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Activation of the JAK/STAT pathway by ceramide in cultured human fibroblasts

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Abstract Endogenous ceramide (CER) was generated by treatment of cultured fibroblasts with sphingomyelinase (SMase) from *Bacillus cereus*. A 30 min treatment with 0.1–0.3 U/ml SMase induced a dose-dependent increase in the intracellular level of CER. The activation of the transcription factors signal transducer and activator of transcription (STAT) 1 and STAT3 by SMase was investigated by determination of the phosphorylation state by immunoblot, and of DNA binding activity by electrophoretic mobility shift assay. SMase treatment induced a dose-dependent Tyr-phosphorylation of STAT1/3. SMase also enhanced STAT1/3 DNA binding activity in a dose-dependent manner. Concomitantly, SMase enhanced the Tyr-phosphorylation of Janus kinase (JAK) 2, a Tyr-kinase localized upstream of STATs in the JAK/STAT pathway. The Tyr-kinase inhibitor genistein and the JAK inhibitor AG490 both prevented JAK2 Tyr-phosphorylation, together with STAT1 and STAT3 Tyr-phosphorylation and binding activity. The SMase-induced increase in STAT1/3 binding activity was prevented by methyl-β-cyclodextrin, a cholesterol binding agent that causes a loss of compartmentalization of the molecules located in caveolae. This increase was also prevented by the MEK inhibitor PD98059, thus demonstrating the role of the MEK/ERK pathway in this system. Besides ERK, SMase activated other signaling kinases such as JNK and p38. Exogenous natural CER also activated STAT1/3 binding activity, which indicates that most probably, endogenous CER is the second messenger involved in the effect of SMase. These results describe a crosstalk between the SMase/CER and the JAK/STAT signaling pathways and include JAK2 within the range of CER-activated intracellular kinases.

Key words: Sphingomyelinase; Ceramide; Janus kinase; Signal transducer and activator of transcription

1. Introduction

STATs (signal transducers and activators of transcription) are involved in cytokine, growth factor and hormone transduction pathways (review in [1]). Cytokine binding, after dimerization/oligomerization of the receptors, initiates the Tyr-phosphorylation of the receptor-associated Tyr-kinases of the Janus kinase family (JAKs). Activated JAKs then induce the Tyr-phosphorylation of the receptor itself and of cytoplasmic STATs recruited by their SH2 domain [2,3]. Tyr-phosphorylated STATs then undergo homo/heterodimerization [4], a prerequisite for translocation to the nucleus [5]. The Tyr-phosphorylated STATs subsequently bind to specific DNA sequences and exert their transcriptional activity on target genes [6]. We previously demonstrated that STAT1 and STAT3 are activated by oxidative stresses generated by oxidized low density lipoproteins [7] or UV-A radiation [8].

Generation of the sphingolipid ceramide (CER) from sphingomyelin (SM) of the cell membrane has been shown to be an important signaling event involved in cell growth, differentiation and programmed cell death (review in [9]). This pathway is also activated by cellular stresses, including activation of tumor necrosis factor α (TNFα) [10] or interleukin 1 (IL1) [11] receptors, UV radiation [12] or Fas antigen [13]. Downstream of SM hydrolysis, it was demonstrated that CER can stimulate several adenylyl cyclase subtypes [14]. CER can also activate different kinds of kinases, including a Ser/Thr kinase termed CER-activated protein kinase which turns out to be a proline-directed kinase [15]. Fas receptor ligation or cellular treatment with synthetic CER results in activation of the small G proteins Ras, Rac1 and of the stress kinases JNK and p38 [16]. CER generation after TNFα ligand binding also stimulates Raf1, a Ser/Thr kinase located upstream of MEK-MAPKK [17]. In vitro, CER leads to the activation of a Ser/Thr phosphatase related to the PP2A family [18].

In the current studies, we demonstrate crosstalk between the JAK/STAT and the sphingomyelinase (SMase)/CER signaling pathways in that endogenous CER can activate JAK2, and thus STAT1 and STAT3, by Tyr-phosphorylation. In addition, besides JAK2, it appeared that this activation also involves the MEK pathway. Finally, exogenous natural CER also activates STAT1/3 DNA binding activity.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum were from Gibco. The oligonucleotide probes STAT1 (5′-CAT GTT ATG CAT ATT CCT GTA AGT G-3′) and STAT3 (5′-GAT CCT TCT GGG AAT TCC TAG ATC-3′) were synthesized by Eurogentec, Belgium. [9,10-^{3}H]Palmitic acid 50 Ci/mmol was provided by Amersham. [γ-^{32}P]ATP 7000 Ci/mmol was from ICN. The anti-STAT1 S21120 and anti-STAT3 S21320 antibodies were from Transduction Laboratories. The anti-phosphotyrosine 4G10 and anti-JAK2 antibodies were obtained from Upstate Biotechnology. The JAK inhibitor AG490 and the MEK inhibitor PD98059 were from France

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Abbreviations: SM, sphingomyelin; SMase, sphingomyelinase; CER, ceramide; JAK, Janus kinase; STAT, signal transducer and activator of transcription

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Biochem. SMase from *Bacillus cereus*, CER from bovine brain and all other chemicals were of Sigma grade.

2.2. Cell culture

MRC5 human fibroblasts were purchased from BioMérieux, France. The cells were maintained in DMEM supplemented with 10% fetal calf serum. All experiments were performed on subconfluent cultures, 3 days after seeding. The cells were preincubated for 24 h in medium devoid of serum and supplemented with 0.1% bovine serum albumin. It was checked that the preincubation with albumin down-regulated the studied transduction pathway. The cells were then incubated with 0.1–0.3 U/ml SMase for 30 min before the preparation of cellular extracts for immunoblot analysis or nuclear extracts for electromobility shift assay. Natural CER was dissolved in ethanol/dodecane 98/2 (v/v) according to Ji et al. [19] before addition to the medium for 1 h. The final concentrations of ethanol and dodecane were 0.5% and 0.01% respectively.

2.3. Generation of endogenous ceramide

Cells were incubated overnight with 0.5 μCi/ml palmitic acid. After washing, the cells were incubated for 30 min with 0.1–0.3 U/ml SMase. After harvesting, lipid extraction was performed by the method of Bligh and Dyer [20]. CER and SM were then separated by thin layer chromatography using chloroform/methanol/H_2O 65/25/4 v/v and petroleum ether/diethyl ether/acetic acid 80/20/1 v/v as solvent [21]. After visualization in iodine vapor and identification by known standards, the radioactivity of the CER spot was determined by liquid scintillation counting. Results are expressed as dpm CER/μg protein.

2.4. Immunoprecipitation and immunoblot analysis

Cells were lysed in 20 mM Tris pH 7.9, 400 mM NaCl, 1 mM EDTA, 20% glycerol, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml leupeptin and aprotinin and 0.1 mM Na_3VO_4, and clarified by centrifugation. Immunoprecipitation was performed on 150 μg protein with 1.5 μg of the anti-phosphotyrosine antibody 4G10 and 5 mg protein A-Sepharose at 4°C overnight. Immunoprecipitates were solubilized and analyzed by SDS–PAGE. After transfer of proteins onto a nitrocellulose membrane, blots were revealed with anti-JAK2, anti-STAT1 and anti-STAT3 antibodies. Visualization was performed with the enhanced chemiluminescence kit from Amersham.

2.5. Preparation of nuclear extracts

Nuclear extracts were prepared according to Dignam et al. [22]. Cells were resuspended in 10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.1% Nonidet P-40 and the protease inhibitors PMSF 0.2 mM, aprotinin 2 mM, antipain, pepstatin, benzamidine and leupeptin 1 μg/ml. After homogenization with a Dounce homogenizer and a 10 min incubation at 4°C, nuclei were collected by centrifugation at 2000×*g* for 30 min. The pellet was resuspended in 20 mM HEPES pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 25% glycerol and the above cited antiproteases. The nuclear proteins were extracted by incubation at 4°C during 30 min. After centrifugation at 13 000×*g* for 15 min, the supernatant was kept at −80°C.

2.6. Electrophoretic mobility shift assay

The double-stranded oligonucleotides were end-labeled using T4 kinase and [γ-^{32}P]ATP. 7 μg of nuclear extract was incubated with 100 000 dpm of labeled probe (0.5 ng) in the presence of 1 μg of poly(dIdC) at room temperature for 20 min, followed by separation of the mixture on a 6% non-denaturing polyacrylamide gel in Tris 50 mM/glycine 0.38 M/EDTA 2 mM buffer at pH 7.5.

Each experiment was repeated three times. Protein bands and DNA complexes were quantified using a Molecular Dynamics densitometer. The coefficient of analytical variability did not exceed 10–15%.

3. Results

3.1. Generation of endogenous CER by SMase

We first checked that the treatment of intact cells with neutral SMase effectively induced the production of CER. The data in Fig. 1 show that, with tritiated palmitic acid as precursor, a 30 min incubation with 0.1–0.3 U/ml SMase increased the production of CER in a dose-dependent manner. An about eight-fold increase was found for 0.3 U/ml SMase.

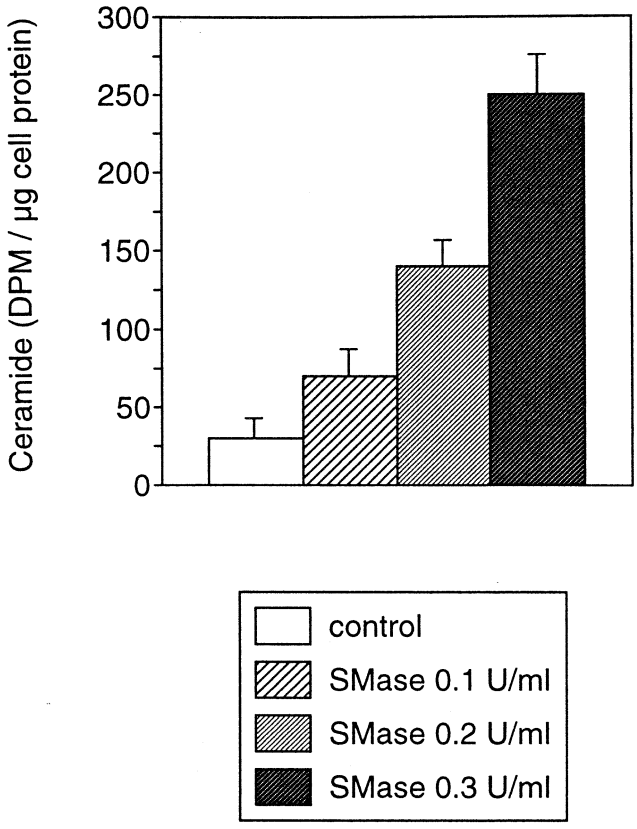

Fig. 1. SMase induces the generation of endogenous CER. MRC5 fibroblasts were preincubated overnight with 0.5 μCi/ml palmitic acid in medium supplemented with 0.1% bovine serum albumin. After washing, the cells were incubated for 30 min with 0.1–0.3 U/ml SMase. After harvesting, lipid extraction was performed and the lipid extract analyzed by thin layer chromatography. Results are expressed in dpm CER/μg protein. Means ± S.D. from a typical experiment in triplicate. This experiment was repeated three times with similar results.

3.2. SMase enhances STAT1 and STAT3 Tyr-phosphorylation and binding activity in a dose-dependent manner

The next experiment was designed to investigate the dose-dependent effect of a 30 min treatment with SMase. Studies of the Tyr-phosphorylation state by immunoblot indicate that SMase dose-dependently increased the phosphorylation of both STATs (Fig. 2A) with a somewhat more marked effect for STAT1. Concomitantly, SMase also augmented the binding activity of both STATs in a dose-dependent manner (Fig. 2B).

3.3. SMase activates STATs by means of JAK2

In the cytokine transduction pathway, the Tyr-phosphorylation of STATs is under the control of the JAK family [1]. We thus looked upstream of STATs for the activation of JAK2, a ubiquitous kinase of the JAK family. The results in Fig. 2C demonstrate first, that SMase induced the Tyr-phosphorylation of JAK2, and second, that if the phosphorylation of JAK2 was prevented by genistein or AG490, the Tyr-phosphorylation of STATs was no longer observed. In parallel, as expected, the STAT1 and STAT3 binding activ-

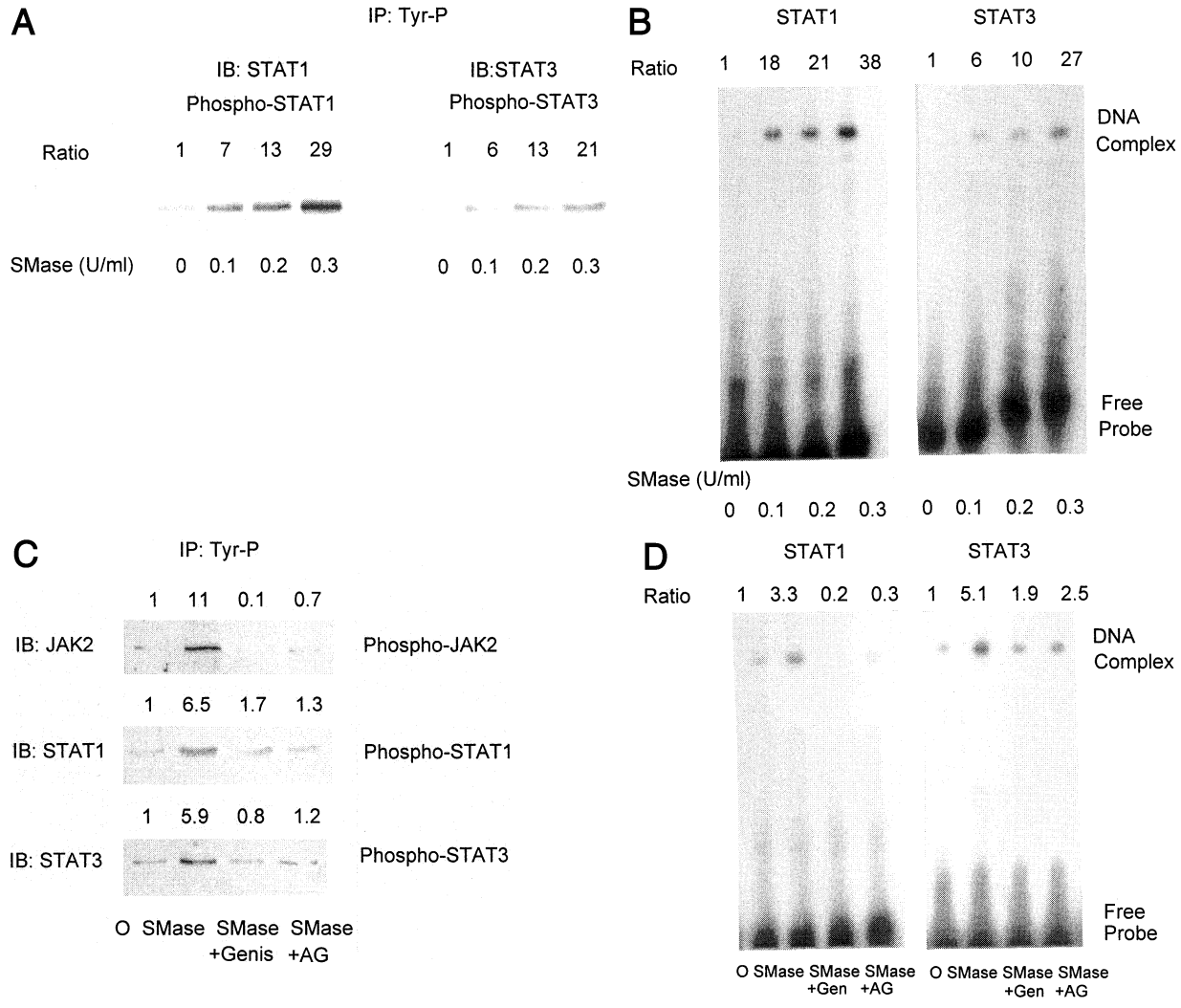

Fig. 2. SMase enhances STAT1 and STAT3 tyrosine-phosphorylation (A) and DNA binding activity (B). The CER-induced Tyr-phosphorylation (C) and binding activity (D) of STAT1 and STAT3 are prevented by genistein and AG490. MRC5 fibroblasts were preincubated for 24 h in medium supplemented with 0.1% bovine serum albumin and incubated for 30 min with 0.1–0.3 U/ml SMase. 5×10^{-5} M genistein and 2×10^{-5} M AG490 were added 30 min before a further incubation of 30 min with 0.2 U/ml SMase. Cellular or nuclear extracts were then prepared. STAT1 and STAT3 phosphorylation state was determined by immunoprecipitation (IP) with an anti-TyrP antibody followed by immunoblotting (IB) with an anti-STAT1 or -STAT3 antibody. STAT1 and STAT3 DNA binding activities were studied by electrophoretic mobility shift assay. Results are from a typical experiment. This experiment was repeated three times.

ities were no longer stimulated (Fig. 2D). Genistein and AG490 had no effect when added alone (data not shown).

3.4. The caveola inhibitor methyl-β-cyclodextrin prevents the SMase-induced STAT1 and STAT3 activation

Since SM is localized within caveolae [23], which are plasma membrane domains enriched in cholesterol, the next experiment was designed to test the effect of the cholesterol binding agent methyl-β-cyclodextrin, which has been reported to cause the loss of compartmentalization of molecules located in caveolae [24]. It was noted (Fig. 3A) that while SMase and methyl-β-cyclodextrin alone exhibited little effect on the cell morphology, the combination of the two factors induced a marked modification of the cell shape, indicating some perturbation within the membrane architecture. Furthermore, after treatment with methyl-β-cyclodextrin, the SMase-induced STAT1 and STAT3 activation was no longer observed (Fig. 3B).

3.5. The activation of STATs by SMase also involves ERK

Since it is known that the Tyr-phosphorylation of STATs is required for DNA binding, whereas Ser-phosphorylation by ERK regulates the transactivating potential [25], we also looked for any effect of SMase on ERK activity. Fig. 4A demonstrates that 0.2 U/ml SMase markedly activated ERK. In addition, the MEK inhibitor PD98059, which exhibited no effect by itself (data not shown), prevented the SMase-induced activation of both STATs (Fig. 4B), thus demonstrating the role of the MEK/ERK pathway in this phenomenon. The data from Fig. 4C indicate that besides ERK, SMase also activated the kinases JNK and p38.

3.6. Exogenous natural CER enhances STAT1/3 binding activity

A dispersion of exogenous CER was introduced in the culture medium in ethanol/dodecane solution in order to deliver hydrophobic CER to cells [19]. It was found (Fig. 5) that 200

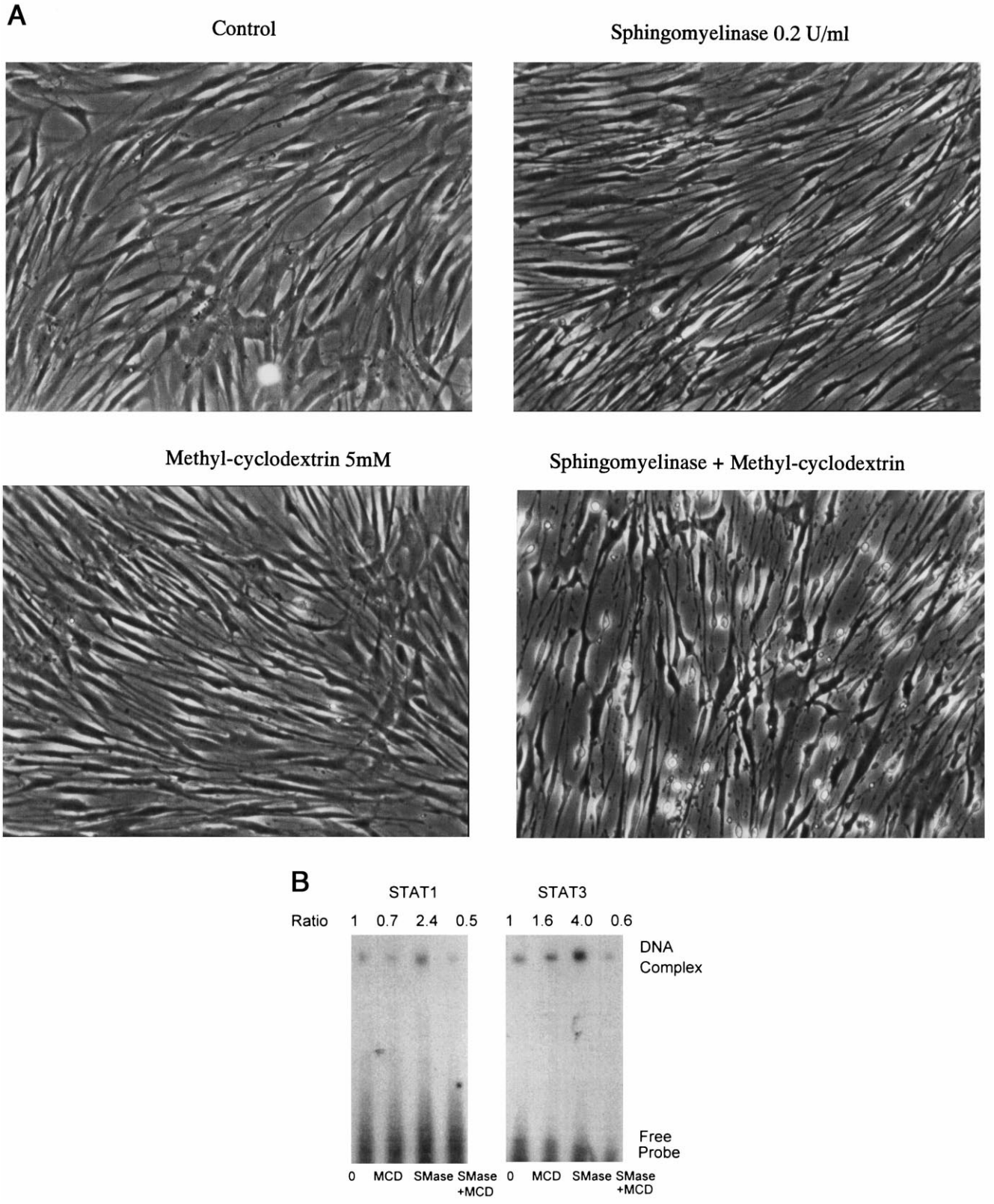

Fig. 3. Effect of SMase and methyl-β-cyclodextrin on cell morphology. The caveola inhibitor methyl-β-cyclodextrin prevents the SMase-induced STAT1 and STAT3 activation. MRC5 fibroblasts were preincubated for 24 h in medium supplemented with 0.1% bovine serum albumin and pretreated for 30 min with 5 mM methyl-β-cyclodextrin. After washing, the cells were incubated with 0.2 U/ml SMase for 30 min. STAT1 and STAT3 DNA binding activities were studied by electrophoretic mobility shift assay. Results are from a typical experiment. This experiment was repeated three times.

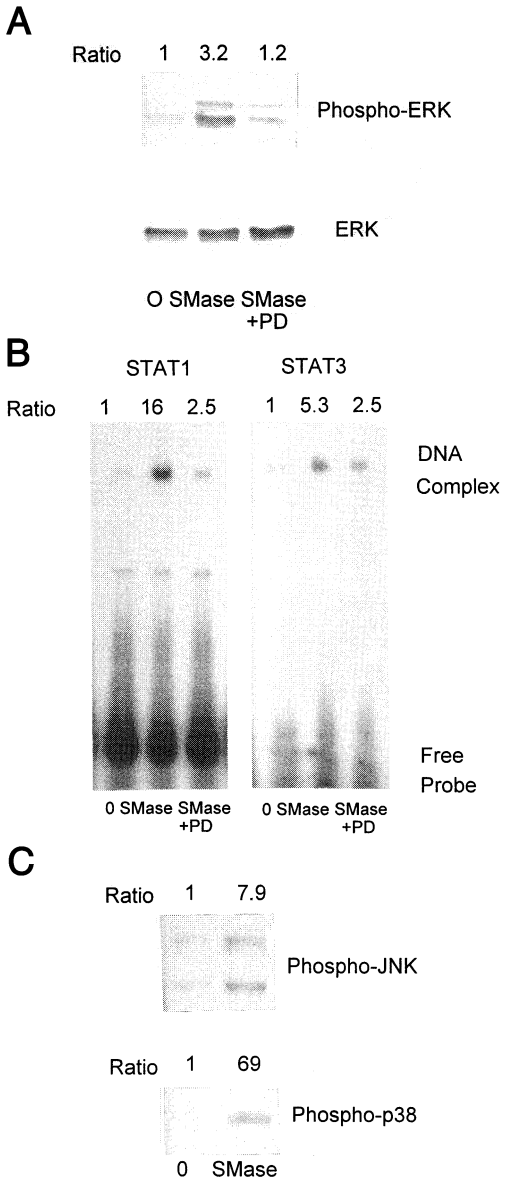

Fig. 4. The MEK inhibitor PD98059 prevents the activation of STAT1 and STAT3 by SMase (A, B). SMase activates JNK and p38 (C). MRC5 fibroblasts were preincubated for 24 h in medium supplemented with 0.1% bovine serum albumin. 10^{-5} M PD98059 was then added 30 min before a further 30 min incubation with 0.2 U/ml SMase. Cellular or nuclear extracts were then prepared. ERK, JNK and p38 phosphorylation states were determined by immunoblot with a specific antibody (A, C). STAT1 and STAT3 DNA binding activities were studied by electrophoretic mobility shift assay (B). Results are from a typical experiment. This experiment was repeated three times.

nM and 500 nM CER increased STAT1/3 activity in a dose-dependent manner, thus demonstrating that most probably, the second messenger CER is involved in the action of SMase.

4. Discussion

We first demonstrated that the second messenger CER can be produced by treatment of cultured fibroblasts with neutral SMase of bacterial origin (Fig. 1). This is an interesting alternative to the utilization of exogenous CER and resolves the problem of cellular penetration and delivery to intracellular targets. Indeed, it has been reported that the generation of CER from SM is highly compartmentalized and takes place in SM-rich plasma membrane domains with the characteristics of caveolae [23]. That caveolae are involved in membrane internalization [26] may thus facilitate the delivery of CER within the cell and the activation of signaling kinases and transcription factors. In our experimental conditions, the fact that methyl-β-cyclodextrin, a cholesterol binding agent which induces a loss of compartmentalization of the molecules located within caveolae, prevented the SMase-induced activation of STAT1 and STAT3 (Fig. 3B) indicates that the generation of CER most probably takes place in caveolae. In this regard, it is of note that exogenous natural CER exhibited the same effect as SMase (Figs. 2B and 5).

Once generated, CER activated STAT1 and STAT3 DNA binding activity in a dose-dependent manner (Fig. 2B). This activation is due, as expected, to the Tyr-phosphorylation of the transcription factors (Fig. 2A). Activation of transcription factors is a general mechanism whereby CER exerts its action on cellular proliferation, differentiation and apoptosis. It has been reported that CER activates AP1 by increasing c-jun mRNA synthesis [27], or NF-κB by inducing the processing of p105 to p50, a component of the NF-κB transcription factor [28]. Exogenous SMase also stimulates NF-κB activity, an effect inhibited by low temperature or hypertonicity, two inhibitors of receptor internalization [29]. Concerning the mechanism of action of CER, it has been proposed that this second messenger primarily acts by inducing an oxidative stress, via a hyperproduction of reactive oxygen species by the mitochondrial electron transport chain [30]. Indeed, C2-CER directly stimulates the production of hydrogen peroxide by mitochondrial preparations [30,31]. It has also been reported that the CER-induced activation of NF-κB and AP1 is mediated by mitochondrial-derived reactive oxygen species [32].

Several lines of evidence indicate that the activation of tran-

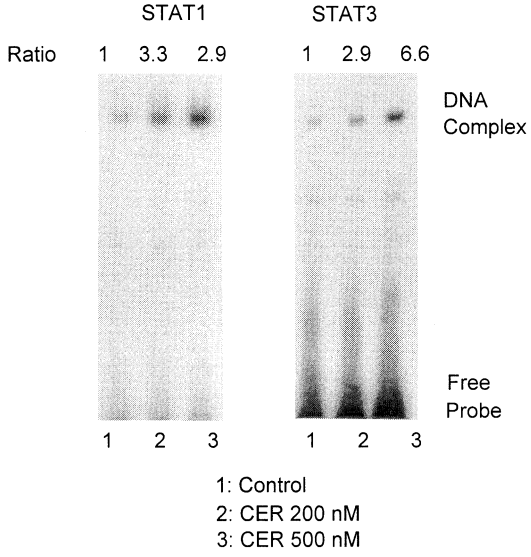

Fig. 5. Exogenous natural CER enhances STAT1 and STAT3 binding activity. MRC5 fibroblasts were preincubated for 24 h in medium supplemented with 0.1% bovine serum albumin. CER was then added in ethanol/dodecane 98/2 v/v solution for 1 h. STAT1 and STAT3 DNA binding activities were studied by electrophoretic mobility shift assay. Results are from a typical experiment. This experiment was repeated three times.

scription factors by SMase might be achieved by means of its action on stress-activated kinases. Reunanen et al. [33] reported that the expression of the matrix metalloprotease MMP1 is under the control of CER via an AP1 *cis* element, and that the CER-induced activation involves ERK1/2, SAPK/JNK and p38. Similarly, Brennen et al. [34] demonstrated that the activation of the transcription factor GADD 153 by CER results from the stimulation of the small G protein Rac1 and the kinases JNK and p38. In our experimental model, the JAK inhibitor AG940 (Fig. 2C,D) and the MEK inhibitor PD98540 (Fig. 3A,B) both prevented the SMase-induced activation of STAT1/3, which demonstrates that SMase stimulates not only the Tyr-phosphorylation of STAT1 and STAT3 by means of JAK2, but also their Ser-phosphorylation by means of ERK. It is of note that in our experimental conditions, besides JAK2 and ERK, SMase also induced a stimulation of other signaling kinases such as JNK and p38 (Fig. 3C).

The current studies point to the pleiotropic nature of CER-mediated signaling pathways by including JAK2 within the range of CER-activated intracellular kinases. The stimulation of JAK2 activity then in turn enhances STAT1 and STAT3 Tyr-phosphorylation and DNA binding activity, which identifies STAT transcription factors as downstream targets of CER. Previous work from our laboratory demonstrated that UV-A radiation induces CER production [35], and concomitantly enhances STAT1 binding activity [8], which suggests that physio-pathological stimuli generating endogenous CER also stimulate the JAK/STAT pathway. Since the SMase/CER signaling pathway is triggered by some inflammatory cytokines such as TNFα and IL1, the activation of the JAK/STAT pathway might provide, besides the other well-known CER-activated transduction pathways, an additional efficient mechanism to regulate the inflammatory response.

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