W; Osram, Berlin, Germany) mounted in a SUSS lamphouse equipped with a shutter to control exposure time. Cells were then lysed in buffer A homogenized by 10 passes through a 26-gauge needle fitted to a 1-ml syringe and centrifuged for 10 min at 14,000 × g, and the supernatant was taken for immunoprecipitation and subjected to SDS/PAGE. The antibodies for c-Raf, p42 and p44 isoforms of MAPK, MAPK kinase (MEK), and protein kinase C (PKC)-ζ have been characterized in detail elsewhere (16). After fixation in 25% isopropanol/10% acetic acid, the gels were dried and exposed to Hyperfilm MP (Amersham) at −70°C.

MAPK Activity Assay. MAPK activity assays were performed as described (15). In brief, stimulated cells were lysed and homogenized in kinase buffer (20 mM Tris-HCl, pH 7.5/1 mM EGTA/2 mM MnCl₂/0.1 mM sodium orthovanadate/25 μg of leupeptin per ml/200 units of aprotinin per ml/1 μM phenylmethylsulfonyl fluoride) by 10 passes through a 26-gauge needle, centrifuged for 5 min at 14,000 × g, and the supernatant was taken for immunoprecipitation (14). Immunoprecipitates were subjected for 10 min at 37°C in a 50-μl kinase reaction containing 10 mM Tris-HCl (pH 7.5), 10 mM MnCl₂, 150 mM NaCl, 2 mM dithiothreitol, 1% Triton X-100, 1 μM ATP, 1 μCi of [γ-32P]ATP, and 100 ng of purified recombinant kinase-negative MEK (MEKk). The reaction was stopped by addition of SDS/sample buffer and subjected to SDS/PAGE. After fixation in 25% isopropanol/10% acetic acid, the gels were dried and exposed to Hyperfilm MP or analyzed with a PhosphorImager (Molecular Dynamics).

Chemicals. [14C]Serine, [γ-32P]ATP, and [32P]orthophosphate were from Amersham, [γ-32P]ATP, and [32P]orthophosphate were from Amersham, C6- and C16-ceramide were from Calbiochem, IL-1β was provided by CIBA-Geigy, and protein A-Sepharose 4B-CL was from Pharmacia. The photoaffinity labeling reagent [125I]TID-ceramide was synthesized as described (17).

Table 1. Stimulation of ceramide production and sphingomyelin hydrolysis by IL-1β

<table>
<thead>
<tr>
<th>Addition</th>
<th>Ceramide, % of control</th>
<th>Sphingomyelin, % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 5</td>
<td>100 ± 2</td>
</tr>
<tr>
<td>IL-1β</td>
<td>536 ± 25*</td>
<td>67 ± 9**</td>
</tr>
</tbody>
</table>

Mesangial cells were labeled for 24 h with [14C]serine and stimulated for 10 min with 1 nM IL-1β. Lipids were extracted and separated on TLC as described. Spots corresponding to ceramide and sphingomyelin were analyzed and quantitated. Results are expressed as percentage of the respective control values and are means ± SD (n = 3). Significant differences from corresponding control: * P < 0.001; ** P < 0.01 (by ANOVA).

Fig. 1. Specific binding of [125I]TID-ceramide to c-Raf in vivo. (A) Mesangial cells were stimulated for 5 min with [125I]TID-ceramide then directly lysed (−) or photolyzed before lysis (+), and equal amounts of protein were taken from immunoprecipitation using specific antibodies against phosphotyrosine (PY; at a dilution of 1:200), p42mapk and p44mapk (MAPK; 1:100 each), PKC-ζ (1:100), MEK (1:100), c-Raf (1:250), and MEK kinase (1:100). Immunoprecipitates were separated on SDS/PAGE and exposed to Hyperfilm MP at −70°C. (B) Exogenous ceramide and IL-1β pretreatment of mesangial cells decrease [125I]TID-ceramide binding to c-Raf. Mesangial cells were stimulated for 5 min with 50 nM [125I]TID-ceramide (0.2 mCi/ml) and directly lysed (lane 2) or photolyzed before lysis (lanes 1 and 3–6). Binding of [125I]TID-ceramide was inhibited by addition of 450 nM (lane 4) or 50 nM (lane 5) exogenous unlabeled ceramide or by pretreatment of the cells with 1 nM of IL-1β for 2 min before TID-ceramide addition (lane 6). Thereafter c-Raf was immunoprecipitated (lanes 2–6; lane 1, preimmune serum), subjected to SDS/PAGE, and exposed to Hyperfilm MP at −70°C.
Fig. 2. (A) Stimulation of c-Raf activity by C6-ceramide and C16-ceramide in vitro. c-Raf was immunoprecipitated from quiescent mesangial cells. Immunoprecipitates were incubated in vitro for 15 min at 30°C with the indicated concentrations of C6 and C16 ceramide before the addition of 100 ng of recombinant MEKk and 1 μCi of [γ-32P]ATP for 10 min. Samples were subjected to SDS/PAGE, exposed to Hyperfilm MP, and quantitated on a PhosphorImager. Data are expressed as percentage of the respective control values and are means of three independent experiments (the SD ranges from 12 to 34%). (Inset) Autoradiograph of one representative experiment. (B) Stimulation of c-Raf activity by C6-ceramide and C16-ceramide in vivo. Quiescent intact mesangial cells were stimulated with the indicated concentrations C6- and C16-ceramide for 5 min. Thereafter cell lysates were prepared and c-Raf was immunoprecipitated. Immunoprecipitates were incubated for 10 min at 30°C with 100 ng of recombinant MEKk and 1 μCi of [γ-32P]ATP. Samples were subjected to SDS/PAGE, exposed to Hyperfilm MP, and quantitated on a PhosphorImager. Data are expressed as percentage of the respective control values and are means of two independent experiments giving similar results. (Inset) Autoradiograph of one representative experiment. (C) Effect of C6- and C16-ceramides on MAPK activity in mesangial cells. Quiescent mesangial cells were stimulated with the indicated concentrations C6- and C16-ceramide for 5 min. Thereafter myelin basic protein phosphorylation was measured as described.

RESULTS

Upon exposure to IL-1β, mesangial cells hydrolyze sphingomyelin to generate ceramide (Table 1) and display a rapid activation of MAPK, which is sustained for >24 h (5). To identify possible molecular targets of ceramide signaling in mesangial cells, we prepared a photoaffinity labeling analog of ceramide of high [125I]iodine-specific radioactivity (>2000 Ci/mmol) (17). Incubation of intact cells with [125I]ITID-ceramide for 5 min followed by homogenization and sequential immunoprecipitation of the different members of the MAPK module with specific polyclonal antibodies reveals a selective
labeling of protein kinase c-Raf (Fig. 1A), which is increased up to 243 ± 37% (mean ± SD, n = 4) upon UV-induced labeling. There is no labeling of the p42 and p44 isoforms of MAPK nor of their upstream activator MEK, MEK kinase, PKC-ζ, or any protein immunoprecipitated with an anti-phosphotyrosine antibody (PY) as shown in Fig. 1A. All antibodies used in this study have been shown to immunoprecipitate their respective antigens (14–16). Labeling by \[^{125}\text{I}\]TID-ceramide is inhibited by exogenous addition of increasing concentrations of unlabeled ceramide (Fig. 1B) as well as by endogenous ceramide produced by IL-1β prestimulation of the cells (Fig. 1B). These data establish that IL-1β-induced ceramide specifically binds to c-Raf in mesangial cells. Comparable data were obtained for tumor necrosis factor α-stimulated mesangial cells (data not shown).

Next we investigated whether ceramide binding to c-Raf also has functional consequences. Fig. 2A shows that C6 and C16 analogs of ceramide, in a concentration-dependent manner, increase protein kinase c-Raf activity in an immunocomplex kinase assay in vitro. Moreover, C6 and C16 analogs also activate protein kinase c-Raf in vivo when added to intact cells and subsequent evaluation of c-Raf activity by an immunocomplex kinase assay (Fig. 2B). Fig. 2C demonstrates that ceramide analogs not only activate c-Raf but that the signal is further processed along the MAPK cascade and causes an increased activity of the p42 and p44 isoforms of MAPK. Ceramide-stimulated activation of MAPK is inhibited by PD 098059 (18), a synthetic inhibitor of MEK (data not shown). To prove that IL-1β indeed uses this signaling pathway to activate c-Raf in mesangial cells, we examined c-Raf phosphorylation and activity. As shown in Fig. 3, IL-1β induces a rapid phosphorylation and activation of c-Raf within 5 min and subsequent stimulation of MAPK (5).

**FIG. 3.** IL-1β-induced phosphorylation and activation of c-Raf in vivo. (A) Quiescent mesangial cells were labeled with \[^{32}\text{P}\] and were stimulated with IL-1β for the indicated time periods. c-Raf was immunoprecipitated, subjected to SDS/PAGE, and exposed to Hyperfilm MP. (B) Cells were treated with vehicle (control) or IL-1β (1 nM) for 5 min, and c-Raf activity in mesangial cell homogenates was determined as described. Results are expressed as percentage of control and are means ± SD of three independent experiments.

**DISCUSSION**

The serine/threonine kinase c-Raf is a well-studied signaling molecule that is responsible for phosphorylation and activation of MEK in the classical MAPK cascade (19). The mechanism of activation of protein kinase c-Raf has been studied extensively, and it is now clear that this is a multistep event. In a first step, c-Raf translocates to the plasma membrane and associates with Ras-GTP. However, this association with Ras-GTP is not sufficient for c-Raf activation but is required for its recruitment to the plasma membrane (20, 21). In a second step, c-Raf is activated by an unknown mechanism that may comprise tyrosine and/or serine/threonine phosphorylation of c-Raf (14, 19, 22–25) or the interaction with another membrane cofactor such as a lipid (18, 24, 25). The c-Raf amino terminus contains a highly conserved region (CR-1) that encompasses a zinc-finger motif analogous to the lipid-binding domain of PKC (19), and it is tempting to speculate that c-Raf is activated by binding of a lipid second messenger in a way similar to the mechanism of activation of PKC by 1,2-diacylglycerol.

The present data clearly demonstrate that ceramide specifically binds to c-Raf and stimulates its kinase activity. These observations extend previous reports on putative lipid factors extractable with chloroform/methanol from membranes of activated cells that markedly enhance c-Raf enzymatic activity (19, 25) and identify ceramide as a lipid second messenger that acts as a major direct activator of c-Raf in IL-1β-triggered signal propagation. Ceramide has gained recognition as an important signaling molecule regulating fundamental biological processes, like cell proliferation and differentiation, oncogenesis, and immune and inflammatory processes (8, 9). Ceramide is produced by sphingomyelin hydrolysis by both neutral and acid sphingomyelinases. With respect to immediate targets of ceramide, suggestions for several ceramide-activated enzymes have been forwarded. These include a 97-kDa proline-directed serine/threonine protein kinase (26, 27), a ceramide-activated protein phosphatase (28) and PKC-ζ (29). Recently Yao et al. (30) observed that a 97-kDa ceramide-activated protein kinase phosphorlyates c-Raf on Thr-269 and increases its activity toward MEK. Furthermore, in intact HL-60 cells, ceramide-activated protein kinase forms complexes with c-Raf and, in response to tumor necrosis factor α, phosphorlylates and activates c-Raf. In IL-1β-stimulated mesangial cells, ceramide definitely does not bind to PKC-ζ and there is also no ceramide-binding protein in the range of 97 kDa visible in the cell homogenates (data not shown). In contrast, our data define c-Raf as a ceramide-activated protein kinase, thus suggesting that there is more than one ceramide-activated protein kinase and that ceramide may have cell-type specific targets.

Previous reports suggested that short-chain ceramide analogs are biologically more active than their long-chain equivalents likely due to the increased solubility of the former. Based on this background, the observation that the C16-ceramides were more potent the C6-ceramides in activating c-Raf is rather unexpected. Moreover, we observed that stimulation of c-Raf activity in vivo occurs at lower concentrations of both ceramide derivatives than in vitro.

A possible explanation could be that C16-ceramide was a natural product, whereas C6-ceramide was a synthetic compound. In addition to chain length and solubility in aqueous solution, other factors may be important for biological activity, especially the degree of unsaturation of the alkyl chains.

In summary, IL-1β signaling in mesangial cells involves ceramide generation and subsequent direct binding to and activation of c-Raf. Two important aspects of this study are the identification of ceramide as the long-living lipid activator of c-Raf and the identification of protein kinase c-Raf as another, yet molecularly defined, member of an emerging family of ceramide-activated protein kinases. Activation of protein ki-
nase c-Raf by ceramide is probably only one possible way to activate this kinase, especially in response to cytokines like IL-1β or tumor necrosis factor α. Activation of c-Raf by phosphorylation is an alternative mechanism used by tyrosine kinase receptor agonists or G-protein-coupled receptors, which do not trigger sphingomyelin hydrolysis.

Some pressing questions that immediately arise are concerned with the molecular mechanism of ceramide binding to and activation of c-Raf and the possibility that ceramide stimulation of c-Raf also signals to other MAPK modules like the stress-activated protein kinases (31), as has recently been suggested for HL-60 cells (32).

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