Induction of stress-activated protein kinases/c-Jun N-terminal kinases by the p55 tumour necrosis factor receptor does not require sphingomyelinases

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Ceramide has been implicated in the activation of stress-activated protein kinases/c-Jun N-terminal kinases (SAPK/JNK). Binding of tumour necrosis factor (TNF) to its 55 kDa receptor (TR55) leads to the generation of ceramide through activation of either acid or neutral sphingomyelinase (A/N-SMase) as well as to potent activation of SAPK/JNK. We have examined a putative role of both N- and A-SMase in the TR55-dependent activation of SAPK/JNK. The analysis of TR55 deletion mutants expressed in 70Z/3 pre-B cells revealed that activation of SAPK/JNK occurs independently of N-SMase. Although both SAPK/JNK and A-SMase are activated by the death domain of TR55, pharmacological prevention of the TR55-dependent activation

of A-SMase, or proteolytic degradation of A-SMase in 70Z/3 cells, did not impair SAPK/JNK activation, indicating that SAPK/JNK are not secondary to A-SMase. In addition, proteolytic degradation of A-SMase also did not affect SAPK/JNK activation by ultraviolet (UV-C) irradiation, arguing against a general role of A-SMase in stress-mediated responses. Furthermore, fibroblasts from Niemann–Pick A patients deficient in A-SMase did not show altered activation of SAPK/JNK in response to either TNF or UV-C. These results suggest that TR55 can activate SAPK/JNK without direct participation of sphingomyelinases or ceramide.

INTRODUCTION

Tumour necrosis factor (TNF), a cytokine produced mainly by activated macrophages, mediates pleiotropic inflammatory and immunoregulatory responses, as well as cytotoxicity, anti-viral activity and stimulation of cell growth (for review, see [1]). Induction of these responses by TNF is initiated through two distinct cell surface receptors of 55 kDa (TR55) and 75 kDa (TR75) apparent molecular mass (reviewed in [2,3]). Several studies indicate that a large majority of TNF activities can be mediated solely by TR55 [4–6]. A C-terminal region of TR55 that sequentially initiates the activation of a phosphatidylcholine-specific phospholipase C and an acid sphingomyelinase (A-SMase), generating ceramide [7,8], is most likely identical with the death domain, which is responsible for mediating the cytotoxic effects of TNF [8–10] as well as for inducing the transcription factor nuclear factor- κ B (NF- κ B) [11]. A second, independent signalling pathway initiated by TR55 involves a membrane-bound neutral sphingomyelinase (N-SMase; [12]; for review see [13,14]), which is activated by recruitment of the Trp-Asp (WD)-repeat protein FAN (factor associated with N-SMase activation) [15] to the N-SMase activation domain (NSD) of TR55 [16]. N-SMase may be responsible for the described activation of the protein kinase Raf-1 by TNF [17], most likely by liberating ceramide from plasma membrane sphingomyelin, thereby stimulating ceramide-activated protein kinase/kinase suppressor of Ras (CAPK/KSR) [18,19], which in turn phosphorylates Raf-1 [20]. Subsequently, members of the mitogen-activated protein kinase family are stimulated [21], ultimately leading to a proliferative and pro-inflammatory response (reviewed in [22]).

Another class of enzymes that are strongly activated by TNF

has been termed stress-activated protein kinases (SAPK; [23]) or c-Jun N-terminal kinases (JNK; [24]). SAPK/JNK are potently induced by environmental stresses, such as inhibitors of protein synthesis, inflammatory cytokines, changes in osmolarity, heat shock and UV irradiation [23,24], and have been implicated in the induction of programmed cell death [25–27]. However, several studies have recently demonstrated that SAPK/JNK activation by TR55 is mediated through the protein, TNF receptor-associated factor 2 (TRAF2) and that it occurs in a non-cytotoxic manner [28–30]. Clearly, additional studies are required to fully elucidate the function of SAPK/JNK in the signal-transduction pathways initiated by TR55.

Exogenously applied analogues of the second messenger ceramide are both capable of activating SAPK/JNK and of inducing cell death [26,31]. Therefore ceramide produced intracellularly by sphingomyelinases may contribute to the induction of apoptosis (reviewed in [32]) and may also have a function in the stimulation of SAPK/JNK. In the present study, we examine the role of both N- and A-SMase in the activation of SAPK/JNK by TR55.

Through analysis of a panel of TR55 deletion mutants expressed in 70Z/3 pre-B cells, we show that TNF-dependent activation of N-SMase does not correlate with the induction of SAPK/JNK, which is mediated through the death domain. Although the death domain of TR55 does activate both A-SMase and SAPK/JNK, we provide evidence that these two responses are initiated independently. First, both enzymes display a significantly different dose–response relationship towards TNF. Secondly, the TNF-mediated activation of A-SMase can be blocked and A-SMase can even be proteolytically degraded without compromising SAPK/JNK activation in 70Z/3 cells. This also holds true for SAPK/JNK activation by irradiation

Abbreviations used: A-SMase, acid sphingomyelinase; GST, glutathione S-transferase; NF- κ B, nuclear factor- κ B; N-SMase, neutral sphingomyelinase; NSD, N-SMase activation domain; TNF, tumour necrosis factor; TR, tumour necrosis factor receptor; SAPK/JNK, stress-activated protein kinases/c-Jun N-terminal kinases; TRAF2, TNF receptor-associated factor 2; TRADD, TNF receptor-associated protein with death domain; FADD, Fas-associated protein with death domain.

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with UV-C, which is also unaffected by proteolytic degradation of A-SMase. Thirdly, fibroblasts from Niemann–Pick A patients that display drastically reduced levels of A-SMase activity show a normal activation of SAPK/JNK in response to either TNF or UV-C. Our results indicate that TR55 can induce SAPK/JNK and sphingomyelinases through distinct, non-overlapping pathways in the above cell types.

EXPERIMENTAL

Plasmids and reagents

Highly purified human recombinant TNF was provided by Dr. G. Adolf (Bender & Co, Vienna, Austria). The cDNA for glutathione S-transferase (GST)–Jun (1–166) was provided by Dr. P. Angel (DKFZ, Heidelberg, Germany). The SAPK/JNK-antibody, JNK1 (C17), was purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A. The xanthogenate tricyclodecan-9-yl (D609, [33]) was purchased from MoBiTec, Göttingen, Germany. Desipramine and imipramine were obtained from Sigma–Aldrich Chemie GmbH, Deisenhofen, Germany. C₂-Ceramide (*N*-acetyl sphingosine) and C₂-dihydroceramide (*N*-acetyldihydro sphingosine) were purchased from Biomol, Hamburg, Germany.

Cell culture and transfections

The murine pre-B cell line 70Z/3 was originally obtained from American Type Culture Collection. Transfectants stably expressing wild-type and mutant TR55 have been described elsewhere [6,8,16]. Fibroblasts from Niemann–Pick patients (cell line F91/11) and control fibroblasts (cell line F91/17) were provided by Dr. K. Sandhoff (Institut für Organische Chemie und Biochemie, Bonn, Germany).

Cells were maintained in a mixture of Click's/RPMI 1640 (1:1, v/v) supplemented with 10% (v/v) fetal calf serum, 10 mM glutamine and 50 µg/ml each of streptomycin and penicillin in a humidified incubator containing 5% (w/v) CO₂.

Stimulation of cells

Treatment of cells with human recombinant TNF was performed at a concentration of 100 ng/ml for the indicated times. To activate SAPK/JNK by UV-C irradiation, cells were exposed to a dose of 5×10^4 µJ/cm² at 254 nm in a UV cross-linker (Spectronics Corp.) and incubated for a further 20 min at 37 °C, unless indicated otherwise, before being harvested. For treatment of cells with synthetic ceramides, C₂-ceramide and C₂-dihydroceramide were dissolved in DMSO and added to the medium at a concentration of 100 µmol/l. The final concentration of DMSO was 1% (v/v).

Assays for activation of SAPK/JNK

Treated cells (10⁷) were washed once with cold PBS, resuspended in 500 µl of cold lysis buffer [25 mM Hepes, pH 7.55/10 mM NaF/100 mM NaCl/1.5 mM MgCl₂/0.5 mM EGTA/0.25 mM EDTA/0.1% (v/v) Nonidet P-40/20 mM β-glycerophosphate/1 mM vanadate/1 mM PMSF/10 µg/ml aprotinin–leupeptin–pepstatin], left for 10 min on ice and homogenized by repeated passing through a 27 gauge needle. After centrifugation, 100 µg of protein was subjected to immunoprecipitation with 1 µg of JNK1 antibody. Phosphorylation assays were performed on immunoprecipitated proteins in 20 µl of kinase buffer (50 mM Hepes, pH 7.55/100 mM KCl/25 mM β-glycerophosphate/10 mM MgCl₂/1 mM MnCl₂/1 mM vanadate/0.5 mM EGTA/

20 µM ATP) in the presence of 5 µg of GST–Jun and 0.5 µCi of [γ -³³P]ATP (specific activity > 2000 Ci/mmol; Du Pont NEN) per reaction for 20 min at 30 °C. The reactions were terminated by addition of 5 µl of 5× loading buffer and boiled for 5 min. The samples were subjected to 12% (w/v) SDS/PAGE, visualized by autoradiography and quantified in a phosphor-imager (Fuji). Bacterially expressed GST–Jun fusion protein was prepared and purified by standard methods.

Assays for N-SMase

The micellar SMase assay using exogenous radiolabelled sphingomyelin was performed as described [8]. Briefly, cells were treated in triplicates in 0.5 ml medium with 100 ng/ml human recombinant TNF for the indicated times. To measure N-SMase, cells were homogenized as described, except that 0.5% (w/v) CHAPS was substituted for 0.2% (v/v) Triton X-100 in the lysis buffer described in [8]. Radioactive phosphocholine produced from [*N*-methyl-¹⁴C]sphingomyelin (labelled in the choline moiety; Amersham CFA566) was identified by TLC and routinely determined in the aqueous phase by scintillation counting.

Assays for A-SMase

To measure A-SMase, cells treated with TNF or UV-C were homogenized in 200 µl of 0.2% (v/v) Triton X-100. The amount of radioactive phosphocholine produced was measured as described [8].

Preparation of nuclear extracts and electrophoretic mobility-shift assays

Nuclear extracts were prepared as described [34]. Electrophoretic mobility-shift assays were performed by incubating 6 µg of nuclear extract with 4 µg of poly(dI-dC) (Pharmacia) in binding buffer [5 mM Hepes (pH 7.8)/5 mM MgCl₂/50 mM KCl/0.5 mM dithiothreitol/10% glycerol] in a total volume of 20 µl for 20 min at room temperature. Then end-labelled double-stranded oligonucleotide probe [NF-κB-specific oligonucleotide containing two tandemly arranged NF-κB binding sites of the HIV-1 long terminal repeat enhancer (5'-ATCAGGGACTTTC-CGCTGGGGACTTTCCG-3'), (1–5) × 10⁴ c.p.m.] was added and the reaction mixture was incubated for 7 min. The samples were separated on native 6% polyacrylamide gels in low-ionic strength buffer (0.25 × Tris/borate/EDTA).

Cell cycle analysis

Cells were washed twice with cold PBS/5 mM EDTA and resuspended in 1 ml of PBS/5 mM EDTA. Cells were fixed by drop-wise addition of 1 ml of ethanol and incubation at room temperature for 30 min, harvested and resuspended in 0.5 ml of PBS/5 mM EDTA. After digestion with 20 µl of RNase A (1 mg/ml) for 30 min at room temperature, cells were incubated for 1 h in 0.5 ml of staining solution (500 µg/ml propidium iodide in PBS/5 mM EDTA). Cell cycle analysis was performed by flow cytometry using a FACSCalibur Analyzer (Becton Dickinson).

RESULTS

Activation of SAPK/JNK and N-SMase is mediated independently and by distinct domains of TR55

To evaluate whether N-SMase and SAPK/JNK are activated through a common signalling pathway, we employed a set of

Figure 1 Non-participation of N-SMase in the induction of SAPK/JNK by TR55

70Z/3 cells expressing wild-type or deletion mutants of TR55 and parental 70Z/3 cells were stimulated with 100 ng/ml TNF for the indicated times and analysed for activation of SAPK/JNK, N-SMase and A-SMase as described in the Experimental section. As a positive control for activation of SAPK/JNK, cells were left untreated or exposed to UV-C irradiation ($5 \times 10^4 \mu\text{J}/\text{cm}^2$ at 254 nm) and further incubated for 20 min at 37 °C to allow for maximal activation of SAPK/JNK. Phosphorylation of GST-Jun is expressed relative to untreated cells (values have been rounded to whole numbers). A schematic representation of the receptors expressed in each cell line indicates the deleted regions (drawn in faint lines). TM, transmembrane domain, NSD, N-SMase activation domain, DD, death domain. SMase activities are expressed as percentage of basal activity in untreated cells, which was between 96 and 165 $\text{pmol} \cdot \text{h}^{-1} \cdot \text{mg}^{-1}$ of phosphocholine production for N-SMase and 0.25–2 $\text{nmol} \cdot \text{h}^{-1} \cdot \text{mg}^{-1}$ for A-SMase. The values shown for activation of N-SMase and A-SMase represent the means from triplicate determinations performed in parallel; error bars indicate the respective standard deviations. The appearance of a double band of phosphorylated GST-Jun is inherent to the preparation of substrate. The marginal activation of SAPK/JNK in cells expressing mutated forms of TR55 is non-specific, as it was also observed in untreated cells and does not follow the time course of activation (D. Adam, A. Ruff, A. Strelow, K. Wiegmann and M. Krönke, unpublished work). One out of several experiments with similar results is shown ($n = 3$ –9 for SAPK/JNK, 2–4 for N-SMase and A-SMase).

murine 70Z/3 pre-B cell lines that stably express the intact human TR55 or various deletion mutants thereof [6,8,16]. Parental 70Z/3 cells do not express endogenous TR55 but are capable of displaying TNF-specific responses after transfection of the human TR55 [6]. All mutant receptors contain deletions that destroy the death domain and are unable to signal activation of A-SMase. The mutant receptors TR55 Δ 394, TR55 Δ 320 and TR55 Δ 212–308/346 still carry a functional NSD that permits signalling through N-SMase, whereas the mutant TR55 Δ 308–340 carries a deletion that inactivates both domains and therefore prevents signalling through either pathway [16].

Parental and transfected 70Z/3 cells were stimulated with TNF and analysed for activation of SAPK/JNK. Additionally, the cell lines were analysed once more for activation of N-SMase and A-SMase to verify stability of the previously described response patterns to TNF [6,8,16]. 70Z/3TR55 cells expressing the intact TR55 displayed a rapid and pronounced increase in the activity of SAPK/JNK, N-SMase and A-SMase (Figure 1). In contrast, untransfected 70Z/3 cells did not show any significant change in SAPK/JNK or N-/A-SMase activity due to their lack of endogenous TR55. Similarly, we did not detect activation of SAPK/JNK in the transfectant 70Z/3TR55 Δ 308–340 which lacks the NSD and therefore cannot activate N-SMase. However, we also did not observe any significant induction of SAPK/JNK after TNF treatment of the transfectants 70Z/3TR55 Δ 394, 70Z/3TR55 Δ 320 and 70Z/3TR55 Δ 212–308/346 in which the activation of N-SMase in response to TNF was fully intact (Figure 1). To ensure that the activation of SAPK/JNK was not compromised in the examined transfectants for some non-specific reason, all cell lines were subjected to treatment with UV-C irradiation. In each case, a significant induction of SAPK/JNK was observed (Figure 1). These results clearly demonstrate that the activation of N-SMase by TR55 is not coupled to the induction of SAPK/JNK. Therefore, a role of N-SMase, or of ceramide produced by N-SMase, in the activation of SAPK/JNK appears unlikely. Rather, and in agreement with previous reports [28–30], the activation of SAPK/JNK by TR55 seems to be mediated exclusively through the death domain, without involvement of the NSD.

Activation of SAPK/JNK and A-SMase by TNF occurs with distinct dose–response relationships

The same deletions in the death domain of TR55 that abolish the activation of SAPK/JNK also lead to an inability of the corresponding 70Z/3 transfectants to activate A-SMase ([8,16]; see Figure 1). To clarify whether A-SMase constitutes an upstream signal from the death domain to SAPK/JNK, we first examined the sensitivity of both enzymes to TNF. In 70Z/3TR55 cells that express the intact TR55, SAPK/JNK were markedly activated by TNF concentrations as low as 2.5 ng/ml (Figure 2A). Increasing the TNF concentration to 100 ng/ml progressively enhanced this activation 2.5-fold. In contrast, A-SMase was not stimulated by TNF concentrations below 75 ng/ml (Figure 2B). Full activation of A-SMase was achieved at 100 ng/ml TNF. Thus, SAPK/JNK are activated at low concentrations of TNF which are insufficient to stimulate the activation of A-SMase. These results do not support a requirement of A-SMase activation for stimulation of SAPK/JNK in 70Z/3 cells.

Pharmacological inhibition of A-SMase does not affect TNF-induced activation of SAPK/JNK

D609 has been described as a specific inhibitor of phosphatidylcholine-specific phospholipase C [7]. D609 also prevents the

Figure 2 Different sensitivity of SAPK/JNK and A-SMase towards activation by TNF

(A) 70Z/3TR55 cells were treated with the indicated concentrations of TNF for 7 min and SAPK/JNK activation was determined. Phosphorylation of GST–Jun is expressed relative to untreated cells. (B) 70Z/3TR55 cells were stimulated for 3 min with the indicated concentrations of TNF or were left untreated (results not shown). Activation of A-SMase was measured as described in the Experimental section. A-SMase activities are expressed as a percentage of basal activity in untreated cells, which was between 2.2 and 3.0 nmol·h⁻¹·mg⁻¹ of phosphocholine production. The values shown for activation of A-SMase represent the means from triplicate determinations performed in parallel; error bars indicate the respective standard deviations.

activation of A-SMase, which might be downstream of phosphatidylcholine-specific phospholipase C [7]. As shown in Figure 3A, pretreatment of 70Z/3TR55 cells for 1 h with 100 μ g/ml D609 completely inhibited the activation of A-SMase by TNF. In contrast, the activation of SAPK/JNK by TNF was entirely insensitive to pretreatment with D609, suggesting that TR55 activates SAPK/JNK and A-SMase through different mechanisms.

The tricyclic antidepressants desipramine and imipramine have been shown to cause a rapid and irreversible reduction in A-SMase activity by inducing proteolytic degradation of the enzyme [35]. Pretreatment of 70Z/3TR55 cells for 2 h with 50 μ M desipramine or imipramine reduced the basal activity of A-SMase to 31% and 34% respectively of the basal activity of untreated cells, which, however, had no inhibitory effect on the stimulation of SAPK/JNK by TNF (Figure 3B). Notably, the residual A-SMase activity still proved sensitive to TNF treatment. In summary, these results indicate that down-modulation of inducible or basal activity of A-SMase has no impact on the TNF-induced stimulation of SAPK/JNK in this cell line.

Induction of SAPK/JNK by UV irradiation does not involve A-SMase

To explore the possibility that stress stimuli other than TNF might depend on A-SMase, we studied the effect of imipramine on the UV-C-induced phosphorylation of GST–Jun by SAPK/JNK in 70Z/3TR55 cells. UV-C induced a comparable pattern of GST–Jun phosphorylation in untreated cells or cells treated with 50 μ M imipramine for 2 h (Figure 4). Imipramine-pretreated cells showed a basal A-SMase activity of 38% compared with untreated cells (Figure 4). However, this reduction in A-SMase basal activity was not reflected by the activity of SAPK/JNK. Notably, A-SMase was not stimulated by UV-C within 3 min, which is the time frame for maximal activation by TNF (see Figure 2). Thus, the induction of SAPK/JNK by UV irradiation appears to occur independently of A-SMase in 70Z/3 cells.

Figure 3 Effect of inhibition of the TNF-dependent activation of A-SMase or proteolytic degradation of A-SMase on the activation of SAPK/JNK by TR55

(A) 70Z/3TR55 cells were left untreated (incubation in medium for 2 h, top panel), or preincubated with D609 (incubation in medium for 1 h followed by incubation for 1 h in medium with 100 μ g/ml D609, bottom panel). (B) 70Z/3TR55 cells were preincubated in medium with imipramine (50 μ M for 2 h, top panel) or desipramine (50 μ M for 2 h, bottom panel). Aliquots of cells were then uniformly incubated for a total of 30 min at 37 °C with 100 ng/ml TNF being added for the indicated times and the activity of SAPK/JNK and A-SMase was determined. Phosphorylation of GST-Jun is expressed relative to untreated cells (values have been rounded to whole numbers). The basal A-SMase activity in untreated cells was 1.9 nmol $\cdot$ h⁻¹ $\cdot$ mg⁻¹ of phosphocholine production. One out of four experiments giving similar results is shown.

Ceramide induces cell death and NF- κ B, but not SAPK/JNK, in 70Z/3 cells

Next, we wanted to examine whether the observed independence in the activation patterns of A-SMase and SAPK/JNK in 70Z/3TR55 cells was reflected by an unresponsiveness of SAPK/JNK to ceramide in this cell type. As shown in Figure 5A, treatment of cells with either TNF or UV-C led to a pronounced induction of SAPK/JNK. In contrast, synthetic C₂-ceramide

Figure 4 Effect of proteolytic degradation of A-SMase on the activation of SAPK/JNK by UV-C

70Z/3TR55 cells were left untreated (incubation in medium for 2 h, upper panel) or preincubated in medium with imipramine (50 μ M for 2 h, lower panel). The cells were divided into aliquots before they were irradiated with UV-C (5×10^4 μ J/cm²) and further incubated at 37 °C for the indicated times. The total time of incubation at 37 °C for all aliquots uniformly was 30 min before the activities of SAPK/JNK and A-SMase were determined. Phosphorylation of GST-Jun is expressed relative to untreated cells (values have been rounded to whole numbers). The basal A-SMase activity in untreated cells was 1.7 nmol $\cdot$ h⁻¹ $\cdot$ mg⁻¹ of phosphocholine production. One out of two experiments giving similar results is shown.

failed to specifically induce SAPK/JNK when compared with biologically inactive C₂-dihydroceramide, regardless of whether the lipids were delivered into the cells as a solution in DMSO (Figure 5A) or bound to serum proteins (results not shown). When the same treatment was applied to cells in assays for nuclear translocation of NF- κ B, a modest but reproducible induction of NF- κ B could be observed with C₂-ceramide, whereas C₂-dihydroceramide did not induce NF- κ B (Figure 5B). Moreover, treatment of cells with C₂-ceramide for 20 h strongly induced cell death, as measured by DNA fragmentation and changes in granularity/size of the cells, whereas C₂-dihydroceramide was largely ineffective (Figure 5C). These results demonstrate that whereas ceramide does elicit cellular responses in 70Z/3 cells, it does not activate SAPK/JNK in this cell type.

Niemann–Pick A-derived fibroblasts deficient for A-SMase show normal induction of SAPK/JNK by UV irradiation or TNF

Niemann–Pick disease is a lysosomal storage disorder that results from a deficiency of A-SMase activity [36]. We analysed fibroblasts from Niemann–Pick type A patients (cell line F91/11) that contain less than 1% of the basal A-SMase activity that was determined in control fibroblasts (cell line F91/17; results not shown). As shown in Figure 6(A), Niemann–Pick A fibroblasts responded to treatment with either TNF or UV-C with a significant activation of SAPK/JNK, which was similar to that of A-SMase-proficient control fibroblasts from healthy patients (Figure 6B). Thus, the stress-stimuli TNF or UV-C also do not utilize A-SMase for activation of SAPK/JNK in fibroblasts derived from Niemann–Pick A patients.

Figure 5 Induction by ceramide of cell death and NF- κ B, but not SAPK/JNK, in 70Z/3TR55 cells

(A) Aliquots of 70Z/3TR55 cells were uniformly incubated for a total of 60 min at 37 °C in medium with either C₂-ceramide or C₂-dihydroceramide added for the indicated times, both at 100 μ M in 1% DMSO, and the activity of SAPK/JNK was determined. The time points at 0 min represent cells incubated in medium with 1% DMSO for 60 min. For positive controls, cells were stimulated with 100 ng/ml TNF for 10 min or exposed to UV-C irradiation as described in the legend to Figure 1. (B) 70Z/3TR55 cells were treated with C₂-ceramide or C₂-dihydroceramide as in (A), nuclear extracts were prepared and 6 μ g of protein per lane was analysed for the presence of NF- κ B. For positive controls, cells were stimulated with TNF (100 ng/ml for 20 min). (C) 70Z/3TR55 cells were treated with either 1% DMSO (control), C₂-ceramide or C₂-dihydroceramide (100 μ M in 1% DMSO) and stained for cell cycle analysis after 20 h. The percentage of hypodiploid apoptotic cells is indicated. In addition, the corresponding density plots of gated cells showing changes in granularity and cell size are indicated to provide a second measure of cell death.

Figure 6 Response of A-SMase-deficient fibroblasts from patients with Niemann–Pick disease type A to SAPK/JNK activation by TNF or UV-C

(A) F91/11 fibroblasts from Niemann–Pick type A patients (residual A-SMase activity of 1% compared with control fibroblasts) were treated with TNF (100 ng/ml) for the indicated times or irradiated with UV-C (5×10^4 μ J/cm²) followed by incubation at 37 °C for the indicated times before the activity of SAPK/JNK was determined. (B) Parallel analysis using control fibroblasts proficient for A-SMase (cell line F91/17). Phosphorylation of GST–Jun is expressed relative to untreated cells (values have been rounded to whole numbers). One out of three experiments with similar results is shown. As can be seen, fibroblasts from patients with Niemann–Pick disease type A responded normally to the treatment.

DISCUSSION

The activation of sphingomyelinases by TR55 and subsequent production of ceramide has been implicated in cellular responses such as cell death or the production of pro-inflammatory

mediators [22,32]. In this study, we have analysed the possible involvement of sphingomyelinases in the induction of SAPK/JNK by TR55.

The results described here favour independent roles of SMases

and SAPK/JNK in the signalling pathways of TR55. The analysis of TR55 deletion mutants in 70Z/3 cells clearly suggests that N-SMase is not involved in activation of SAPK/JNK in these cells. Bradshaw et al. [37] have recently examined N-SMase and SAPK/JNK in T-cells. The addition of exogenous C_2 -ceramide or of bacterial N-SMase from *Staphylococcus aureus* did not activate SAPK/JNK within 10 min, a time-frame usually observed with other stimuli [37]. Apparently, N-SMase and ceramide produced by N-SMase do not connect to signalling cascades leading to the activation of SAPK/JNK in the above cell types.

With regard to a possible function of A-SMase in SAPK/JNK activation, our results demonstrate that stimulation of SAPK/JNK by TR55 does not generally depend on prior activation of A-SMase. In the cell types examined, we do not observe an impaired activation of SAPK/JNK when A-SMase is inherently deficient (as in fibroblasts from patients with Niemann–Pick disease) or inhibited or degraded by pharmacological agents (70Z/3 pre-B cells). Therefore a block in A-SMase signalling has no apparent effect on the activation of SAPK/JNK by TR55 in these cells. Although these observations indicate an independent activation of SAPK/JNK and N-/A-SMase by TR55, at this point we cannot rule out the possibility that A-SMase may be required for the induction of SAPK/JNK in other cell types.

The possibility of a cell-type-specific requirement for A-SMase may provide a solution for contradictory observations concerning the coupling of ceramide to SAPK/JNK activation. For example, various SAPK/JNK-inducing stresses like ionizing radiation, hydrogen peroxide, UV-C irradiation, heat shock and TNF all lead to elevated production of ceramide in U937 (human monoblastic leukaemia) and BAE (bovine aortic endothelial) cells [26]. Also, exogenously applied C_2 -ceramide can result in the induction of SAPK/JNK in these cell lines [26]. Whereas these findings suggest a role of ceramide (and of sphingomyelinases) in the initiation of a stress response through SAPK/JNK in these specific cell lines, our own results demonstrate that TR55 and UV-C can induce SAPK/JNK entirely without involvement of sphingomyelinases in other cell types. In addition, our results show that exogenously applied C_2 -ceramide does not induce SAPK/JNK in 70Z/3 pre-B cells, whereas induction of cell death and NF- κ B still occurs, further supporting the assumption that sphingomyelinases or ceramide are not absolutely required for activation of SAPK/JNK in specific cell types. The notion of cell-type-specific effects of sphingomyelinases and ceramide is additionally supported by reports showing that SAPK/JNK are also not induced by exogenous ceramide in yet other cell types, e.g. in endothelial cells [17,38] or in murine stromal cells [39].

We would like to emphasize that our results do not exclude the possibility that *de novo* ceramide synthesis by ceramide synthase may bypass the requirement for A-SMase. However, it should be noted that, unlike A-SMase, ceramide synthase is activated only after hours of stimulation and for prolonged periods of time [40].

A putative interaction between A-SMase and SAPK/JNK may also depend on the examined receptor system that triggers the signal. For Fas/APO-1, it has recently been shown that proteolytic degradation of A-SMase by imipramine abolishes the activation of SAPK/JNK and p38 by this receptor [41]. In Fas/APO-1 signalling, inhibition of caspases by cytokine response modifier A (Crma) or treatment with Ac-YVAD-chloromethyl ketone blocked stimulation of A-SMase, release of ceramide, activation of SAPK/JNK and p38- as well as Fas/APO-1-triggered apoptosis, suggesting a model in which Fas sequentially activates caspases and A-SMase, resulting in the release of ceramide, which in turn activates SAPK/JNK/p38

[41]. Additional support for this model comes from the observation that a distinct pathway leading from Fas to apoptosis sequentially involves the Fas-death-domain-associated protein, SAPK/JNK and caspases [42].

For TR55, however, studies from several groups indicate that this receptor activates SAPK/JNK through a non-apoptotic pathway, that is, by recruiting the protein TRAF2 into the receptor complex without a requirement for factors such as Fas-associated protein with death domain (FADD) or involvement of caspases. Consequently, an involvement of SAPK/JNK in the apoptotic response of TR55 appeared unlikely in these studies [28–30]. With regard to a model of how TR55 might activate SAPK/JNK, Song et al. [43] have recently demonstrated that overexpression of TRAF2 activated both SAPK/JNK and nuclear translocation of NF- κ B, whereas expression of the TRAF2-associated kinase NF- κ B-inducing kinase (NIK) only stimulated activation of NF- κ B but not of SAPK/JNK. Therefore, a distinct TRAF2-interacting kinase may trigger a signalling cascade culminating in JNK activation, independently from the pathway leading from TRAF2 to translocation of NF- κ B. This kinase, however, has not yet been identified.

Considering that A-SMase is not required for activation of SAPK/JNK in 70Z/3 cells and Niemann–Pick fibroblasts, and that A-SMase-deficient Niemann–Pick fibroblasts can still induce TRAF2-mediated [28] nuclear translocation of NF- κ B after treatment with TNF [44,45], it is plausible to assume that A-SMase does not belong to a signalling pathway involving TRAF2 in these cell types. Rather, A-SMase may be part of an apoptotic pathway through the adapter proteins TNF receptor-associated protein with death domain (TRADD) and FADD (for review, see [46]) and may signal through pathways independent from SAPK/JNK. This assumption is supported by experiments from our own laboratory which demonstrate that overexpression of TRADD and/or FADD enhances TNF-dependent activation of A-SMase in 293 cells [47]. In contrast, overexpression of TRAF2 or of dominant negative TRAF2 had no effect on the induction of A-SMase by TNF, underscoring the independent induction of both A-SMase and SAPK/JNK by TR55 [47].

In summary, the differences in the relationship between A-SMase and SAPK/JNK described for Fas/APO-1 and TR55 may be explained by the fact that the two receptors recruit different sets of associated proteins and therefore utilize different signalling pathways to elicit similar responses, including SAPK/JNK activation. For Fas/APO-1, the activation of A-SMase may be an integral part of the SAPK/JNK and apoptotic pathway. For TR55, however, A-SMase is obviously not an essential component in the activation of SAPK/JNK.

The results presented in this study provide evidence that sphingomyelinases are not critical for the induction of SAPK/JNK by TR55 in 70Z/3 pre-B cells and in Niemann–Pick A fibroblasts. The possibility remains that A-SMase may be important in the activation of SAPK/JNK in other cell types, e.g. in those that depend on SAPK/JNK for initiation of apoptosis. A possible role of A-SMase in the apoptotic response of TR55 via TRADD/caspases and the exact positioning of A-SMase will be addressed in further studies.

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