Tumor Necrosis Factor Induces the Loss of Sphingosine Kinase-1 by a Cathepsin B-dependent Mechanism*

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Tarek A. Taha‡, Kazuyuki Kitatani§, Jacek Bielawski§, Wonhwa Cho¶, Yusuf A. Hannun¶, and Lina M. Obeid¶¶**

From the ‡Division of General Internal Medicine, Ralph H. Johnson Veterans Administration Hospital, Charleston, South Carolina 29401, ‡Departments of Medicine and §Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, South Carolina 29425, and ¶Department of Chemistry, University of Illinois at Chicago, Chicago, Illinois 60607

Sphingosine kinase-1 (SK1) has emerged as a key component of cytokine responses, including roles in apoptosis, yet the specific mechanisms by which cytokines regulate SK1 in the apoptotic responses have not been studied. In this study, we show that prolonged treatment of MCF-7 cells with tumor necrosis factor (TNF) induces a dose- and time-dependent decrease in SK1 protein. Inhibition of the upstream caspase 8 by IETD significantly rescued TNF effects on SK1, yet the caspase 7 inhibitor DEVD failed to have any effect, suggesting that the decline in SK1 occurs downstream of the initiator caspase but upstream of the effector caspase. In addition to caspase activation, TNF caused disruption of lysosomes with relocation of the cysteine protease cathepsin B into the cytosol. Down-regulation of cathepsin B using small interfering RNA significantly restored SK1 levels following exposure to TNF, suggesting that SK1 loss was dependent on cathepsin B activity. The regulation of SK1 by the lysosomal protease was further supported by the colocalization of SK1 with the lysosome and cathepsin B in cells and the loss of the colocalization following exposure to TNF. The ability of cathepsin B to regulate SK1 was further corroborated by an in vitro approach where recombinant cathepsin B cleaved SK1 at multiple sites to produce several cleavage fragments. Therefore, these studies show that SK1 down-regulation by TNF is dependent on the “lysosomal pathway” of apoptosis and specifically on cathepsin B, which functions as an SK1 protease in cells.

A growing body of evidence implicates the enzyme sphingosine kinase 1 (SK1) and the lipid it produces, sphingosine-1-phosphate (S1P), in cellular pathways of proliferation, cell survival, and apoptosis. SK1 has been shown to rescue cells from stimuli that normally induce cell death, such as death receptor ligands and antineoplastic agents. Of particular interest has been the proinflammatory cytokine TNF-α (TNF), which is a well known pleotropic cytokine capable of activating survival as well as death pathways in various cell types. Interestingly, the induction of SK1 activity and increased production of S1P have been recently shown to occur following acute stimulation by TNF (4, 5). Furthermore, enhanced S1P production provides a protective effect from the cytotoxic effects of TNF in human umbilical vein endothelial cells (4).

A central feature of TNF signaling in multiple cell types is the induction of apoptosis, which typically involves the binding of the cytokine to its receptor (TNF receptor 1), followed by the recruitment of adapter molecules TNF receptor-1-associated death domain protein and Fas-associated death domain protein and activation of caspase enzymes. In addition to the activation of the mitochondrial pathway of apoptosis, TNF can also mobilize proteases from the lysosome (6). In particular, cathepsins B and D have been implicated as effector proteases in the TNF cascade of cell death (7–9). These proteases can induce cell demise by stimulating mitochondrial permeabilization (10, 11). The BH3-only protein Bid is a target for both cathepsins B and D, and cleavage of Bid by these proteases can drive mitochondrial release of cytochrome c (7, 12).

The same TNF receptor 1 can also recruit the adapter protein TRAF2, which has recently been shown to interact directly with and activate SK1 at the membrane (5). The activation of SK1 can then drive NF-κB translocation to the nucleus and the induction of antiapoptotic and proinflammatory genes. Therefore, SK1 is an important determinant of cell fate after TNF stimulation and inhibition of SK1 activity sensitizes cells to the cytotoxic effects of the cytokine (4). Nevertheless, whereas TNF acutely activates SK1 within minutes, the long term effects of the cytokine on SK1 have not been investigated, particularly at time points where the proteases of death pathways are activated.

Recently, our studies showed that in the Molt-4 leukemia cell line genotoxic stress induces a decrease in SK1 protein and SK activity in a p53-dependent manner (13). Furthermore, the decline of SK1 occurred via a cysteine protease-mediated pathway. Specific inhibitors to proteases known to be activated in the apoptotic response showed a significant role for both caspases and cathepsin B in SK1 loss. Interestingly, the activation of all of these proteases following exposure to TNF has been documented in several cell types. These data prompted us to examine the regulation of SK1 by TNF-activated mediators of cell death in order to gain insight into the mechanisms of SK1 loss.

In this study, we investigate the effects of TNF on SK1 function. TNF caused a decrease in SK1 protein in MCF-7 cells. Furthermore, a pool of SK1 was found to be closely associated with Rac1 in the cytoplasm and activated Rac1 was found in the nucleus following TNF stimulation. This work provides a new role for SK1 in the regulation of apoptosis by TNF and suggests that SK1 could be a novel target for the development of therapeutic strategies to combat TNF-induced cell death.

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** To whom correspondence should be addressed: Dept. of Medicine, Medical University of South Carolina, 114 Doughty St., P. O. Box 250779, Charleston, SC 29425, Tel.: 843-876-5169; Fax: 843-876-5172; E-mail: obeid@musc.edu.

† The abbreviations used are: SK1, sphingosine kinase 1; TNF, tumor necrosis factor; S1P, sphingosine-1-phosphate; siRNA, small interfering RNA; Lamp, lysosome-associated membrane protein; PBS, phosphate-buffered saline; GFP, green fluorescent protein; Z, benzyloxycarbonyl; fmk, fluoromethyl ketone.

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with cathepsin B in vivo and SK1 regulation occurred downstream of cathepsin B in the TNF response. SK1 also served as a substrate for cleavage by the protease in vitro.

MATERIALS AND METHODS

Chemicals and Reagents—TNF-α was purchased from Peprotech Inc. Recombinant cathepsin B and the cathepsin B activity detection kit were from Calbiochem. Mouse monoclonal anti-cathepsin B (Ab-1) was from Ozyme, a rabbit polyclonal anti-cathepsin B was from Athens Research and Technology, Inc. The human cathepsin B siRNA and mouse monoclonal anti-Lamp-1 (sc-18821) were from Santa Cruz Biotechnology. LysoTracker Red and MitoTracker Red were purchased from Molecular Probes. The rabbit polyclonal anti-SK1 antibody has been described previously (14).

Cell Culture—MCF-7 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Trypan Blue Exclusion Assay—Cells were seeded in RPMI 1640 medium containing 10% fetal bovine serum and then treated with 25 ng/ml TNF for 20 h. Floating and adherent cells were harvested by trypsinization and centrifuged at 1000 × g at 4 °C for 5 min. The cell pellet was resuspended in 0.2% trypsin blue solution diluted in RPMI 1640 medium containing 10% fetal bovine serum. Cells were counted microscopically, and trypan blue positive cells were scored as a percentage of total cell number.

Transient Transfection—MCF-7 cells were seeded in 35-mm dishes and transfected with pEGFP-SK1 using the Effectene transfection reagent (Qiagen). For each dish, 0.2 μg of DNA, 100 μl of EC buffer, and 1.6 μl of enhancer were used. The cells were analyzed 24 h after transfection.

Immunoblotting—Cells were collected by centrifugation at 3000 × g for 5 min at 4 °C. They were lysed in lysis buffer (20 mM HEPES, 50 mM NaCl, 1 mM EGTA, 5 mM β-glycerophosphate, 28.8 mM sodium pyrophosphate, 0.1 mM sodium orthovanadate, 1% Triton X-100, and 0.1% protease inhibitor mixture (Sigma)) for 30 min on ice. The lysate was homogenized with a 21-gauge needle and centrifuged at 18,000 × g for 30 min at 4 °C. Protein concentration was determined on the supernatant using the method of Bradford. Equal amount of protein was resuspended in 4X Laemmli sample buffer, boiled for 5 min, and applied to a polyacrylamide gel electrophoresis. Following transfer to nitrocellulose, the membranes were blocked overnight in 5% nonfat milk in Tris-buffered saline (TBS) for 1 h of the appropriate antibodies in 3% bovine serum albumin for 1–2 h, washed three times with TBS and 0.1% Tween 20. The membranes were probed with primary antibodies for 1–2 h, washed three times with TBS, and then probed with secondary antibodies for 1 h. The membranes were then rinsed three times with TBS-buffered saline + 0.1% Tween 20, and the signal was detected using enhanced chemiluminescence (Amersham Biosciences) or Supersignal West Dura Extended Duration Substrate (Pierce) according to the manufacturer's instructions.

Immunofluorescence—MCF-7 cells in 35-mm confocal dishes were washed twice with PBS and fixed in cold (−20 °C) methanol for 5 min at room temperature. The cells were then washed with PBS three times, blocked, and permeabilized with 3% bovine serum albumin, 0.05% Triton X-100 for 20 min. After washing three times with PBS, the cells were treated with 5 μg/ml of the appropriate antibodies in 3% bovine serum albumin for 1–2 h. They were washed with PBS three times and then treated for 1 h with fluorescent secondary antibodies (Alexa Flour 488 anti-mouse secondary antibody from Molecular Probes and rhodamine-conjugated anti-rabbit secondary antibody from Jackson ImmunoResearch Laboratories). After washing off the secondary antibodies three times with PBS, the cells were visualized under a Zeiss LSM 510 confocal microscope (Alexa Flour: excitation 488 nm, emission: BP 505–530 nm; rhodamine: excitation 543 nm, emission: LP 560 nm).

Mass Spectrometric Analysis of S1P Levels—Measurement of S1P was done as described previously (15) with a C18 column used instead of a C8 column. Data are normalized to total protein in each sample.

In Vivo Cathespin B Assay—To assay for cathespin B activity in MCF-7 cells, the cathepsin B detection kit was used. A cell-permeable Cresyl Violet-labeled cathepsin B substrate was added to the cells, which were then incubated at 37 °C for 30 min. The signal was visualized under a Zeiss LSM 510 confocal microscope (excitation 543 nm, emission: LP 560 nm).

RESULTS

TNF Induces the Down-regulation of Sphingosine Kinase 1—To determine the effects of TNF on SK1, MCF-7 cells were treated with 25 ng/ml TNF and SK1 protein was analyzed by Western blotting. In a time course study (Fig. 1A), SK1 protein showed a noticeable decrease by 16 h. The decline in SK1 continued progressively over the time course, such that by 36 h, SK1 could no longer be detected (Fig. 1A). At time points between 16 and 20 h, cells started to display morphological signs of cell death, such as cell rounding (data not shown), and the number of trypan blue positive cells at 20 h reached 64.2 ± 1.1% in the TNF-treated cells compared with only 4.02 ± 1.3% in the control cells, suggesting that pathways of cell death were highly active at the 16–20 h time points.

The dose dependence of SK1 response to TNF was determined. Upon treatment of MCF-7 cells with increasing concentrations of the cytokine for 16 h, a dose-dependent decline of SK1 was observed (Fig. 1B). Protein levels started to diminish at 10 ng/ml TNF and were significantly decreased at 25 ng/ml. These results indicated that prolonged TNF treatment indeed down-regulates SK1 in MCF-7 cells. For the remainder of the studies, a concentration of 25 ng/ml TNF and time points between 16 and 20 h were used.

Because SK1 is a key enzyme in the synthesis of the prosurvival lipid, S1P, changes in S1P levels following exposure to TNF were investigated as well. As expected, the down-regulation of SK1 by the cytokine was accompanied by a decline in S1P from 2.53 ± 0.87 to 0.53 ± 0.12 pmol/mg protein, suggesting that prolonged TNF exposure attenuates metabolism through the SK1-S1P arm of the sphingolipid metabolic pathway.

Cysteine Proteases Are Involved in SK1 Decrease by TNF—Because TNF is known to activate cysteine proteases in MCF-7 cells, the role of these proteins in the SK1 decline was investigated by pharmacological approaches. Treatment for 21 h with 20 μM pancysteine protease inhibitor Z-VAD-fmk alone had no significant effect on the basal SK1 protein levels (Fig. 2, lane 2). Pretreatment with 20 μM Z-VAD-fmk almost completely reversed the effect of TNF on SK1 (Fig. 2, lane 6), suggesting that cysteine proteases regulate SK1 levels in TNF-stimulated cells. To test the roles of particular caspases known to be activated by TNF, more specific caspase inhibitors were used. Inhibition by IETD of caspase 8, a well known upstream initiator of TNF-activated pathways of apoptosis, resulted in a significant reversal of the TNF effect on SK1 (Fig. 2, lane 7) but not as effectively as Z-VAD-fmk. In contrast, IETD alone did not increase basal SK1 levels (Fig. 2, lane 3). These results suggest that caspase 8 is probably not involved in basal SK1 regulation but that TNF-induced SK1 loss is in part a caspase 8-regulated process.

Another protease known to be activated by TNF in MCF-7

Cathepsin B siRNA—MCF-7 cells were treated with 50 nm cathepsin B siRNA targeting human cathepsin B following the Oligofermamine reagent (Invitrogen) protocol.

In Vivo Cleavage Assays of SK1 by Cathepsin B—For the cathepsin B assay, 1.0 μl of recombinant SK1 (2 μg/μl) was added to 5 μl of 3× cathepsin B reaction buffer (150 mM sodium acetate, pH 6.0, 12 mM EDTA, and 24 mM dithiothreitol) and 5.0 μl of recombinant cathepsin B (0.02 units/μl) to achieve the final concentrations required for the assay. Whenever required, cathepsin B was diluted in 50 mM sodium acetate, pH 5.0, 1 mM EDTA prior to its use in the assay. The final volume of the assay was brought to 15 μl with dH2O. The cleavage reaction was performed by incubation at 37 °C for the times indicated in the text. Following incubation, 15 μl of 2× sample buffer was added to the samples. They were boiled and subjected to SDS-PAGE. The proteins were transferred to Problott polyvinylidene difluoride membrane (Applied Biosystems) and stained with Coomassie Brilliant Blue until the bands were visible. The membrane was then destained with 50% methanol and left to dry at room temperature.
cells is caspase 7. Unlike caspase 8, caspase 7 is an effector rather than an initiator caspase. To examine the possible involvement of caspase 7 in SK1 loss, the inhibitor DEVD was used. At 20 \( \mu \)M, DEVD had no effect on SK1 in control cells (Fig. 2, lane 4) as well as in TNF-treated cells (Fig. 2, lane 8). This suggests that caspase 7 is not involved in SK1 regulation by TNF.

**TNF Induces the Release of Active Cathepsin B from Lysosomes into the Cytosol**—In addition to the activation of caspases, TNF can mobilize multiple proteases such as cathepsins from the lysosomal compartment in a caspase 8-dependent manner (7–9, 11, 16). Therefore, the effects of TNF on lysosomal function and cathepsin B localization in MCF-7 cells were investigated. Cathepsin B localization was examined, since we have previously implicated this protease in SK1 regulation by genotoxic stress (13). Lysosomes were visualized by staining for Lamp-1, a lysosome-associated membrane protein. Cathepsin B and Lamp-1 showed a punctate distribution throughout the cytoplasm of untreated MCF-7 cells with a distinctively higher concentration of the two proteins in the perinuclear region (Fig. 3, A and B). At 16 h following exposure to TNF, the time at which SK1 loss was observed, there was a clear disruption of the lysosome as indicated by the disappearance of the strong perinuclear pattern of Lamp-1 (Fig. 3F). The disruption of the lysosome was further confirmed by the shift in Lysotracker staining from a punctate (Fig. 3C) to a diffuse cytosolic signal (Fig. 3G). As expected, the disruption of the lysosome was accompanied by the relocation of cathepsin B, which lost its distinct perinuclear signal to a cytosolic punctate pattern that extended throughout the cell (Fig. 3F).

Cathepsin B is pH-sensitive, so it was important to establish whether the enzyme was catalytically active once released into the cytosol from its perinuclear location. Enzyme activity was determined in a cell-based assay using a cell-permeable cathepsin B substrate that fluoresces upon cleavage by the protease. As shown in Fig. 3, D and H, the perinuclear punctate activity of cathepsin B seen in control cells became diffuse throughout the cell upon treatment with TNF, demonstrating active protease in the cytosol.

**Cathepsin B Is Required for the Decrease of SK1 by TNF**—To investigate whether the decrease in SK1 was mediated by the release of cathepsin B into the cytosol, an siRNA approach was used to silence cathepsin B expression. Initially, a time course of cathepsin B siRNA was performed and it was determined that around 60% reduction of the active form of the protease could be achieved at 37 h post-treatment. The knockdown reached 75% by 48 h and was maintained for up to 70 h at least (Fig. 4A). This indicated that the half-life of active cathepsin B in MCF-7 cells is less than 37 h. We next evaluated the effect of silencing cathepsin B on the TNF response. Upon incubation of the cells with siRNA against the protease for 72 h followed by TNF treatment for 17 h, we observed a significant rescue of SK1 protein from down-regulation by the cytokine (Fig. 4B, lanes 3 and 4). The cells transfected with scrambled siRNA, however, showed a loss of SK1 that was similar to what was noted in Fig. 1 (Fig. 4B, lanes 1 and 2). These data strongly suggest that cathepsin B contributes to the decrease of SK1 by TNF.

**Cathepsin B and Lysosomes Colocalize with SK1**—Because cathepsin B appears to be required for SK1 loss, we next
examined the localization of the two proteins in MCF-7 cells. Two approaches were employed. The first, shown in Fig. 5A, illustrates the distribution of GFP-tagged SK1, LysoTracker, and a cathepsin B fluorogenic substrate in MCF-7 cells. Interestingly, although GFP-SK1 exhibited a general cytosolic distribution as reported previously (17), the protein also showed a punctate pattern in certain locales where SK1 was concentrated in unidentified cellular compartments. Upon staining with LysoTracker and the cathepsin B fluorogenic substrate, some of these more concentrated regions of SK1 displayed a close association with the lysosome and the cathepsin B-positive compartments, particularly in the perinuclear region (Fig. 5A, top and middle panels). This was indicated by the yellow ring of overlay surrounding the red staining of the lysosomes and cathepsin B. To determine whether this effect was seen with other cytoplasmic organelles, GFP-SK1 localization was also analyzed in the presence of MitoTracker, a mitochondrial marker (Fig. 5A, lower panel). In this case, no overlay was observed, implying that SK1 is unlikely to be associated with the mitochondrion. In fact, MitoTracker displayed a clear negative staining pattern with respect to GFP-SK1.

The second approach used to determine the topological relationships among SK1, cathepsin B, and the lysosome was an immunohistochemical approach with fixed MCF-7 cells (Fig. 5B). Here, staining for the endogenous proteins was performed and Lamp-1 was used to probe for lysosomes. Similar to overexpressed SK1, a pool of the endogenous enzyme showed partial colocalization with both cathepsin B (Fig. 5B, top panel) and lamp-1 (Fig. 5B, lower panel), especially in the perinuclear regions of the cell. From these results, it appeared that, although SK1 is primarily a cytosolic protein, a pool of the enzyme is closely associated with the lysosomal compartment or a closely related compartment as well as with cathepsin B.

Having established the colocalization of SK1 with Lamp-1 and cathepsin B, we next examined how these proteins interact following exposure to TNF. Upon treatment with the cytokine, the loss of the punctate signal for LysoTracker (indicating lysosome disruption) was accompanied by a significant decrease in the perinuclear punctate GFP-SK1 signal seen in
Fig. 5. Colocalization of cathepsin B and lysosomes with SK1. A, MCF-7 cells transiently transfected with GFP-SK1 were treated for 30 min with LysoTracker (upper panel), cathepsin B fluorogenic substrate (middle panel), or MitoTracker (lower panel). Arrows in the upper and middle panels indicate areas of colocalization. B, methanol-fixed MCF-7 cells were probed for endogenous SK1 and cathepsin B (upper panel) or
control cells (Fig. 5C). Examination of endogenous SK1 also revealed that the pool of the enzyme residing near the nucleus (Fig. 5D, upper left panel) disappeared when the Lamp-1 signal was lost (Fig. 5D, upper right panel). In the panel showing cathepsin B staining (Fig. 5D, lower right panel), the cell showed some lysosomes that have not yet disrupted. Associated with these intact lysosomes was an SK1 signal. However, in areas where the perinuclear cathepsin B signal was not present, SK1 staining was lacking as well, raising the possibility that SK1 has already been proteolyzed.

**Cathepsin B Cleaves SK1 in Vitro**—Having established that cathepsin B is required for SK1 loss and that the two proteins colocalize in cells, it became important to determine whether SK1 is a substrate for cathepsin B. To this end, the ability of the protease to cleave SK1 in vitro was tested. Upon incubation of 2 μg of recombinant SK1 with 0.66 μM (0.1 units in 15 μl of reaction volume) cathepsin B, a time-dependent loss of SK1 was seen along with the appearance of several cleavage fragments: one band at ~31 kDa; one band at ~23 kDa; two bands at ~18 kDa; and one band at ~13 kDa (Fig. 6). At this concentration of cathepsin B, a 20-min incubation of SK1 with the protease was long enough to produce four of the five detectable cleavage products: the 31-kDa band; the 23-kDa band; and the two 18-kDa bands. Over time, the higher of the two 18-kDa bands disappeared and the lower one became more prominent. The 13-kDa fragment was the latest to appear (beginning around 60 min). These results indicate that SK1 is a direct substrate for cathepsin B. Moreover, cathepsin B cleaves SK1 at multiple sites in an apparently sequential manner.

**DISCUSSION**

In this study, we examined the prolonged effects of TNF on SK1 in MCF-7 cells. The cytokine induced a dose-dependent loss of SK1 protein at time points where proteases of cell death pathways were active. The decline in SK1 occurred downstream of the lysosomal cysteine protease cathepsin B, and inhibition of this protease reversed TNF effects on SK1. Moreover, cathepsin B may be directly involved in the loss of SK1 because the protease cleaves SK1 in vitro. Furthermore, confocal microscopy showed a close association of endogenous and overexpressed SK1 with cathepsin B and the lysosome, placing a pool of SK1 in close proximity to cathepsin B and lending support to SK1 regulation by the protease.

Although the mitochondrion is thought to be the compartment upon which many cell death signals converge and commit cells to die, the lysosome is another key organelle whose role in programmed cell death is beginning to be appreciated. Initially thought to merely house hydrolytic proteases, the lysosome is now known to be biochemically regulated in various forms of cell death (6). Disruption of its membrane results in the release of many proteases, the most notable of which are the cathepsins. Cathepsins B and D can each trigger mitochondrial membrane permeabilization when they relocate from the lysosome to the cytosol and may therefore serve as transducer proteases between the lysosome and the mitochondrion (8, 12). In this respect, they operate upstream of “the point of no return” in programmed cell death and modulation of their activities and localization may impact cell survival. In this study, we show that SK1 regulation by TNF is dependent on cathepsin B activity, hence rendering SK1 a component of the lysosomal pathway of apoptosis. Moreover, inhibition of the initiator caspase 8 but not the effector caspase 7 partly reversed the TNF effect on SK1. These results potentially place SK1 at a critical point in programmed cell death, downstream of initiator caspases and the lysosome and upstream of effector caspases and the mitochondrion and therefore upstream of the commitment step of programmed cell death. This model is further supported by the colocalization of SK1 with cathepsin B and lysosomes but not with mitochondria, suggesting that dysfunction of the lysosomal membrane may be a trigger for SK1 loss. Indeed, confocal microscopy showed that, following stimulation by TNF, a concentrated pool of SK1 remained in the perinuclear region in areas where lysosomes were intact but not in regions where lysosomes were disrupted and cathepsin B was released (Fig. 5D). Based on these data, SK1 loss may be part of a death signal from the lysosome to the mitochondrion and interference with SK1 function may also be a trigger for mitochondrial membrane permeabilization. This is well supported by our previous finding that down-regulation of SK1 is sufficient to drive cell death in MCF-7 cells (13).

Numerous studies have shown that enhanced SK activity promotes cell survival and proliferation (17) and that S1P, the product of the SK reaction, can also rescue from the effects of several apoptotic stimuli such as ceramide and chemotherapy (13, 18). A recent study by Xia et al. (5) demonstrated an important functional role of SK1 as a TRAF2-interacting protein that mediates NF-κB-driven prosurvival responses to TNF. The cytokine induced translocation of SK1 to the plasma membrane followed by its activation within minutes, which is the same time required by TNF to mobilize NF-κB. SK1 activity returned to base line quickly as did NF-κB activity. Acute activation of SK1 by TNF has also been shown to mediate induction of cyclooxygenase-2 and the production of prostaglandin E2, known prosurvival factors (15). In addition to its ability to activate antiapoptotic responses via NF-κB mobilization, TNF is a well-known prodeath cytokine that activates several proteases involved in programmed cell death, the most notable of which are caspases and lysosomal proteases (6, 19). The dependence of TNF-mediated cell death on cathepsin B activity has been previously described in several cell systems (8, 11, 20). Furthermore, the cathepsin B inhibitor Spi2A has recently been reported to be a target gene for transcription by NF-κB after TNF (21). Spi2A inhibits cathepsin B released into the endogenous SK1 and Lamp-1 (lower panel). Inset in the lower panel shows a higher magnification of the nuclear and perinuclear regions within the cell. White arrows indicate regions of overlay. C, GFP-SK1-transfected MCF-7 cells stained with LysoTracker before (left panel) and after (right panel) TNF treatment. White arrow in the control panel indicates the areas of colocalization between the lysosome and GFP-SK1. D, when the perinuclear Lamp-1 and cathepsin B signals seen in the control cells (left panels) are lost following TNF stimulation, the perinuclear SK1 staining is lost as well (right panels). Lower right panel shows some regions where the perinuclear cathepsin B signal has not been lost yet (white arrows), and accompanied with the cathepsin B signal is an overlying SK1 signal (inset).
cytosol during the death phase of the TNF response. This correlates well with studies where TNF cytotoxicity is enhanced when the cytokine is used in combination with a transcriptional or a translational inhibitor, which presumably attenuates NF-kB driven antiapoptotic gene up-regulation. In these instances, cathepsin B activity during the death phase of the TNF response may be augmented (due to the absence of SpI2A) and SK1 loss may become more dramatic. Therefore, it seems that during the survival phase of the TNF response, mechanisms that enhance SK1 activity (by translocation to the plasma membrane) and attenuate cathepsin B activity (via SpI2A up-regulation) are mobilized, whereas in the death phase of the response, cathepsin B activation (due to release into the cytosol) and SK1 down-regulation (by cathepsin B) are key events taking place. The balance between these two processes may determine the net fate of the cell following prolonged exposure to TNF. The data presented in this work support the involvement of a cathepsin B-SK1 axis in the later phases of the TNF response.

In conclusion, this study has provided insight into the regulation of SK1 following prolonged exposure to TNF and has implicated SK1 modulation by a lysosome-dependent pathway mediated via cathepsin B. In addition, these studies may place SK1 between the lysosome and the mitochondrion in pathways that enhance SK1 activity (by translocation to the mitochondrion) and attenuate cathepsin B activity (via mechanisms that enhance SK1 activity by translocation to the mitochondrion). Furthermore, these studies may place SK1 modulation by a lysosome-dependent pathway in the regulation of programmed cell death.

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