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Novel synergistic mechanism for sst2 somatostatin and TNFα receptors to induce apoptosis: crosstalk between NF-κB and JNK pathways

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Somatostatin is a multifunctional hormone that modulates cell proliferation, differentiation and apoptosis. Mechanisms for somatostatin-induced apoptosis are at present mostly unsolved. Therefore, we investigated whether somatostatin receptor subtype 2 (sst2) induces apoptosis in the nontransformed murine fibroblastic NIH3T3 cells. Somatostatin receptor subtype 2 expression induced an executioner caspase-mediated apoptosis through a tyrosine phosphatase SHP-1 (Src homology domain phosphatase-1)-dependent stimulation of nuclear factor kappa B (NF-κB) activity and subsequent inhibition of the mitogen-activated protein kinase JNK. Tumor necrosis factor α (TNFα) stimulated both NF-κB and c-Jun NH2-terminal kinase (JNK) activities, which had opposite action on cell survival. Importantly, sst2 sensitized NIH3T3 cells to TNFα-induced apoptosis by (1) upregulating TNFα receptor protein expression, and sensitizing to TNFα-induced caspase-8 activation; (2) enhancing TNFα-mediated activation of NF-κB, resulting in JNK inhibition and subsequent executioner caspase activation and cell death. We have here unraveled a novel signaling mechanism for a G protein-coupled receptor, which directly triggers apoptosis and crosstalks with a death receptor to enhance death ligand-induced apoptosis.
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Somatostatin is a regulatory peptide which is mostly expressed in the central and peripheral nervous system, in the gastro-intestinal tract and in the pancreas. Somatostatin acts via a family of five G protein-coupled receptors (GPCR), somatostatin receptor subtypes 1–5 (sst1–sst5) that are variably expressed throughout numerous tissues ranging from the central nervous system to the endocrine and immune systems.[1] Somatostatin or its analogues promote growth inhibition of various normal and tumor cells, both *in vitro* and *in vivo*. Somatostatin affects cell growth indirectly by inhibiting either trophic or growth factor synthesis and/or secretion, or their respective intracellular pathways. Somatostatin antiproliferative action also results from a direct blockade of cell cycle and/or induction of apoptosis.[2] Whereas somatostatin effect on cell cycle arrest has been extensively studied, mechanisms for somatostatin-induced apoptosis and receptor subtype implication are only partially elucidated. Somatostatin has been shown to induce cell death in both normal and tumoral cell models[3–9]. Among the five somatostatin receptors, sst3 and sst2 have been found to play a critical role in somatostatin-induced apoptosis of normal and tumor cells, respectively.[3,5,10] Mechanisms for somatostatin apoptotic action were shown to rely on the mitochondrial pathway through activation either of sst2 or sst3. However, signaling pathways coupling sst2 receptor to apoptosis have been partially elucidated.

We have previously demonstrated that expression of sst2 in the nontransformated murine fibroblastic NIH3T3 cells results in a cell growth inhibition, which was dependent on the activation of the tyrosine phosphatase Src homology domain phosphatase-1 (SHP-1).[11] sst2 antiproliferative action was evident in the absence of any addition of exogenous somatostatin and occurred as a consequence of an autocrine sst2-dependent loop whereby sst2 induces the expression of its own ligand, somatostatin.[11] We aimed to explore mechanisms for sst2 proapoptotic action using this cell model, and we therefore investigated whether sst2 expression induces cell death in NIH3T3 cells. We were also interested to investigate whether sst2 expression sensitizes NIH3T3 cells to apoptosis induced by the death ligand tumor necrosis factor α (TNFα).

Results

sst2 induces an executioner caspase-dependent apoptosis. We previously demonstrated in NIH3T3 cells stably transfected with the human sst2 cDNA (NIH3T3/sst2), that the autocrine sst2-somatostatin loop results in cell

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Abbreviations: EMSA, electrophoretic mobility shift assay; GPCR, G protein-coupled receptor; IL-1, interleukin-1; IκB, inhibitory factor-kappa B; JNK, c-Jun NH2-terminal kinase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; NF-κB, nuclear factor-kappa B; PAF-R, platelet-activating factor-receptor; PARP, poly(ADP ribose) polymerase; SHP-1, Src homology domain phosphatase-1; sst1–5, somatostatin receptor subtypes 1–5; TNF, tumor necrosis factor; TNFR1, tumor necrosis factor receptor 1; TUNEL, terminal deoxynucleotidyltransferase (TdT)-mediated dUTP-biotin nick end labeling
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growth inhibition.[11] We therefore investigated whether sst2 induces cell death in this previously described and characterized model.[11,12] We quantified apoptosis by using the terminal deoxynucleotidyltransferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) technique. After 5-h cell serum starvation, apoptosis in sst2-expressing cells was 3.6-±1.2-fold higher than in mock cells (5.6±0.8 *versus* 1.5±0.5% of apoptotic cells, respectively) (Figure 1a). Cell treatment with the specific executioner caspase competitive inhibitor DEVD-CHO (uncleavable substrate)[13] abrogated sst2-mediated apoptosis, and reverted apoptosis levels in NIH3T3/sst2 to those observed in mock cells (NIH3T3/mock) (Figure 1a). By using a cell viability assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT)), we showed as well that sst2 decreased cell viability in NIH3T3/sst2 to 72±1.3% of mock cells (Figure 1b). These results demonstrated that expression of sst2 in NIH3T3 cells stimulates an executioner caspase-dependent apoptosis.

sst2 sensitizes NIH3T3 cells to TNFα-induced apoptosis. We then examined whether sst2 sensitizes NIH3T3 cells to TNFα-induced cell death by using the TUNEL technique. As shown in Figure 1a, apoptosis was increased in NIH3T3/mock cells by 5.3±2.4-fold after a 5-h treatment with TNFα. Interestingly, TNFα-induced cell death was further enhanced in sst2-expressing NIH3T3 cells, as the percentage of TNFα-treated apoptotic cells was increased by 2.3±0.8-fold in NIH3T3/sst2 *versus* NIH3T3/mock cells (19.0±1.5 *versus* 8.2±2.8%, of apoptotic cells, respectively) (Figure 1a). We therefore concluded that sst2 sensitized NIH3T3/sst2 cells to TNFα-induced apoptosis because sst2 and TNFα effects on apoptosis were more than additive.

Similarly, a 24-h treatment with TNFα decreased cell viability more robustly in NIH3T3/sst2 cells than in mock cells (36±0.4 v*ersus* 75±9.9%, of untreated mock cells, respectively) (Figure 1b). To specifically show the difference between mock- and sst2-expressing NIH3T3 cell sensitivity towards TNFα treatment, without considering basal sst2 effect, cell viability was expressed as 100% of the respective TNFα-untreated mock- or sst2-expressing cells (Figure 1b, inset). Using this representation, sst2 sensitization to TNFα-induced decrease of cell viability was evident (53±1.7 *versus* 75±9.9%, for TNFα-treated sst2- *versus* mock-expressing cells, respectively) (Figure 1b, inset). A dose–response with TNFα (0–100 ng/ml) showed that sst2-mediated cell sensitization to TNFα-induced cell survival decrease was maximal at 10 ng/ml TNFα (data not shown). Moreover, sst2 shifted the TNFα dose–response curve to the left ($IC_{50}=1.3$ *versus* 12 ng/ml for NIH3T3/sst2 *versus* NIH3T3/mock cells).

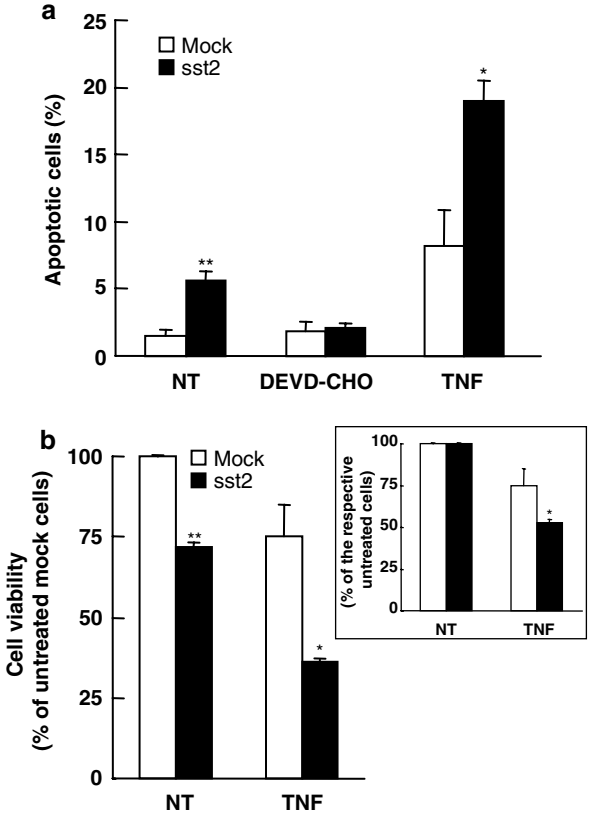

Figure 1 Quantification of basal and TNFα-induced cell death in NIH3T3 cells expressing or not sst2. (**a**) TUNEL assay. Mock- and sst2-transfected NIH3T3 were treated for 5 h in the presence of 10 μM DEVD-CHO (DEVD-CHO) or its absence (NT), or with 10 ng/ml TNFα (TNF). Apoptotic cells were detected by TUNEL. The data are expressed as mean±S.E.M. ($n=3$). (**b**) MTT assay. Mock- and sst2-transfected NIH3T3 cells were treated or not (NT) for 24 h with 10 ng/ml TNFα (TNF). Data points represent the mean±S.E.M. ($n=3$) and are expressed as the percentage of the cell viability observed in mock-transfected cells (**b**) or in the respective TNFα-untreated mock- and sst2-transfected cells (**b**, insert). (**a, b**) $^*P<0.05$, $^{**}P<0.01$ for sst2-transfected cells *versus* mock-transfected cells

sst2 both stimulates and sensitizes NIH3T3 cells to a TNFα-induced executioner caspase activity. To investigate whether sst2-induced apoptosis and cell sensitization to TNFα-induced apoptosis correlate with sst2-mediated stimulation of an executioner caspase activity, we measured, in a quantitative assay using Z-Asp-Glu-Val-Asp-para-nitroaniline (DEVD-pNA) as a substrate, the DEVDase executioner caspase activity in NIH3T3/mock and NIH3T3/sst2 cells (Figure 2a and b). sst2 expression increased DEVDase activity in NIH3T3/sst2 cells (1.8-±0.1-fold of NIH3T3/mock cells; Figure 2a), which was abrogated in the presence of 10 μM of executioner caspase inhibitor DEVD-CHO showing the specificity of this assay (Figure 2a). Besides, cell treatment with increasing concentrations of BIM23627, a specific antagonist of sst2,[14] dose dependently inhibited sst2-mediated activation of executioner caspases, which returned to levels observed in mock cells (for 100 nM of BIM23627). This antagonist did not affect NIH3T3/mock cell DEVDase activity (Figure 2b). To further avoid the possibility that the results observed in NIH 3T3/sst2 cells may be due to clonal effects, the same results have been repeated in another independent sst2-expressing NIH3T3 clone named sst2.1. This clone has been previously characterized.[11] As previously observed in NIH3T3/sst2 cells, sst2 expression in NIH3T3/sst2.1 cells increased DEVDase activity when compared with NIH3T3/mock cells (1.8±0.3-fold of NIH3T3/mock cells; Figure 2a). These results indicated that the increase of DEVDase activity observed in sst2-expressing cells is indeed sst2-mediated and specific to sst2 expression.

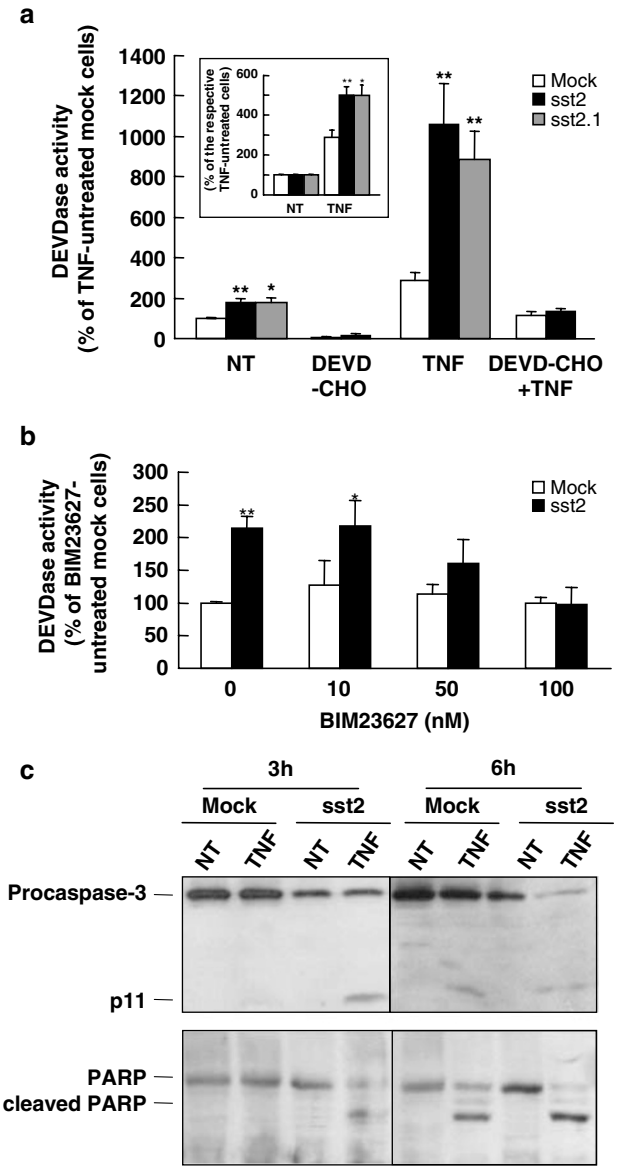

Figure 2 Basal and TNFα-induced executioner caspase activity in NIH3T3 cells expressing or not sst2. Mock- and sst2-transfected NIH3T3 cells (Mock, sst2 and sst2.1 clones) were treated or not (NT) with TNFα (TNF) for 3 h (**a**, **c**) or 6 h (**c**), in the presence or not of 10 μM DEVD-CHO (**a**), or treated with 0–100 nM of the sst2 antagonist BIM23627 (**b**). (**a**, **b**) Executioner caspase activity assay. Results are presented as the percentage of the executioner caspase activity measured in TNFα-untreated (**a**) or BIM23627-untreated mock-transfected cells (**b**), or in the respective TNFα-untreated mock- and sst2-transfected cells (**a**, insert), and are expressed as mean ± S.E.M. ($n=4$). *$P<0.05$, **$P<0.01$ for sst2-transfected cells *versus* mock-transfected cells. (**c**) Immunoblots using anti-caspase-3 (upper panel) and anti-PARP (lower panel) antibodies ($n=3$)

A 3-h cell treatment with TNFα stimulated DEVDase activity by 2.9 ± 0.4-fold in NIH3T3/mock cells, which was further enhanced by sst2 expression in both clones (10.6 ± 2.0- and 8.95 ± 1.5-fold of TNFα-untreated mock cells for NIH3T3/sst2 and NIH3T3/sst2.1 cells, respectively) (Figure 2a). When represented as the fold- increase over the respective TNFα-untreated mock- or sst2-expressing cells, sst2 indeed enhanced TNFα-induced DEVDase activity by 1.7 ± 0.3- and 1.6 ± 0.1-fold in NIH3T3/sst2 and NIH3T3/sst2.1 cells, respectively, *versus* mock-expressing cells (Figure 2a, inset). Pretreatment with the executioner caspase competitive inhibitor DEVD-CHO abolished TNFα-induced executioner caspase activation in both NIH3T3/mock and NIH3T3/sst2 cells (Figure 2a). These results indicated that sst2 both stimulated and sensitized NIH3T3 cells to TNFα-induced executioner caspase activation. They were further confirmed by Western blot using either an anti-caspase-3 antibody, which recognizes both the procaspase and the cleaved active caspase-3 p11 subunit (Figure 2c, upper panel), or an anti-poly(ADP ribose) polymerase (PARP) (a caspase substrate) antibody, which similarly recognizes the full and the cleaved form of PARP (Figure 2c, lower panel). Neither the p11-cleaved active subunit of the caspase-3 nor the cleaved form of PARP was observed in TNFα-untreated mock- or sst2-transfected NIH3T3 cells (Figure 2c), probably because this technique is not as sensitive as the DEVDase activity assay to detect low levels of executioner caspase activation. In contrast, procaspase-3 and PARP were cleaved after a 6-h treatment with TNFα in both NIH3T3/mock and NIH3T3/sst2 cells, whereas these cleavages were observed only in sst2-expressing cells after a 3-h treatment, therefore confirming, at this time point, sst2-mediated cell sensitization to TNFα-induced executioner caspase activation.

sst2 acts on the TNFα-triggered signaling pathway. TNFα-triggered apoptosis involves, as an upstream event, the activation of the TNFα receptor 1 (TNFR1) and of the initiator caspase-8.[15] To study the molecular mechanisms implicated in sst2-mediated cell sensitization to TNFα-induced apoptosis, we investigated whether sst2 sensitizes cells to TNFα-induced caspase-8 activity by Western blot, using an antibody that recognizes the caspase-8 proform (Figure 3a). The intensity of the caspase-8 proform band was more potently decreased by a 2-h cell treatment with TNFα in NIH3T3/sst2 than in mock cells, as compared to the respective TNFα-untreated cells. This result was confirmed by measuring, in a quantitative assay using IETD-pNA as a substrate, an increase in IETDase TNFα-induced caspase-8 activity in sst2-expressing NIH3T3 cells as compared to mock cells (data not shown). Moreover, pretreatment with the competitive caspase-8 inhibitor IETD-CHO decreased TNFα-induced executioner caspase activation in both NIH3T3/mock and NIH3T3/sst2 cells (Figure 3b).

We then explored whether sst2 might upregulate TNFα ligand and receptor mRNA and/or protein expression, by using real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis and Western blotting, respectively. We were unable to detect TNFα mRNA in both NIH3T3/sst2 and NIH3T3/mock cells (data not shown). In addition, TNFR1 mRNA expression was not affected by sst2 transfection in NIH3T3 cells (Figure 3c). However, expression of the TNFR1 protein was dramatically upregulated in NIH3T3/sst2 (6.5 ± 1.9-fold; $P<0.05$) as compared to mock cells (Figure 3d).

SHP-1 is a critical effector for sst2-induced executioner caspase activation. We previously reported that the

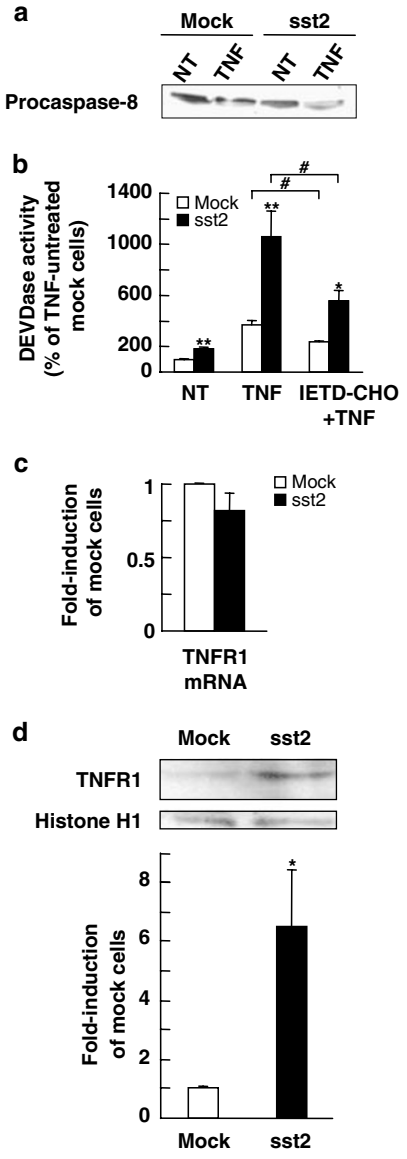

Figure 3 Effect of sst2 transfection on TNFα-triggered signaling pathway in NIH3T3 cells. Mock- and sst2-transfected NIH3T3 cells were treated or not (NT) with TNFα (TNF) for 2 h (**a**) or 3 h (**b**), in the presence or not of 10 μM IETD-CHO (IETD-CHO + TNF) (**b**). (**a**) Immunoblot using anti-caspase-8 antibody ($n=3$). (**b**) Executioner caspase activity. Results are presented as the percentage of the executioner caspase activity measured in TNFα-untreated mock-transfected cells and are expressed as mean ± S.E.M. ($n=3$). $^{*}P<0.05$, $^{**}P<0.01$ for sst2-transfected cells *versus* mock-transfected cells. $^{\#}P<0.05$ for IETD-CHO-treated *versus* IETD-CHO-untreated cells. (**c**) Real-time quantitative RT-PCR was performed to determine changes in mRNA expression for TNFR1. Results are expressed as fold-induction over mRNA expression observed in mock-transfected NIH3T3 cells and represent the mean ± S.E.M. ($n=4$). (**d**) Immunoblot using the TNFR1 antibody on whole NIH3T3 cell protein extracts. The expression of histone H1 was used as an internal loading control. Immunoblots were analyzed by densitometry, and results of four independent experiments are expressed as fold-induction over protein expression observed in mock-transfected NIH3T3 cells and are presented as mean ± S.E.M. ($n=4$). $^{*}P<0.05$, $^{**}P<0.01$ for sst2-transfected cells *versus* mock-transfected cells

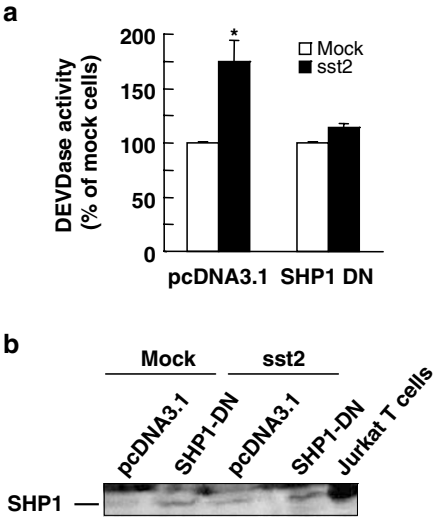

Figure 4 Effect of SHP-1 activity on sst2-induced activation of the executioner caspase activity in NIH3T3 cells. Mock- and sst2-transfected NIH3T3 cells were transiently transfected with cDNA encoding for the catalytically inactive SHP-1 (SHP-1 DN), or with the empty plasmid (pcDNA3.1). (**a**) Executioner caspase activity assay. Results are presented as the percentage of the executioner caspase activity measured in mock-transfected NIH3T3 cells and are expressed as mean ± S.E.M. ($n=4$). $^{*}P<0.05$, $^{**}P<0.01$ for sst2-transfected cells *versus* mock-transfected cells. (**b**) Immunoblot using an anti-SHP-1 antibody. The lower band of the doublet in lanes 1–5 corresponds to SHP-1. The upper band is unspecific towards SHP-1. Jurkat T-cell lysate was used as positive control for SHP-1 expression

tyrosine phosphatase SHP-1 is a critical sst2-signaling molecule in several models including NIH3T3 cells.[11,16,17] Therefore, we explored the implication of SHP-1 in sst2-induced executioner caspase activation. NIH3T3/mock and NIH3T3/sst2 cells were transiently transfected either with cDNA encoding the catalytically inactive C453S SHP-1 form (SHP-1 DN), or with the empty pcDNA3.1 vector. As expected, in pcDNA3.1-transfected NIH3T3/sst2 cells, sst2 stimulated DEVDase activity by 1.7 ± 0.2-fold, as compared to NIH3T3/mock cells (Figure 4a). Transfection of SHP-1 DN abrogated sst2 effect on DEVDase activity and restored the level of DEVDase activity in NIH3T3/sst2 cells to the one observed in SHP-1 DN-transfected NIH3T3/mock cells. As a transfection efficiency control, expression levels of SHP-1 DN in both mock- and sst2-expressing cells were compared in a Western blot (Figure 4b). These results demonstrated a critical role for SHP-1 in sst2-induced executioner caspase activation in NIH3T3 cells.

sst2 stimulates NF-κB and sensitizes NIH3T3 cells to a TNFα-induced NF-κB activation. The transcription factor nuclear factor-kappa B (NF-κB) is a known regulator of apoptosis acting as a proapoptotic or antiapoptotic factor depending on the cell context.[18] We therefore assessed whether sst2 regulates NF-κB-binding activity in an electrophoretic mobility shift assay (EMSA) using the consensus NF-κB-binding element as a probe and nuclear extracts of NIH3T3/sst2 and NIH3T3/mock cells. Surprisingly, basal NF-κB-binding activity was increased by 4.9 ± 0.5-fold ($P<0.05$) in NIH3T3/sst2 cells as compared to mock cells, as demonstrated by the appearance of a shifted complex (1) (Figure 5a, left). Addition of a 100-fold molar

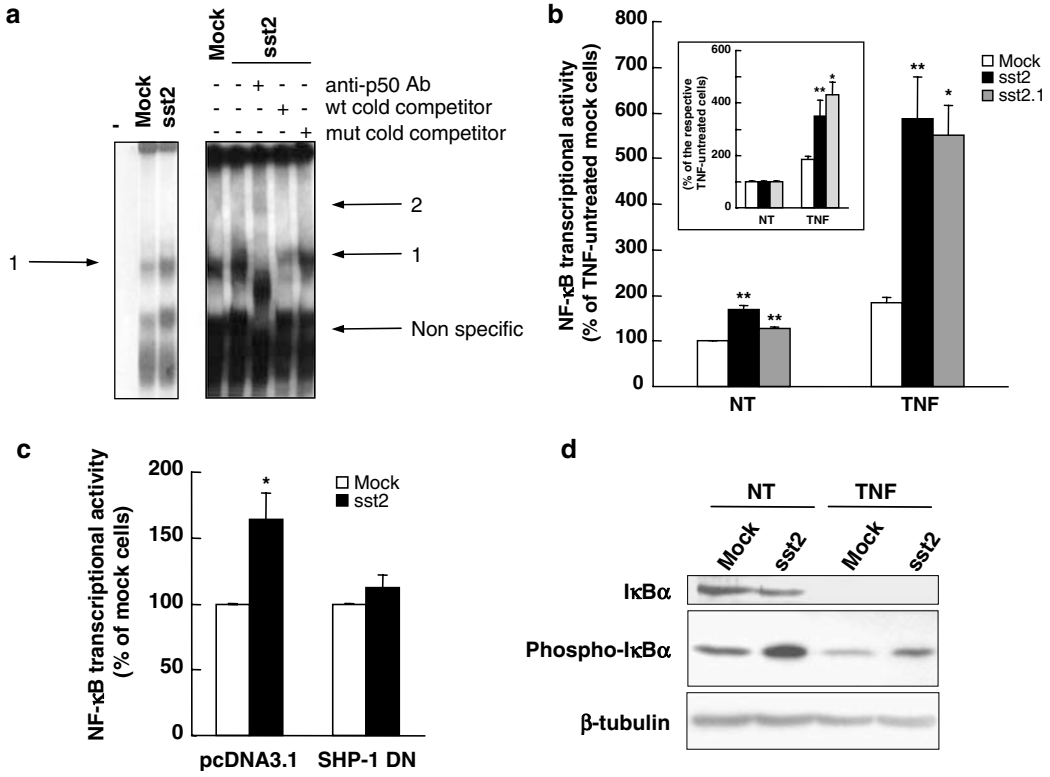

Figure 5 Effect of sst2 transfection on NF-κB activity in NIH3T3 cells. (**a**) To measure the level of NF-κB-binding activity, nuclear extracts (10 μg) of mock- and sst2-transfected NIH3T3 cells were subjected to EMSA using consensus double-stranded probe for NF-κB. Arrow 1 refers to NF-κB complex. An anti-p50 antibody (anti-p50 Ab) was added to the binding reaction and supershifted the NF-κB complex (arrow 2). Nuclear extracts were also incubated with the unlabeled wild-type or mutated oligonucleotides (wt or mut cold competitor) and assayed for NF-κB-binding activity; lane (−): probe only ($n=3$). The right panel corresponds to a higher time exposure. (**b**, **c**) Mock- and sst2-transfected cells were co-transfected with a mixture of NF-κB-Luc or pTAL vector, and pCMVβGal vector (**b**, **c**), ± SHP-1 DN or pcDNA3.1 vector (**c**). (**b**) Cells (Mock, sst2 and sst2.1 clones) were treated or not (NT) with TNFα (TNF) for 5 h and used for a luciferase activity assay. (**c**) SHP-1 DN- or pcDNA3.1-transfected cells were used for a luciferase activity assay. Transfection level of the SHP-1 DN protein was assessed in a Western blot, as described in Figure 4b (not shown). Results are presented as fold-induction of TNFα-untreated mock cells (**b**) or of the respective TNFα-untreated mock and sst2-transfected cells (**b**, insert) or of the mock cells (**c**), and are expressed as mean ± S.E.M. ($n=4$). *$P<0.05$, **$P<0.01$ for sst2-transfected cells *versus* mock-transfected cells. (**d**) Mock- and sst2-transfected NIH3T3 cells were treated or not (NT) for 3 h with TNFα (TNF). Immunoblots using anti-IκBα, or anti-phosphoIκBα antibodies on whole NIH3T3 cell protein extracts. The expression of β-tubulin was used as an internal loading control ($n=3$)

excess of the wild-type, but not mutated, unlabeled cold probe disrupted the sst2-induced NF-κB–DNA complex, which confirmed the specificity of this complex (Figure 5a, right). Furthermore, we confirmed the presence of NF-κB as part of the sst2-induced complex, as preincubation of NIH3T3/sst2 nuclear extracts with an anti-p50 antibody disrupted NF-κB–DNA complex, whereas a higher molecular weight supershifted complex appeared (Figure 5a, right).

NF-κB transcriptional activity was also assessed using a luciferase reporter gene under the transcriptional control of NF-κB-response elements. sst2 expression increased NF-κB transcriptional activity by 1.7 ± 0.07- and 1.3 ± 0.03-fold in NIH3T3/sst2 and NIH3T3/sst2.1 cells, respectively, as compared to mock cells (Figure 5b), confirming that sst2 indeed stimulated NF-κB activity. To explore the implication of SHP-1, mock- and sst2-expressing NIH3T3 cells were transiently transfected with SHP-1 DN or the empty pcDNA3.1 vector. In pcDNA3.1-transfected NIH3T3/sst2 cells, sst2 stimulated NF-κB transcriptional activity by 1.6 ± 0.2-fold, as compared to mock cells (Figure 5c). Transfection of SHP-1 DN abrogated sst2-induced NF-κB transcriptional activity in NIH3T3/sst2 cells to levels observed in NIH3T3/mock cells (Figure 5c). These results demonstrated a critical role for SHP-1 in sst2-induced NF-κB transcriptional activity.

NF-κB is known to be activated by diverse stimuli, including TNFα.[18] Whereas a 5-h treatment with TNFα weakly stimulated luciferase activity (1.8 ± 0.1-fold of NIH3T3/mock cells), sst2 enhanced this effect (5.9 ± 0.9- and 5.5 ± 0.6-fold for NIH3T3/sst2 and NIH3T3/sst2.1 cells, respectively) (Figure 5b). When represented as the percentage of the respective TNFα-untreated mock- or sst2-expressing cells (Figure 5b, insert), NF-κB transcriptional activity was significantly more potently increased in sst2- *versus* mock-expressing cells (3.5 ± 0.6- and 4.3 ± 0.5-fold for NIH3T3/sst2 cells and NIH3T3/sst2.1 cells, respectively *versus* 1.8 ± 0.1-fold for NIH3T3/mock cells), demonstrating that sst2 sensitizes cells to NF-κB transcriptional activation by TNFα.

Mechanisms for sst2- and TNFα-induced NF-κB activation were investigated. Proteasome-dependent degradation of inhibitory factor-kappa B (IκB) α, occurring as a consequence of serine phosphorylation of IκBα, is a mechanism for

activation of NF-κB.[18] We therefore assessed IκBα protein expression and serine phosphorylation in Western blots using antibodies recognizing either the total protein or its serine32-phosphorylated form (Figure 5d, upper and middle panels, respectively). sst2 expression in NIH3T3 cells treated or not with TNFα increased IκBα serine phosphorylation as compared to mock cells, and consequently downregulated IκBα expression. Furthermore, IκBα was completely degraded in both TNFα-treated, but not in untreated, mock- and sst2-expressing NIH3T3 cells, which is consistent with a downstream activation of NF-κB in the TNFα-treated conditions. These results are consistent with sst2-mediated activation of NF-κB and cell sensitization to TNFα-induced NF-κB activity as a result of IκBα phosphorylation and degradation.

NF-κB is a critical effector for sst2-induced apoptosis and is implicated in sst2-induced sensitization to TNFα. The demonstration that sst2 induces NF-κB transcription activity suggests a possible role for NF-κB in sst2-induced apoptosis and cell sensitization to TNFα-mediated apoptosis. To test this hypothesis, we investigated whether cell treatment with NF-κB inhibitors affects sst2-induced cell death, as measured in an executioner caspase activity assay. Mock- and sst2-transfected cells were treated or not with either Helenalin (a plant-derived sesquiterpene lactone, which specifically and irreversibly alkylates p65 subunit of NF-κB to block DNA binding[19]), or with SN50 (which acts as a dominant-negative peptide for p65 nuclear translocation[20]) or SN50M (mutated inactive form of SN50 peptide), or were transfected with a cDNA of IκBαSR (an undegradable form of IκBα, which constitutively inactivates NF-κB pathway[21]). Inhibitory effects of these molecules was first confirmed on sst2-induced NF-κB transcriptional activity using the gene reporter luciferase assay (Figure 6a, c and e) and then assessed on sst2-induced executioner caspase activity (Figure 6b, d and f). Whereas sst2 increased DEVDase activity in NIH3T3/sst2 cells as previously shown in Figure 2a, NF-κB inhibitors, although having no significant action on NIH3T3/mock cells, reverted sst2 effect and decreased executioner caspase activity levels in NIH3T3/sst2 cells to those observed in NIH3T3/mock cells (Figure 6b, d and f).

Involvement of NF-κB in sst2-dependent cell sensitization to TNFα-induced apoptosis was then assessed using Helenalin (Figure 6g and h). Helenalin effectively abrogated both sst2- and TNFα-induced NF-κB transcriptional activity, as measured in the gene reporter luciferase assay (Figure 6g). Whereas TNFα indeed increased, in the presence of DMSO, the executioner caspase activity by 3.9 ± 0.7- and 11.2 ± 2.2-fold in NIH3T3/mock and NIH3T3/sst2 cells, respectively, Helenalin abrogated both TNFα-induced and sst2-mediated cell sensitization to TNFα-induced executioner caspase activity (Figure 6h).

These results indicated that activation of NF-κB is proapoptotic in NIH3T3 cells and is implicated in sst2-induced apoptosis and sensitization to TNFα.

sst2-induced NF-κB activation decreases JNK phosphorylation. The c-Jun NH2-terminal kinase (JNK) pathway is implicated in the control of cell death, and TNFα has been reported to stimulate JNK activity.[22] We first investigated whether TNFα indeed stimulates JNK activity in NIH3T3 cells. JNK phosphorylation was assessed by Western blot using the anti-phospho(Thr183/Thr185)JNK antibody, which recognizes the p46JNK1 and p54JNK2 forms. As observed in Figure 7a, a 3-h cell treatment with TNFα increased JNK phosphorylation in NIH3T3/mock cells. Strikingly, sst2 also affected both basal and TNFα-induced JNK activity, as observed by the decreased phosphorylation level of both JNK 54 and 46 kDa forms in NIH3T3/sst2 and NIH3T3/sst2.1 cells as compared to NIH3T3/mock cells (Figure 7a).

Moreover, recent studies described a negative crosstalk between the NF-κB and JNK pathways based on the ability of NF-κB to inhibit JNK-regulated apoptosis in response to TNFα.[18,22] Because NF-κB activity is increased in sst2-expressing cells, we therefore asked whether NF-κB is involved in sst2-mediated inhibition of JNK activity. NIH3T3/mock and NIH3T3/sst2 cells were pretreated or not with the potent NF-κB inhibitor Helenalin, and then treated or not for 3 h with TNFα. Strikingly, Helenalin increased JNK phosphorylation in both mock- and sst2-expressing cells whether or not treated with TNFα (Figure 7b, left and right panels), indicating that NF-κB is indeed a negative regulator of JNK activity in these cells. Helenalin reverted sst2-mediated inhibition of JNK phosphorylation in NIH3T3/sst2 cells whether or not treated with TNFα, and restored these levels to those observed in Helenalin-treated NIH3T3/mock cells (Figure 7b).

Inversely, we assessed whether JNK also regulates NF-κB activity by using a specific JNK inhibitor SP600125[23] and showed that cell pretreatment with 10 μM SP600125 did not affect NF-κB activity in both NIH3T3/mock and NIH3T3/sst2 cells whether or not treated with TNFα (Figure 7c). The potency of SP600125 to inhibit JNK autophosphorylation was confirmed by Western blotting (Figure 7d).

These results demonstrated that (1) sst2 has opposite effects on NF-κB and JNK activities in NIH3T3/sst2 cells, (2) inhibition of NF-κB upregulates JNK phosphorylation in both NIH3T3/mock and NIH3T3/sst2 cells whether or not treated with TNFα, and (3) sst2-activated NF-κB lies upstream of JNK and is a critical effector of sst2-induced inhibition of JNK.

Inhibition of JNK mimics sst2-induced apoptosis. Because sst2-dependent activation of NF-κB in NIH3T3 cells both induced apoptosis and negatively regulated JNK activity, we asked whether inhibition of JNK activity is implicated in sst2-induced apoptosis and cell sensitization to TNFα-induced apoptosis. NIH3T3/mock and NIH3T3/sst2 cells were pretreated or not with 10 μM of the specific JNK inhibitor SP600125,[23] and then treated for 3 h with TNFα. DEVDase activity was then assessed (Figure 8). As expected, sst2 and TNFα increased DEVDase activities to 183 ± 19 and 445 ± 41% of DMSO-treated mock cells, respectively, and sst2 enhanced TNFα-induced DEVDase activity to 1514 ± 137% (Figure 8), as previously described (Figure 2a).

Strikingly, cell pretreatment with SP600125 increased DEVDase activity in NIH3T3/mock to 234 ± 29% ($P < 0.05$) of DMSO-treated NIH3T3/mock cells, whereas it did not significantly affect DEVDase activity in NIH3T3/sst2 cells

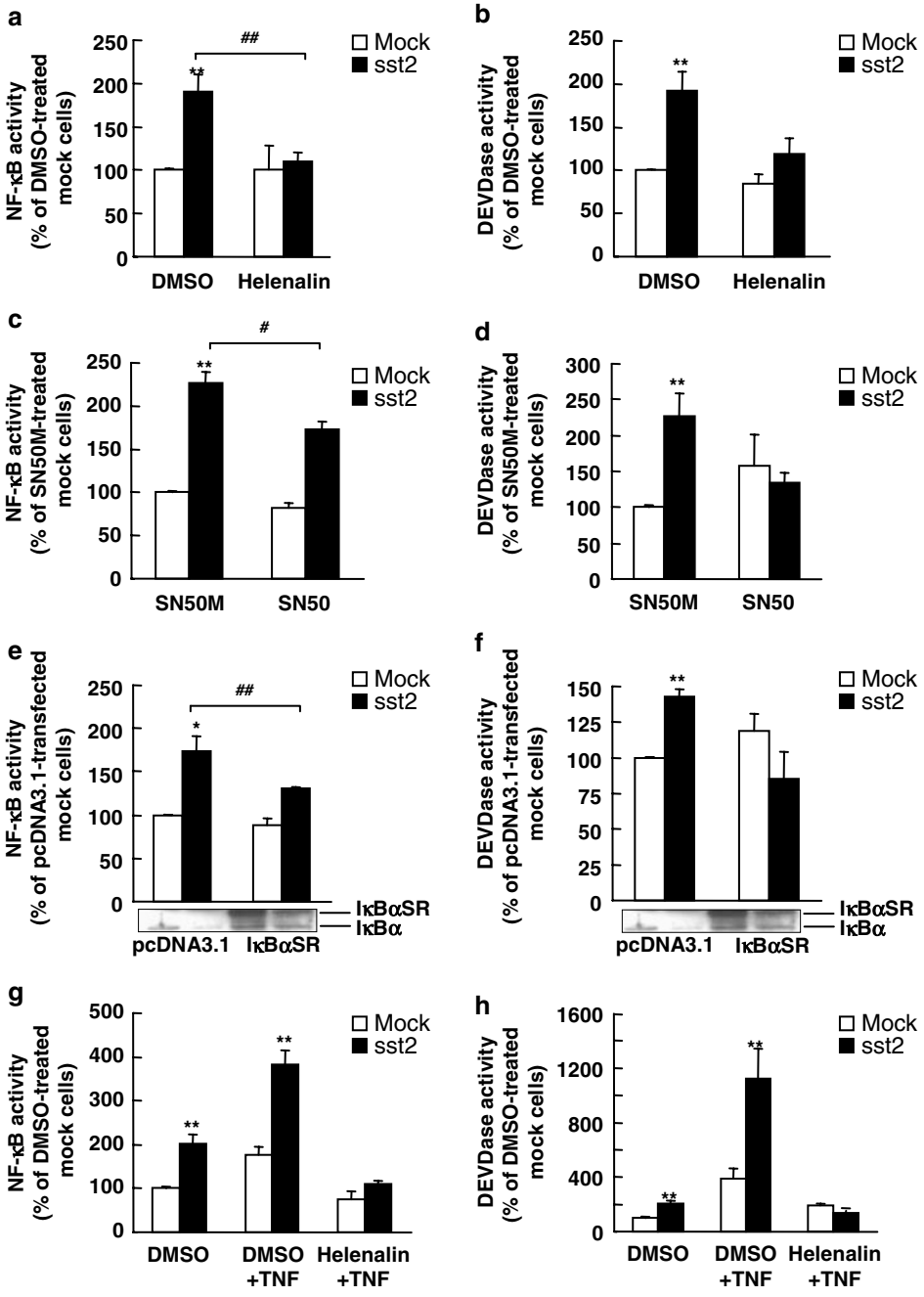

Figure 6 Effect of NF-κB activity on sst2-induced executioner caspase activity in NIH3T3 cells. Mock- and sst2-transfected NIH3T3 cells were pretreated for 1 h with 10 μM Helenalin or DMSO (**a**, **b**, **g**, **h**) and treated or not for 3 h with TNFα (**g**, **h**), or pretreated for 1 h with 25 μg/ml SN50M or SN50 (**c**, **d**), or transfected with pcDNA3.1 or IκBαSR (**e**, **f**). Transfection level of the protein IκBαSR was assessed in a Western blot (**e**, **f**). IκBαSR has a higher molecular weight than IκBα. Transcriptional NF-κB activity assays (**a**, **c**, **e**, **g**) and executioner caspase activity assays (**b**, **d**, **f**, **h**) were performed. Results are presented as the percentage of the transcriptional NF-κB activity (**a**, **c**, **e**, **g**) or of the executioner caspase activity (**b**, **d**, **f**, **h**) measured in DMSO-treated mock cells (**a**, **b**, **g**, **h**) or in SN50M-treated mock cells (**c**, **d**), or in the pcDNA3.1-transfected mock cells (**e**, **f**) and are expressed as mean $\pm$ S.E.M. ($n=3$). $^{*}P<0.05$, $^{**}P<0.01$ for sst2-transfected cells *versus* mock-transfected cells. $^{\#}P<0.05$, $^{\#\#}P<0.01$ for NF-κB inhibitor-treated *versus* NF-κB inhibitor-untreated sst2-transfected cells

(Figure 8). Consequently, the DEVDase activities measured in SP600125-treated NIH3T3/mock and NIH3T3/sst2 cells were not statistically different. Similarly, cell pretreatment with SP600125 significantly increased TNFα-induced DEVDase activity to 821 $\pm$ 145% ($P<0.01$) in NIH3T3/mock (Figure 8), whereas having no effect on TNFα-treated NIH3T3/sst2 cells.

In conclusion, SP600125-mediated JNK inhibition increased cell death and TNFα-induced apoptosis in NIH3T3/mock cells, indicating that JNK plays an antiapoptotic role in this cell type. Furthermore, treatment of NIH3T3/mock cells with SP600125 is able to mimic sst2 proapoptotic action and to enhance TNFα-induced cell death.

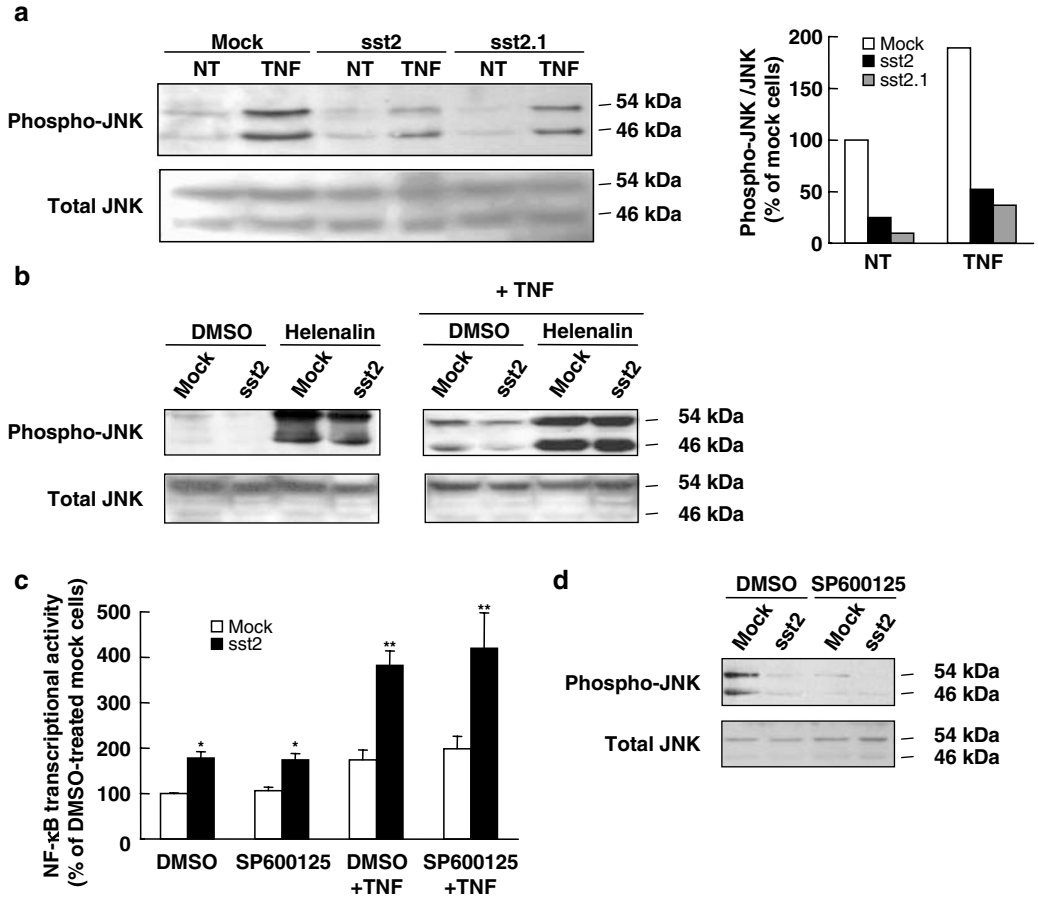

Figure 7 Effect of sst2 transfection on JNK activity in NIH3T3 cells. (**a**) Mock- and sst2-transfected NIH3T3 cells (Mock, sst2 and sst2.1 clones) were treated for 3 h or not (NT) with TNFα (TNF), $n=4$. This immunoblot was analyzed by densitometry (right), and expressed as percent of phospho-JNK/total JNK ratio observed in mock-transfected NIH3T3 cells. (**b**) Mock- and sst2-transfected NIH3T3 cells were pretreated for 1 h with Helenalin or with DMSO, and treated or not for 3 h with TNFα. For immunoblotting, an anti-phosphoJNK antibody was used. The expression of total JNK was used as an internal loading control ($n=3$). (**c**, **d**) Cells were pretreated for 1 h with 10 μM SP600125 or DMSO, treated or not with TNFα for 5 h (TNFα), and used for a transcriptional NF-κB activity assay (**c**). Results are presented as fold-induction of DMSO-treated mock cells and are expressed as mean ± S.E.M. ($n=3$). $^{*}P<0.05$, $^{**}P<0.01$ for sst2-transfected cells *versus* mock-transfected cells. For immunoblotting, an anti-phosphoJNK antibody was used (**d**). The expression of total JNK was used as an internal loading control

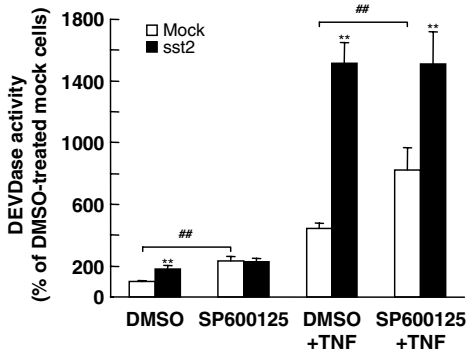

Figure 8 Effect of JNK on sst2-induced executioner caspase activity in NIH3T3 cells. Mock- and sst2-transfected NIH3T3 cells were pretreated for 1 h with SP600125 or with DMSO, and treated or not for 3 h with TNFα. Executioner caspase activity assay. Results are presented as the percentage of the executioner caspase activity measured in DMSO-treated mock cells and are expressed as mean ± S.E.M. ($n=5$). $^{*}P<0.05$, $^{**}P<0.01$ for sst2-transfected cells *versus* mock-transfected cells; $^{\#\#}P<0.01$ for SP600125-treated *versus* DMSO-treated mock-transfected cells

Discussion

We have demonstrated here that sst2 expression induces an executioner caspase-dependent apoptosis in the nontransformed NIH3T3 cells. The sst2 antagonist BIM23627 suppressed sst2-mediated activation of the executioner caspases, demonstrating the specificity of sst2 effect and confirming sst2 implication in somatostatin-induced apoptosis. The role of the sst2 receptor was also addressed on all components of the intracellular pathway described (executioner caspases, NF-κB, JNK) in two independent sst2-expressing NIH3T3 clones, which further avoids the possibility that the results observed may be due to clonal effects. Moreover, somatostatin-directed siRNAs increased sst2-expressing NIH3T3 cell viability (personal data from Cordelier P), further demonstrating that the sst2-dependent autocrine loop, whereby sst2 induces the expression of its own ligand somatostatin, is responsible for sst2-induced apoptosis. This result is consistent with our previous published data, demonstrating that sst2 expression also triggers apoptosis

in the human pancreatic cancer BxPC-3 cells,[10] and with the demonstration of a somatostatin-mediated apoptotic action in the myeloïd cancer HL-60 cells, which only express the subtype 2 of somatostatin receptor.[3] Beside its apoptotic action on cancer cells, somatostatin has also been shown to decrease cell survival in several non-cancer cell models, including intestinal epithelial cells,[4] fibroblasts from Graves' ophtalmopathy[6] and peripheral blood lymphocytes.[7–9] However, mechanisms for somatostatin-induced apoptosis and receptor subtype implication were not described in these models. Therefore, our results provide the first demonstration of an sst2-mediated apoptotic effect in a noncancer cell model.

Molecular mechanism involved in sst2 apoptotic action was shown to rely on the activity of the SH2-containing tyrosine phosphatase SHP-1, which is constitutively activated in sst2-expressing NIH3T3 cells.[11] Strikingly, SHP-1 has been described to be a critical effector of both somatostatin and sst2 apoptotic effects in breast cancer MCF-7 and pancreatic cancer BxPC-3 cells, respectively.[10,24] We observed that activation of the executioner caspases is a critical event for sst2-dependent induction of apoptosis and is dependent on SHP-1 activity. Our model for sst2-dependent activation of SHP-1 whereby SHP-1 recruitment and activation by sst2 relies on the formation of a dynamic complex comprising sst2, the tyrosine kinase Src and the tyrosine phosphatase SHP-2 was previously described.[17]

To investigate the signaling molecules involved in executioner caspase activation through sst2-activated SHP-1, we first examined the mitochondrial pathway. Indeed, the mitochondrial antiapoptotic Bcl-2 is involved in both pancreatic and breast cancer cell resistance to somatostatin-induced cell death, and sst2-dependent downregulation of Bcl-2 is critical for sst2 proapoptotic action.[10,24] However, in NIH3T3 cells, we failed to detect any Bcl-2 protein expression nor to observe any regulation by sst2 of the other pro- and antiapoptotic Bcl-2 family members, including Bak, Bax, Bcl-x_L or Mcl-1 (data not shown). Thus, sst2-induced apoptosis in a non-cancerous model seems to be mediated through an alternate pathway.

Strikingly, beside its proapoptotic action, sst2 markedly enhanced TNFα-induced apoptosis in sst2-expressing NIH3T3 cells, as TNFα apoptotic effect in sst2-expressing cells was more robust than the addition of sst2 and TNFα effects in mock cells. This result is consistent with our previous observations demonstrating that sst2 sensitizes pancreatic cancer cells to death ligand-induced cell death.[10] TNFα induces cell death through its binding to the TNFR1 receptor, which results in the activation of the initiator procaspase-8.[15] Interestingly, sst2 sensitized NIH3T3 cells to TNFα-induced apoptosis by both upregulating the expression of the TNFR1 protein and inducing the subsequent cleavage of the procaspase-8. Interleukin (IL)-6-dependent upregulation of TNFR1 mRNA has previously been shown to be involved in rat hepatocyte sensitization to TNFα-induced apoptosis.[25] Conversely, in our cell models, including NIH3T3 and pancreatic cancer BxPC-3 cells, sst2 did not affect TNFR1 mRNA expression but rather upregulated TNFR1 protein,[10] suggesting that sst2 acts at a post-transcriptional level.

NF-κB plays an important role in response to immune challenge, inflammation and cellular stress. NF-κB corresponds to a hetero- or homodimer of proteins of the NF-κB transcription factor family, including NF-κB1 (p50/p105) and RelA (p65). NF-κB is complexed in an inactive form in the cytoplasm with a family of inhibitors termed IκBs. Degradation of IκB results in translocation of NF-κB to the nucleus where it acts as a transcription factor.[18] NF-κB has been shown to have a controversial role in the regulation of apoptosis and is usually considered as an antiapoptotic molecule.[18] However, compelling data highlight a proapoptotic role for NF-κB. Indeed, induction of apoptosis has been correlated to NF-κB activation in a wide variety of systems including fibroblasts,[26,27] T cells,[28,29] ceramide-activated osteoblasts[30] and dopaminergic neurons derived from Parkinson's disease patients.[31] Cell fate towards apoptosis or survival after NF-κB activation seems to be cell type specific and/or dependent on cell environment. In NIH3T3 cells, we demonstrated that NF-κB is proapoptotic as cell treatment with NF-κB inhibitors decreased executioner caspase activity. In addition to TNFα, sst2 was surprisingly shown to activate NF-κB in sst2-expressing NIH3T3 cells. Although sst2 activation of NF-κB did not exceed two-fold in NIH3T3/sst2 *versus* mock cells, this was sufficient to mediate sst2-induced cell death as apoptosis was abrogated by several NF-κB inhibitors. A few reports describe a GPCR-dependent activation of NF-κB, that usually results in inhibition of cell death. In contrast, activation of NF-κB by sst2 here results in activation of cell death. To our knowledge, apoptosis mediated through a GPCR-dependent activation of NF-κB was thus far only described for the platelet activating factor-receptor (PAF-R) which was shown to enhance chemotherapy-induced cell death in human carcinoma cell lines.[32] Strikingly, we also demonstrated that sst2-dependent cell sensitization to TNFα-induced apoptosis requires NF-κB activation.

We have shown here that SHP-1 is an effector of sst2 proapoptotic action, and is also a critical mediator of sst2-dependent NF-κB activation. Interplay between SHP-1 and NF-κB pathways has previously been reported in B and T cells and astrocytes, and is consistent with a dysregulation of NF-κB observed in mutant motheaten mice that express a catalytically inactive form of SHP-1.[33,34] However, molecular mechanisms for this regulation have not been described yet. Our findings demonstrate that sst2 regulates NF-κB through the phosphorylation and the degradation of its inhibitor IκBα. One possible mechanism involved in SHP-1-mediated activation of NF-κB may require Erk pathway, which in turn initiates IKK activation and subsequent IκBα phosphorylation and degradation. Erk pathway has indeed been reported to regulate bradykinin B2 receptor-mediated NF-κB activation,[35] and we recently demonstrated that SHP-1 is involved in sst2-mediated activation of the Ras/B-Raf/Erk pathway.[36]

Regulation of apoptosis by NF-κB includes transcriptional modifications of proapoptotic proteins, including death ligands and death ligand receptors (FasL, DR4, DR5) or antiapoptotic proteins such as Bcl-x_L, c-IAP1, c-IAP2, FLIP and XIAP.[18] However, we did not observe any sst2-induced regulation of these proteins (data not shown), suggesting that sst2-induced apoptosis through NF-κB involves the activation of an alternate pathway. Several reports indicate an NF-κB-

dependent negative regulation of JNK activation.[37–40] JNK is a member of the mitogen-activated protein kinase family and is identified as three protein kinases, JNK1, JNK2 and a neuronal-specific form JNK3. In NIH3T3 cells, TNFα activates both NF-κB and JNK pathways, which is consistent with previous studies on TNFα signaling.[15] Furthermore, NIH3T3 cell treatment with an NF-κB inhibitor increased JNK activity, which demonstrated that NF-κB exerts a negative control on JNK activation in these cells. Such an NF-κB-dependent negative loop on JNK activation has recently been reported in other cell systems including murine embryonic fibroblasts, 3DO T cells, ovarian epithelial cancer cells and Ewing's sarcoma cells.[37,39,41,42] Surprisingly, sst2 inhibits JNK activity in both TNFα-treated or -untreated cells, which is dependent on sst2-activated NF-κB, as sst2-mediated JNK inhibition is indeed abolished by an NF-κB inhibitor. Thus, the GPCR sst2 is an additional type of receptor, beside death receptor to TNFα and the cytokine receptor to IL-1, which transduces NF-κB-mediated inhibition of JNK.[18,38]

JNK has been implicated in the control of cell death, although its role is complex. In particular, JNK has been shown to either positively or negatively regulate cell death depending on the biological context.[22] JNK-dependent regulation of cell death is transduced by activation of either transcription factors, including c-Jun, or nontranscription factor proteins, such as members of the Bcl-2 family (Bcl-2 and Bcl-x_L).[22] We have demonstrated here that NIH3T3/mock cell treatment with a JNK inhibitor activated executioner caspases to a level similar to that observed in sst2-expressing cells, and therefore mimicked sst2 proapoptotic action. Similarly, in TNFα-treated NIH3T3/mock cells, the JNK inhibitor also mimicked sst2 cell sensitization action on TNFα-induced cell death. We therefore concluded that sst2-mediated inhibition of JNK activity in NIH3T3 cells results in activation of the executioner caspases, consequently inducing cell death. Similarly, an antiapoptotic role for JNK has recently been reported in p65−/− and super-repressor IκB-transfected cells.[38] Indeed, constitutive inhibition of NF-κB in these cells provides an antiapoptotic signal in response to TNFα by enhancing JNK activity, which explains how NF-κB null cells remain viable following TNFα treatment.[38] By contrast, in our model, levels of NF-κB or JNK activation, determining cell fate toward apoptosis or cell survival, were not manipulated by forced expression following cDNA transfection but regulated in a physiological context by the hormone receptor sst2 and/or TNFα.

In conclusion, we have unraveled here a novel mechanism for a GPCR, sst2, to trigger apoptosis, as summarized in Figure 9. This mechanism involves the SHP-1-dependent activation of NF-κB, which consequently results in the inhibition of JNK antiapoptotic action. sst2 also increases NIH3T3 cell sensitivity to the TNFα death ligand-induced apoptosis, by inducing TNFR1 overexpression, affecting in an NF-κB-dependent manner TNFα-activated signaling pathways. We describe here a first demonstration of a regulation of NF-κB and JNK pathways by somatostatin. As numerous studies demonstrate that coordinate stimulation of NF-κB and JNK cascades by inflammatory cytokines, including TNFα or IL-1, contributes to inflammatory diseases, unraveling an action of somatostatin on these pathways is of critical importance to understand somatostatin anti-inflammatory molecular mechanisms in the future.

Somatostatin has been reported to control the growth of fibroblastic-like cells in both physiological and pathological conditions.[6,43] Interestingly, the pathogenesis of immune-driven inflammatory disorders, including rheumatoïd arthritis

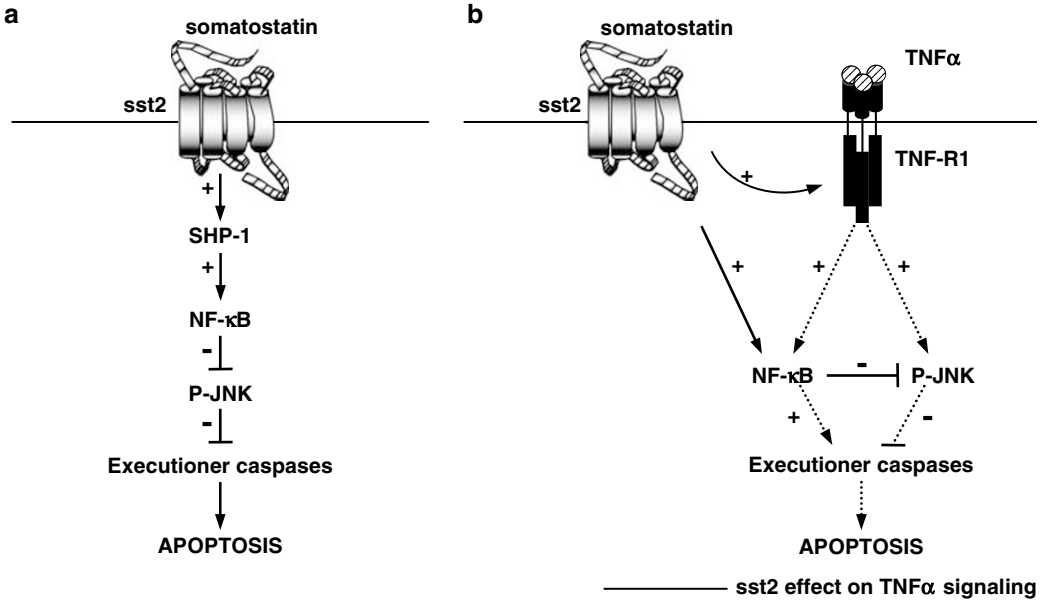

Figure 9 Schematic representation of sst2-induced cell death and cell sensitization to TNFα apoptotic action in NIH3T3 cells. (**a**) SHP-1-mediated NF-κB activation by sst2 inhibits the antiapoptotic action of JNK, resulting in cell apoptosis. (**b**) TNFα stimulates both NF-κB and JNK activities, which have opposite actions on cell survival. sst2-mediated sensitization to TNFα-induced NF-κB activation enhances JNK inhibition by NF-κB, and consequently TNFα-induced cell death. The overexpression of TNFR1 induced by sst2 explains, at least in part, the synergism between sst2 and TNFα

or Graves' disease, is characterized by an excess of fibroblastic-like cell proliferation. The presence of sst2 in Graves' ophtalmopathy fibroblasts may account, at least in part, for the antiproliferative and apoptotic effects of somatostatin in these cells, and for the clinical benefice of somatostatin analogues for the treatment of this disease.[1,6]

Materials and Methods

Cell culture. Murine fibroblastic NIH3T3 cells were transfected either with the human 1.35-kbp sst2 cDNA (NIH3T3/sst2 cells (clone 2/5[11]), NIH3T3/sst2.1 cells (clone 2/1[11])), or with the mock vector (mock cells), and cultured as previously described.[11,12] Cells were treated with 10 ng/ml TNFα (Preprotech Inc.) in the presence of 10 μg/ml of cycloheximide (Sigma). For experiments involving specific inhibitors, cells were pretreated for 1 h with 10 μM DEVD-CHO or IETD-CHO (BIOMOL), 25 μg/ml SN50 or SN50M (SN50Mutated) (BIOMOL), 10 μM Helenalin (BIOMOL), 10 μM SP600125 (BIOMOL), or with 10-50-100 nM BIM23627 (Biomeasure).

TUNEL. Cells were grown (10^5 cells/ml) on slides, and starved for 5 h in the presence of 10 μM DEVD-CHO (DEVD-CHO) (BIOMOL) or its absence (NT), or treated for 5 h with TNFα + cycloheximide. Slides were then fixed in acetone and dried. TUNEL staining was performed using the cell death apopDETEK kit (Enzo Diagnostics), according to the manufacturer's instructions. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol. Slides stained with no TUNEL mixture were used as negative controls.

Image processing. TUNEL reaction staining was visualized using an optical microscope coupled to a Visiolab 2000 image analyzer (Biocom). The percentage of labeled nuclei was evaluated on the basis of more than 1000 cells in 15–20 successive high-power (× 40) fields.

Cell viability assay. Cells were grown (10^4 cells/well), and starved or treated for 24 h with 10 ng/ml TNFα in the presence of 10 μg/ml of cycloheximide. Mitochondrial viability was measured using the MTT (Sigma) colorimetric assay.[10]

Executioner caspase activity assay. Cells were grown (10^5 cells/ml), starved for 15 h and treated or not with TNFα + cycloheximide for 3 h. The executioner caspase activity assay was performed using the Kit Quantipak (BIOMOL) with 50 μg cytosolic extracts and with 200 μM of the specific executioner caspase chromogenic substrate DEVD-pNA, according to the manufacturer's instructions. Samples containing no cell extract were used as negative controls. Inhibition of the executioner caspase or caspase-8 activity was carried out with the specific inhibitor DEVD-CHO or IETD-CHO, respectively (10 μM; BIOMOL).

Preparation of whole-cell extracts and Western blot analysis. To generate whole-cell extracts, cells were grown (10^5 cells/ml), starved for 15 h and treated as indicated. Cells were then lysed in 50 mM Tris HCl, pH 7.4, 100 mM KCl, 10% glycerol, 1 mM ethylene diamine tetraacetic acid (EDTA), 1% Triton X-100 (Sigma), 1 mM dithiothreitol (DTT) and protease inhibitors (Roche Molecular Biochemicals). Soluble proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis . Following probing with a specific primary antibody and then with a horseradish peroxidase-conjugated secondary antibody, the protein bands were detected by enhanced chemiluminescence (Pierce). Primary antibodies used were as follows: anti-caspase-3, anti-IκBα and anti-histone H1 (Santa Cruz Biotechnology), anti-mouse TNFR1 (R&D Systems), anti-SHP-1 (Transduction Laboratories), anti-JNK, anti-phospho-JNK(Thr183/Tyr185) and anti-phosphoIκBα(Ser32) (Cell Signaling), anti-β-tubulin (Sigma), anti-PARP (Roche Molecular Biochemicals), anti-caspase-8 (a kind gift of Dr. G Cohen, Leicester[44]).

Transient transfection of C453S-SHP-1 or IκBαSR cDNA. Cells were grown (10^5 cells/ml) and transfected with 5 μg of pcDNA3.1/C453S-SHP-1, pcDNA3.1/IκBαSR or pcDNA3.1 vector (Invitrogen) using Fugene 6 (Roche Molecular Biochemicals). After a 24-h growth in 10% FCS and 15 h without FCS, cells were used for an executioner caspase activity assay.

Luciferase reporter assay. Cells were grown (5×10^4 cells/ml) in six-well plates and were cotransfected with a mixture of 2 μg pNF-κB-Luc (Clontech) or pTAL vector (Clontech) + 0.5 μg pCMVβGal vector, ±1 μg pcDNA3.1/C453S-SHP-1, pcDNA3.1/IκBαSR or pcDNA3.1 vector (Invitrogen) using FuGENE 6 transfection reagent (Roche Molecular Biochemicals). After a 24-h growth in 10% fetal calf serum, cells were starved and treated as indicated. Cell extracts were prepared using the Reporter Lysis Buffer (Promega). Luciferase activity was measured using a Labsystems Luminoskan 96-well plate luminometer (Thermolab System), and β-galactosidase activity by spectrophometry (MRX Dynex Technologies). Normalization of the luciferase activity measured in each point was first performed with the respective β-galactosidase activity and then with the luciferase activity measured in the respective pTAL-transfected cells. Results were expressed as a ratio (fold-induction) of normalized luciferase activities in treated *versus* untreated cells. pNF-κB-Luc vector contains four tandem copies of the NF-κB consensus sequence κB fused to a TATA-like promoter (P_{TAL}) region from the herpes simplex virus thymidine kinase promoter and the firefly luciferase gene. pTAL vector corresponds to pNF-κB-Luc vector lacking the NF-κB response elements.

EMSA. Cells were grown (10^5 cells/ml) and starved for 24 h. Cell nuclear extracts were prepared and EMSA were performed as described.[45] The consensus double-stranded oligodeoxynucleotide probe for NF-κB (sense 5′-AGTTGAGG*G* GACTTTCCCAGGC-3′) was radioactively labeled using γ-[^{32}P]ATP and T4 polynucleotide kinase (Invitrogen) using standard procedures. Nuclear extracts (10 μg) were preincubated in a final volume of 20 μl (5 mM HEPES, pH 7.8, 5 mM $MgCl_2$, 50 mM KCl, 0.2 mM EDTA, 5 mM DTT, 10% glycerol) with 2 μg of poly(dIdC)-poly(dI-dC) at room temperature for 15 min. Five fmol of ^{32}P-labeled DNA probe (60.000 c.p.m.) were then added for 20 min at room temperature. In competition experiments, 100-fold molar excess of unlabeled wild-type or mutant (sense 5′-AGTTGAGG*C*GACTTTCCCAGGC-3′) competitor oligonucleotides were added to the preincubation reaction (The base in italics and underlined was mutated in the mutated sequence). For supershift experiments, extracts were preincubated with 2 μg of polyclonal anti-NF-κB p50 antibody (Santa Cruz Biotechnologies) for 1 h at 4°C. Protein–DNA complexes were resolved on a 4% nondenaturing polyacrylamide gel containing 2.5% glycerol in 0.5 × Tris/borate/EDTA at 4°C, dried and autoradiographed.

mRNA quantification by real-time quantitative RT-PCR. Cells were grown (10^5 cells/ml) and starved for 24 h. Total RNAs were extracted with RNable (Eurobio) according to the manufacturer's instructions. After a DNase treatment, total RNAs were reverse transcribed using random hexamer primers. Resulting cDNAs were used in a real-time quantitative PCR using Sybr Green as a dye (SYBR Green MasterMix 2 × , Applied Biosystems) and specific primers (MWG Biotech) for 18S (sense 5′-TGCATGGCCGTTCTTAGTTG-3′, antisense 5′-TGGCTGAACGC CACTTGTC-3′), TNF-R1 (sense 5′-TACCTCCTCCGCTTGCAAAT-3′, antisense 5′-GAGTAGACTTCGGGCCTCCAC-3′), and TNFα (sense 5′-TGTCCATTCCTG AGTTCTGCAA-3′, antisense 5′-TCATTCTGAGACAGAGGCAACCT-3′) cDNA on Abiprism 5800 (Applied Biosystems). PCR efficiencies were measured by performing a standard curve for each primer pair (efficiency > 85%). Target gene expression was normalized using the 18S expression as an internal control. Samples incubated without reverse transcriptase were used as negative template controls. cDNAs of murine fibrosarcoma L929 cells were used as a positive control for the expression of TNFR1 and TNFα mRNAs.

Statistical analysis. Statistical analysis was performed by unpaired *t*-test. All values are mean ± S.E.M.

Acknowledgements

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