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Serine 58 of 14-3-3ζ Is a Molecular Switch Regulating ASK1 and Oxidant Stress-Induced Cell Death▽†

Jibin Zhou,[1] Zhili Shao,[1] Risto Kerkela,[1] Hidenori Ichijo,[4] Anthony J. Muslin,[5] Celia Pombo,[6] and Thomas Force[1,2,3]*

Center for Translational Medicine,[1] Program in Cell and Developmental Biology,[2] and Cardiology Division,[3] Thomas Jefferson University, Philadelphia, Pennsylvania; Laboratory of Cell Signaling, Graduate School of Pharmaceutical Sciences, University of Tokyo, Tokyo, Japan[4]; Center for Cardiovascular Research, John Milliken Department of Internal Medicine, Washington University School of Medicine, St. Louis, Missouri[5]; and Laboratory of Cell Signaling and Cancer Research, Department of Physiology, School of Medicine, University of Santiago de Compostela, Santiago de Compostela, Spain[6]

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Oxidant stress is a ubiquitous stressor with negative impacts on multiple cell types. ASK1 is a central mediator of oxidant injury, but while mechanisms of its inhibition, such as sequestration by 14-3-3 proteins and thioredoxin, have been identified, mechanisms of activation have remained obscure and the signaling pathways regulating this are not clear. Here, we report that phosphorylation of 14-3-3ζ at serine 58 (S58) is dynamically regulated in the cell and that the phosphorylation status of S58 is a critical factor regulating oxidant stress-induced cell death. Phosphorylation of S58 releases ASK1 from 14-3-3ζ, and ASK1 then activates stress-activated protein kinases, leading to cell death. While several members of the mammalian sterile 20 (Mst) family of kinases can phosphorylate S58 when overexpressed, we identify Ste20/oxidant stress response kinase 1 (SOK-1), an Mst family member known to be activated by oxidant stress, as a central endogenous regulator of S58 phosphorylation and thereby of ASK1-mediated cell death. Our findings identify a novel pathway that regulates ASK1 activation and oxidant stress-induced cell death.

Oxidant stress plays a central role in a wide variety of pathologies, and a critical mediator of oxidant injury is the protein kinase ASK1 (30). Indeed, ASK1 is required for several types of oxidant stress-induced cell death (32). Its activity is restrained by a large number of complementary mechanisms, a fact that attests to the importance of ASK1 being maintained in an inactive state in the cell. For example, reduced thioredoxin binds to the N-terminal region of ASK1, thereby inhibiting its activity (27). Following oxidant stress and oxidation of thioredoxin, ASK1 is released, allowing its activation. Multiple phosphorylation events, including phosphorylation of ASK1 at S83 by Akt and at S1033 by an unknown mechanism, also negatively regulate ASK1 (6, 41; reviewed in reference 30). Critical to the negative regulation of ASK1 is phosphorylation of S966, which drives the association of ASK1 with 14-3-3 proteins, thereby inhibiting ASK1-mediated activation of downstream signaling and cell death (8, 43). The kinases responsible for S966 phosphorylation are not known, but the protein phosphatase calcineurin has been shown to dephosphorylate S966, leading to dissociation of ASK1 from 14-3-3 (13). Thus, other than calcineurin-mediated dephosphorylation of ASK1, signaling mechanisms positively regulating the release of ASK1 from 14-3-3 proteins are not known, despite intense interest in this kinase as a potential target in cardiovascular and neurologic diseases (30). Therefore, we undertook studies to attempt to identify such a mechanism.

14-3-3 proteins play protective roles in the cell by sequestering proapoptotic factors in a phosphorylation-dependent manner (1, 15, 23). These proapoptotic proteins that are sequestered by 14-3-3 proteins are typically phosphorylated on one or more 14-3-3 binding motifs (18, 39). For example, in addition to ASK1 phosphorylation at S966, (8, 30), Bad is phosphorylated by Akt and ribosomal S6 kinases at several residues, inhibiting its proapoptotic functions (4, 14, 42, 45). Acting in opposition to this is the well-characterized c-Jun N-terminal kinase (JNK)-mediated phosphorylation of serine 184 of 14-3-3 proteins, leading to release of the proapoptotic factors Bax, Bad, FOXO3a, and Abl (29, 33, 40). In addition to S184, the phosphorylation statuses of other 14-3-3 residues can regulate 14-3-3/client interactions, such as T233, which is phosphorylated by CKI, disrupting the 14-3-3/Raf-1 interaction (5).

Although most of the attention to phosphorylation of 14-3-3 has been focused on S184 and T233 (1), S58 has been known to be phosphorylated in situ for some time, and several kinases have been implicated, including protein kinases A and D, Akt, mitogen-activated protein kinase-activated kinase 2 (MK2), and sphingosine-dependent protein kinase 1 (later identified as a cleavage fragment of protein kinase C δ) (9, 16, 17, 24, 25, 44). However, it is not clear which specific kinases mediate phosphorylation under specific circumstances, nor are the biological consequences clear. This is underscored by the fact that both pro- and antiapoptotic kinases have been reported to phosphorylate this residue (23). It does seem clear, however, that S58 phosphorylation disrupts 14-3-3 dimerization and that

* Corresponding author. Mailing address: Center for Translational Medicine, Thomas Jefferson University, College Building, Rm. 316, 1025 Walnut St., Philadelphia, PA 19107. Phone: (215) 503-9520. Fax: (215) 503-5731. E-mail: thomas.force@jefferson.edu.

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this reduces the binding of some proteins (e.g., Raf-1) (28, 34), though probably not all, since Woodcock et al. reported that 14-3-3ζ monomers phosphorylated at S58 remained competent to bind phosphopeptides (37).

Thorson et al. and Wang et al. created 14-3-3 mutants that were deficient in binding phosphopeptides, and Xing et al. employed one of these, 14-3-3ζ(R56A/R60A), to show that it led to enhanced activation of the stress-activated protein kinases, JNKs and p38, and enhanced cell death in response to UVC irradiation, a model of oxidant stress (31, 36, 38). However, since S58 of 14-3-3ζ is in the center of the R56-R60 region, we hypothesized that phosphorylation of S58 might disrupt binding of 14-3-3ζ to ASK1, which is upstream from the JNKs and p38 in the response to oxidant stress. In addition, Preisinger et al. reported that Ste20/oxidant stress response kinase 1 (SOK-1)/STK25, a mammalian sterile 20 (Mst) family member also known as yeast sterile 20 kinase 1 (YSK1), phosphorylated 14-3-3ζ on S58, leading to reorganization of the Golgi structure and cell motility (26). However, we and others had initially identified SOK-1 as a kinase activated by oxidant stress (20–22), and we have also shown that SOK-1 exits the Golgi apparatus following cellular stress (19), potentially giving SOK-1 greater access to the ASK1/14-3-3 complex (which is primarily cytosolic). Thus, we hypothesized that oxidant stress-induced activation of SOK-1 might lead to S58 phosphorylation, release of ASK1 from 14-3-3ζ, activation of JNKs and p38, and cell death. Here, we confirm these hypotheses and identify S58 as a molecular switch regulating ASK1-mediated oxidant stress-induced cell death.

MATERIALS AND METHODS

Antibodies and kinase inhibitors. The antibodies employed included the following: 14-3-3ζ–phospho-Ser 58 (Affinity BioReageagents; PA1-4612); ASK1 (sc7931), SOK-1 (sc-6865), and JNK1 (sc571) (Santa Cruz Biotechnology); ASK1–phospho-Thr845 (Cell Signaling; no. 3765); and JNK-phospho (Promega; V7931). The kinase inhibitors used were JNK inhibitor I and the p38 inhibitor SB239063, both from Calbiochem.

Plasmids. Construction of the 14-3-3ζ mutants was done as follows. pCINeo, harboring M2 epitope-tagged wild-type (WT) 14-3-3ζ cDNA, was constructed as described by Xing et al. (38). pCINeo-M2-14-3-3ζ–S58A and pCINeo-M2–14-3-3ζ–S58D were generated with a QuikChange II XL site-directed mutagenesis kit (Stratagene; no. 200521), following the manufacturer's instructions. The primers, purchased from Sigma, were as follows: SA forward primer, 5′-GTA GGA GCC CGT AGG TCA GCT TGG AGG GTC GTC TCA AGT ATT G-3′, and SA reverse primer, 5′-CAA TAC TTG AGA CGA CCC TCC AAG CTG ACC TAC GGG CTC CTA C-3′; SD forward primer, 5′-GTA GGA GCC CGT AGG TCA GAT TGG AGG GTC GTC TCA AGT ATT G-3′, and SD reverse primer, 5′-CAA TAC TTG AGA CGA CCC TCC AAT CTG ACC TAC GGG CTC CTA C-3′. The coding regions of all three plasmids were sequenced. pcDNA3-HA-ASK1 was constructed as described previously (10). All restriction enzymes were purchased from New England Biolabs.

Cells and cell culture. $ASK1^{-/-}$ and $ASK1^{+/+}$ mouse embryo fibroblasts (MEFs) were generated as described previously (32). The cells were maintained in MEF growth medium (Dulbecco's modified Eagle's medium [DMEM] with 10% fetal calf serum, supplemented with L-glutamine, nonessential amino acids [Gibco; no. 25030-050], and 1% penicillin-streptomycin) in 5% CO_2 at 37°C. NIH 3T3 fibroblasts were from ATCC (CRL-1658) and were maintained in DMEM with 10% bovine calf serum, 1% penicillin-streptomycin in 5% CO_2 at 37°C.

Transfection. Cells were transfected using the FuGENE 6 reagent (Roche; 11 814 443 001) following the manufacturer's instructions. Transfection efficiency with this protocol is 60 to 70%.

Coimmunoprecipitation. Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% NP-40, and protease inhibitor cocktail and phosphatase inhibitor cocktail [P8430 and P2850, respectively; both from Sigma]). EZview Red Anti-FLAG M2 affinity gel beads conjugated with anti-FLAG M2 monoclonal antibody (Sigma; F2426) were used for coimmunoprecipitation, following the manufacturer's instructions. The beads were washed five times in lysis buffer, followed by the addition of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer.

Immunoblotting and densitometry. Following SDS-PAGE and transfer to a nitrocellulose membrane, primary antibody incubations were performed at the following dilutions: 14-3-3ζ–pSer58, 1:1,000; 14-3-3ζ, 1:1,000; ASK1, 1:500; ASK1-pThr845, 1:500; SOK-1, 1:1,000; JNK1, 1:1,000; and JNK-p, 1:4,000. All incubations were done at 4°C overnight. The secondary antibody used was Alexa Fluor 680 (Molecular Probes) at 1:3,000 dilution for 1 h at room temperature. The membranes were scanned with the Odyssey infrared imaging system (Li-Cor). The densities of target bands were quantified using the application software of the Odyssey infrared imaging system. Band density was normalized to that of the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) loading control.

Determination of apoptosis employed terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). Cells were plated on chamber slides for 24 h, serum starved for 2 h, and then treated with 125 μM H_2O_2 for 2 h, followed by an additional incubation for 1 h in culture medium (DMEM) prior to fixation. Identification of apoptotic cells was done with the apoptosis detection kit from Chemicon (S7111), by following the manufacturer's instructions. For experiments in which transfection was required, cells were transfected 24 h after being plated. After an additional 24 h, the cells were exposed to 125 μM H_2O_2 for 3 h and processed as described above.

The percentage of apoptotic cells (i.e., the apoptotic index, or the number of TUNEL-positive cells per 100 cells) was determined by counting the cells in five predefined regions of the slides (the four corners and the middle of each well). The total number of cells counted was ~1,000 to 1,200 per well. In some instances, it was necessary to count up to nine areas of a slide.

siRNA-mediated RNA interference. Small interfering RNAs (siRNAs) for SOK-1 (D-065319-04-0010 and On-Targetplus Smartpool, a pool of four siRNAs—J065319-05, J-065319-06, J065319-07, and J-065319-08) and for Mst2 (D-040440-04-005) were from Dharmacon. Control siRNA was from Ambion (Silencer Negative Control no. 2; AM4613). On the day before transfection, cells were plated in antibiotic-free medium and were incubated overnight. The cells were then transfected using DharmaFect1, T-2001, with a final siRNA concentration of 100 nM of the siRNA of interest or control siRNA, following the manufacturer's instructions. The cells were incubated for an additional 72 hours prior to performing experiments.

Statistical analyses. We employed Student's *t* test to analyze densitometry data. The chi square test was used to determine the significance of the TUNEL studies. A P value of < 0.05 was considered statistically significant. The data are presented as means ± standard errors of the means (SEM).

RESULTS

To explore the potential role of S58 phosphorylation in regulating cell death, we examined cell death induced by the universal stressor oxidant stress in NIH 3T3 fibroblasts. First, we employed a phosphospecific antibody for S58 to examine the mechanisms and consequences of S58 phosphorylation. This antibody indeed appeared to be highly selective for S58-phosphorylated 14-3-3ζ based on the results of competition studies performed by the manufacturer. In these studies, the phosphopeptide used to generate the antibody completely competed out phospho-S58 antibody binding to 14-3-3ζ, whereas the nonphosphopeptide had no effect on binding of the antibody. Furthermore, phospho-S58 antibody binding was eliminated by prior treatment of 14-3-3ζ with λ phosphatase (data available from and provided to us by the manufacturer). We repeated the peptide competition studies and confirmed the selectivity of the antibody for S58-phosphorylated 14-3-3ζ (Fig. 1A).

We then examined the phosphorylation status of S58 and found that it was dynamically regulated in response to oxidant stress induced by H_2O_2 (Fig. 1B and C). To further test the specificity of the phosphoantibody, we expressed WT 14-3-3ζ or 14-3-3ζ with an S58-to-A mutation (SA) prevent-

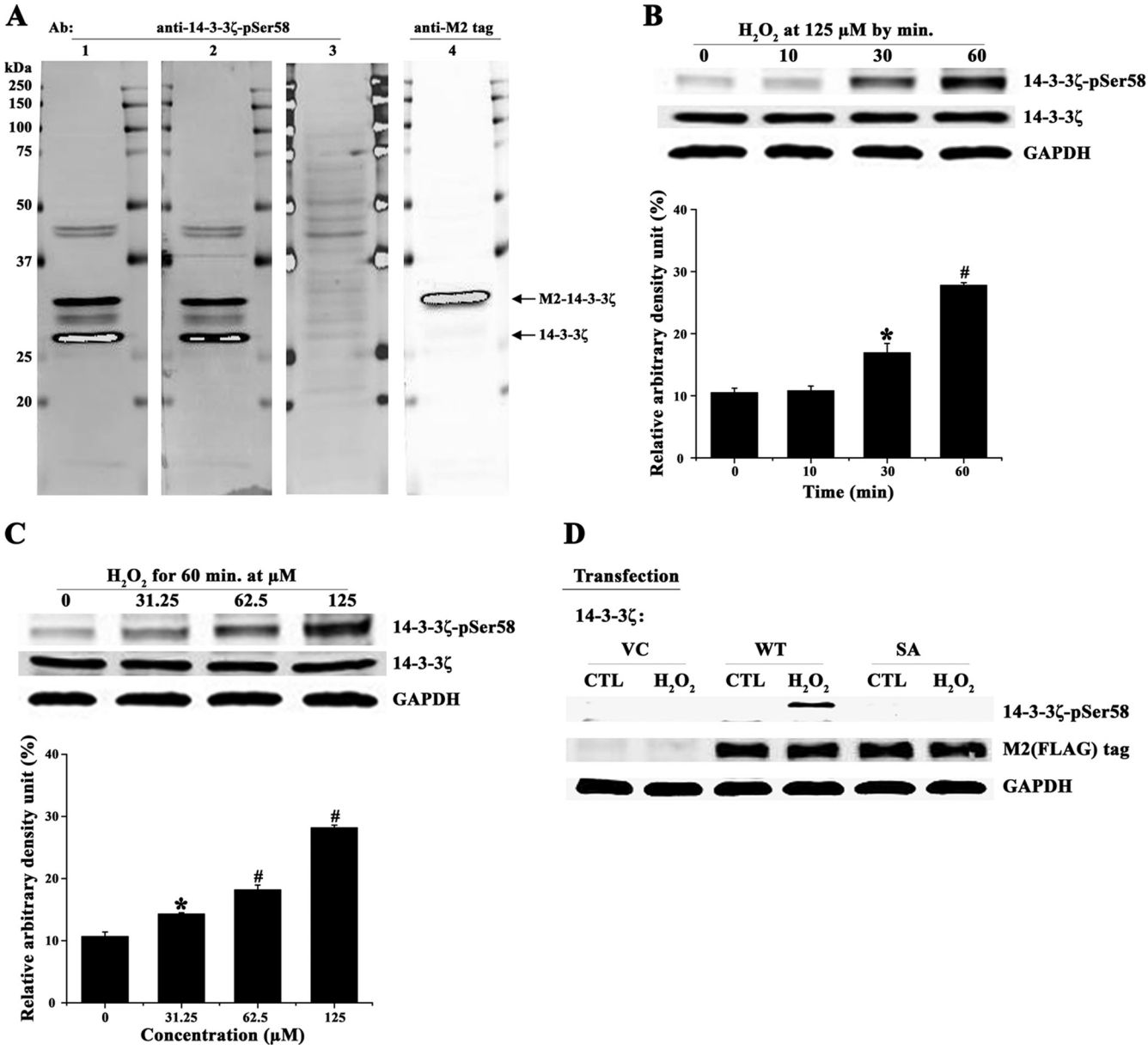
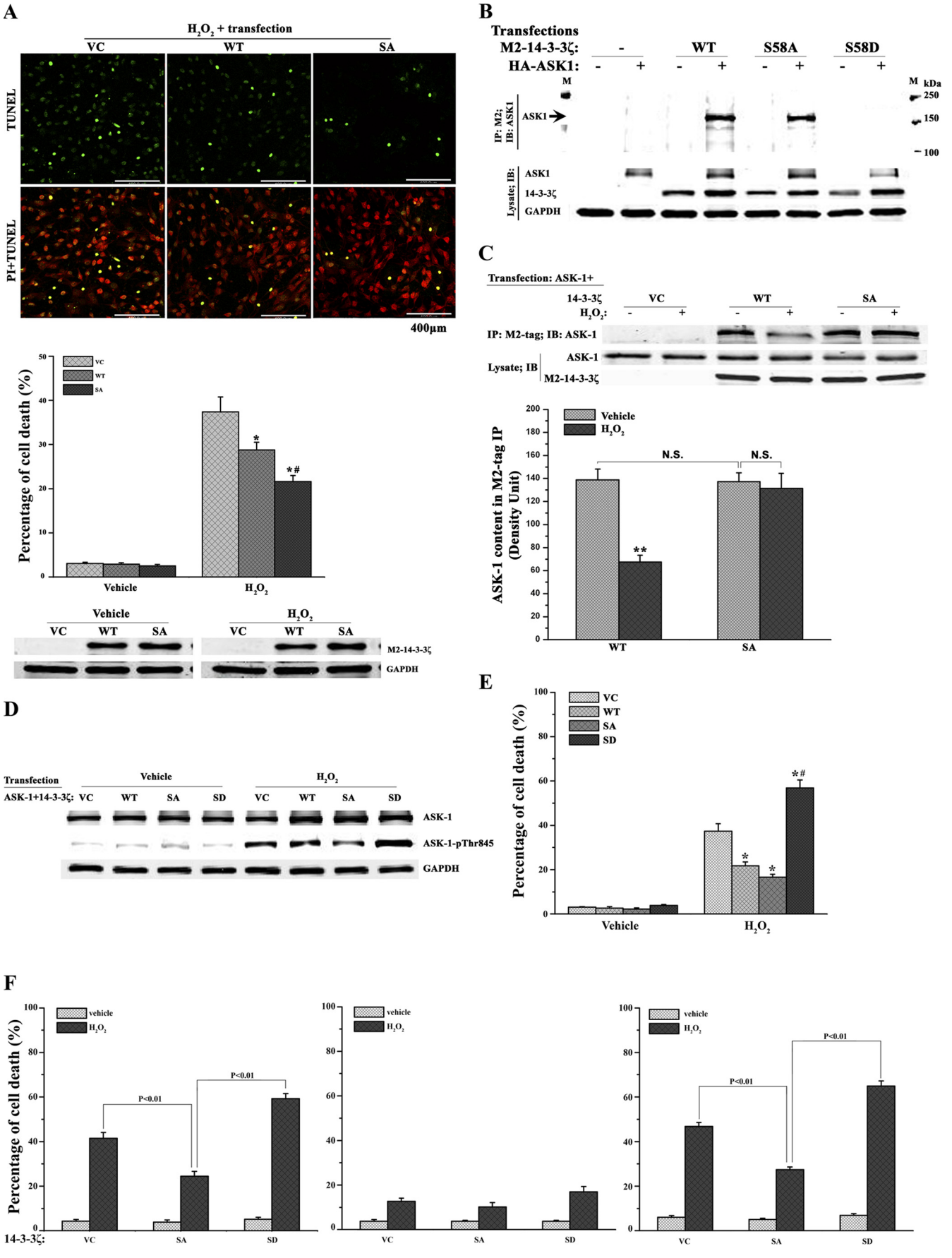


FIG. 1. Phosphorylation of Ser 58 in response to oxidant stress. (A) Peptide competition studies to validate the selectivity of anti-phospho-Ser 58 antibody (Ab). Cells were transfected with vectors harboring WT 14-3-3ζ tagged with HA and M2 epitopes. Twenty-four hours later, the cells were treated with H_2O_2 (125 μM) for 3 h. The lysates were run on SDS-PAGE and then transferred to nitrocellulose. The membranes were then probed overnight with the anti-phospho-Ser 58 Ab in the presence of either no competing peptide (lane 1), unphosphorylated peptide (100 ng/ml) (lane 2), or phosphorylated peptide (100 ng/ml) (lane 3) (phosphopeptide sequence, CGARRSpSerWRVVS). The lysate was also probed with anti-M2 (lane 4) to confirm the location of M2–14-3-3ζ. The location of endogenous 14-3-3ζ is also shown. Incubation with phosphopeptide, but not unphosphorylated peptide, eliminated binding of anti-phospho-Ser 58 Ab. (B) Time course of oxidant stress-induced phosphorylation of Ser 58 of 14-3-3ζ. NIH 3T3 cells were treated with H_2O_2 (125 μM) for the times shown, and then the lysates were blotted with the anti-phospho-Ser 58 Ab, anti-14-3-3ζ, or GAPDH (used throughout as a loading control). Quantitation of three independent experiments is shown below. *, $P < 0.05$ versus time zero; #, $P < 0.01$ versus time zero. The error bars indicate SEM. (C) Dose response of oxidant stress-induced phosphorylation of Ser 58 of 14-3-3ζ. NIH 3T3 cells were treated with H_2O_2 at the concentrations shown for 60 min, and then the lysates were blotted with the anti-phospho-Ser 58 Ab, anti-14-3-3ζ, or GAPDH. Quantitation of three independent experiments is shown below. *, $P < 0.05$ versus 0 μM; #, $P < 0.01$ versus 0 μM. (D) S58 phospho-specific Ab recognizes WT 14-3-3ζ after H_2O_2, but not SA 14-3-3ζ. Cells were transfected with vector control (VC), WT M2–14-3-3ζ, or SA M2–14-3-3ζ and then were exposed to vehicle (CTL) versus H_2O_2. The lysates were immunoblotted with anti-phospho-S58 Ab (top), M2 Ab (middle), or GAPDH Ab (bottom).

ing phosphorylation, and then exposed the cells to H_2O_2. This induced a marked increase in phosphoantibody binding to WT 14-3-3ζ but virtually no binding to SA 14-3-3ζ (Fig. 1D). Thus, oxidant stress triggers S58 phosphorylation, and this phosphorylation can be monitored by the phosphospecific antibody.

We next explored the biological consequences of Ser 58 phosphorylation by inducing oxidant stress in cells expressing


A
H2O2 + transfection
VC
WT
SA
TUNEL
PI+TUNEL
400μm
Percentage of cell death (%)
VC
WT
SA
Vehicle
H2O2
M2-14-3-3ζ
GAPDH
B
Transfections
M2-14-3-3ζ:
HA-ASK1:
WT
S58A
S58D
M
kDa
250
150
100
IP: M2; IB: ASK1
ASK1
Lysate; IB:
14-3-3ζ
GAPDH
C
Transfection: ASK-1+
14-3-3ζ
H2O2:
IP: M2-tag; IB: ASK-1
Lysate; IB
ASK-1
M2-14-3-3ζ
ASK-1 content in M2-tag IP (Density Unit)
N.S.
WT
SA
D
Transfection
ASK-1+14-3-3ζ:
ASK-1
ASK-1-pThr845
GAPDH
E
Percentage of cell death (%)
F
vehicle
P<0.01
14-3-3ζ:

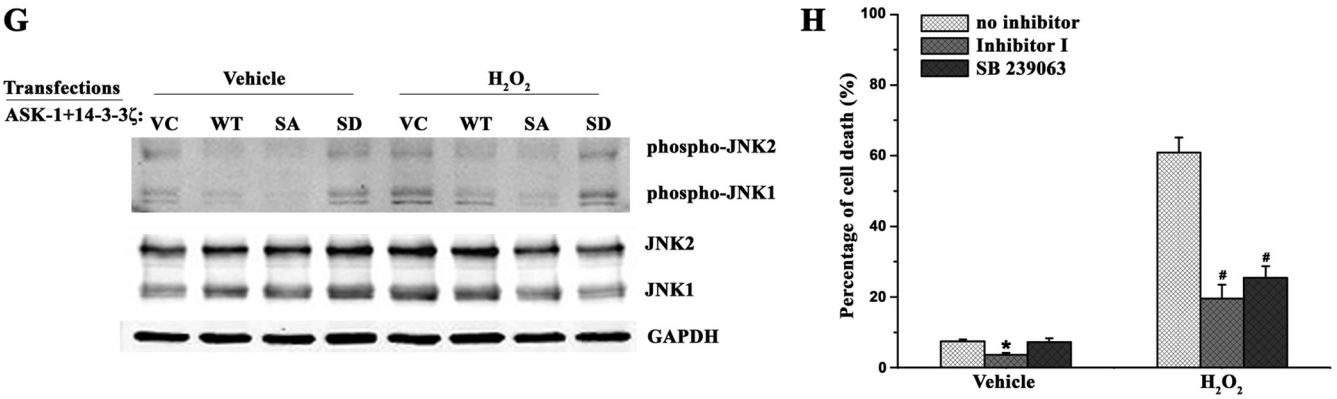


FIG. 2. The Ser 58 phosphorylation status regulates the ASK1/14-3-3ζ interaction, ASK1 activation, and cell survival. (A) Expression of SA 14-3-3ζ protects against oxidant stress-induced cell death. NIH 3T3 cells were transfected with vector (VC), M2-tagged WT 14-3-3ζ, or M2-tagged SA 14-3-3ζ for 24 h. The cells were then exposed to vehicle (H_2O) or H_2O_2 (125 μM) as described in Materials and Methods, followed by staining for TUNEL (top). Quantification of the percent TUNEL-positive cells (middle) demonstrated a reduction in cell death with the WT compared to VC and a more marked reduction with SA 14-3-3ζ. Immunoblotting with anti-M2 antibody confirmed equivalent expression of WT and SA 14-3-3ζ in the experiments (bottom). *, $P < 0.01$ versus VC; #, $P < 0.01$ versus WT. The error bars indicate SEM. (B) The phosphorylation status of 14-3-3ζ regulates the 14-3-3ζ/ASK1 interaction. NIH 3T3 cells were transfected with hemagglutinin (HA)-tagged ASK1 (HA-ASK1 +) or vector (HA-ASK1 −) and with the various M2-tagged 14-3-3 constructs (+) or vector (−) as shown. Twenty-four hours later, the lysates were prepared and then were immunoprecipitated (IP) with M2 antibody (Ab) (pulling down 14-3-3ζ), followed by immunoblotting (IB) with anti-HA Ab (for ASK1). Immunoblots of lysates are shown in below. Note the coimmunoprecipitation of WT and SA 14-3-3ζ with ASK1 (top, lanes 4 and 6 from left) but no coimmunoprecipitation of SD 14-3-3ζ with ASK1 (lane 8 from left), despite comparable levels of expression of SD and WT 14-3-3ζ (bottom, 14-3-3ζ, compare lane 8 to lane 4) and greater expression of SD than SA 14-3-3ζ (bottom, compare lane 8 with lane 6). Thus, mutation of S58 to the phosphomimetic D58 disrupts 14-3-3ζ and ASK1 binding. Molecular mass (M) markers are shown on the right. (C) Oxidant stress induces dissociation of ASK-1 from WT 14-3-3ζ but not from SA 14-3-3ζ. 3T3 cells were transfected with ASK1 and either M2-tagged WT 14-3-3ζ, M2-tagged SA 14-3-3ζ, or vector control (VC). The cells were then treated with H_2O_2 or vehicle (−) for 60 min. The lysates were immunoprecipitated with anti-M2 Ab, and the blots were probed with anti-ASK1. Oxidant stress induced significantly greater dissociation of ASK1 from WT than from SA 14-3-3ζ (compare lanes 4 and 6 from left). Quantification is shown below. **, $P < 0.01$. (D) The phosphorylation status of S58 of 14-3-3ζ regulates the ASK1 activation state. 3T3 cells were transfected with VC versus WT, SA, or SD 14-3-3ζ for 24 h and then were subjected to H_2O_2 (125 μM) for 1 h. The lysates were immunoblotted with an anti-phospho-ASK1 Ab that recognizes phosphorylation of the activation loop Thr residue (T838 in human ASK1 and T845 in mouse ASK1). Expression of SA 14-3-3ζ reduced, and SD 14-3-3ζ enhanced, oxidant stress-induced phosphorylation of T845 (compare lane 6 from left [WT] with lanes 7 and 8). Also note that phosphorylation of ASK1 in SA 14-3-3ζ-transfected cells was less than in VC-transfected cells despite higher levels of expression of ASK1 in SA 14-3-3ζ-transfected cells (compare ASK1 blot lane 5 to lane 7). (E) The serine 58 phosphorylation status regulates oxidant stress-induced cell death. 3T3 cells were transfected with ASK1 and either VC or one of the 14-3-3ζ constructs and then were treated with vehicle or H_2O_2 (125 μM) for an additional 24 h. The percentages of TUNEL-positive cells were then determined. Expression of WT or SA 14-3-3ζ reduced, whereas expression of SD 14-3-3ζ increased, oxidant stress-induced cell death. *, $P < 0.01$ versus VC; #, $P < 0.01$ versus WT and SA 14-3-3ζ. (F) ASK1 is the 14-3-3ζ interactor that is regulated by the S58 14-3-3ζ phosphorylation status and mediates oxidant-induced cell death. (Top) $ASK1^{+/+}$ MEFs were transfected with VC or SA or SD 14-3-3ζ as described above. The cells were then exposed to vehicle (VC) versus H_2O_2 (125 μM), and the percentages of TUNEL-positive cells were determined. In WT MEFs, as in 3T3 cells, SA 14-3-3ζ decreased oxidant stress-induced cell death. (Middle) $ASK1^{-/-}$ MEFs were transfected with VC or SA or SD 14-3-3ζ and then were exposed to vehicle versus H_2O_2 (125 μM). The percentages of TUNEL-positive cells were determined. Deletion of ASK1 nearly abolished oxidant stress-induced cell death, and detrimental effects of SD 14-3-3ζ were not seen in the absence of ASK1. (Bottom) $ASK1^{-/-}$ MEFs were transfected with ASK1 and either VC or SA or SD 14-3-3ζ prior to treatment with H_2O_2 as described above. Reintroduction of ASK1 into the $ASK1^{-/-}$ MEFs restored oxidant stress-induced death, and as in $ASK1^{+/+}$ MEFs, this was significantly reduced by expression of SA but not by expression of SD 14-3-3ζ. (G) Activation of the ASK1 targets, the JNK family of stress-activated kinases, is also regulated by the 14-3-3 phosphorylation status. 3T3 cells were transfected with ASK1 and either VC or WT, SA, or SD 14-3-3ζ. The cells were then treated with vehicle versus H_2O_2 for 1 h. The lysates were immunoblotted with anti-T183/Y185 JNK phosphospecific antibody and with antibodies to total JNK1 and JNK2. Note the lower level of phospho-JNK1 and -JNK2 in WT and SA 14-3-3ζ-transfected cells (top, lanes 6 and 7 from left) compared to VC- or SD 14-3-3ζ-transfected cells (lanes 5 and 8). Thus, as with ASK1, JNK activation is regulated by the S58 phosphorylation status. (H) JNKs and p38-MAPK mediate the increased oxidant stress-induced cell death seen with SD 14-3-3ζ. 3T3 cells were transfected with ASK1 and SD 14-3-3ζ and then were exposed to vehicle versus H_2O_2 (125 μM), either in the presence of the JNK inhibitor peptide (Inhibitor 1; 1 μM), the p38 inhibitor SB239063 (10 μM), or no inhibitor. *, $P < 0.05$ versus no inhibitor; #, $P < 0.01$ versus no inhibitor.

SA versus WT 14-3-3ζ. Expression of either WT or SA 14-3-3ζ partially protected cells from oxidant stress-induced cell death, with expression of SA 14-3-3ζ being more protective (Fig. 2A). Thus, the inability to phosphorylate Ser 58 of 14-3-3ζ reduces oxidant stress-induced cell death.

These findings suggested the possibility that oxidant stress-induced release of a proapoptotic factor from 14-3-3ζ was prevented by the nonphosphorylatable SA mutation. Serine 58 is within a region known to be required for binding to ASK1. Therefore, we coexpressed ASK1 and the various 14-3-3ζ mutants to examine the effects of the phosphorylation status of S58 on the ASK1/14-3-3ζ interaction. ASK1 readily bound to WT and SA 14-3-3ζ, but minimal if any binding to 14-3-3ζ with an S58-to-D (SD) mutation could be detected (Fig. 2B). These data suggest that the phosphorylation status of S58 determines whether ASK1 is sequestered by 14-3-3ζ.

To explore this further, we asked whether the interaction of ASK1 with 14-3-3ζ was dynamically modulated in response to oxidant stress. We found that oxidant stress induced dissociation of ASK1 from WT 14-3-3ζ, but not from SA 14-3-3ζ (Fig.

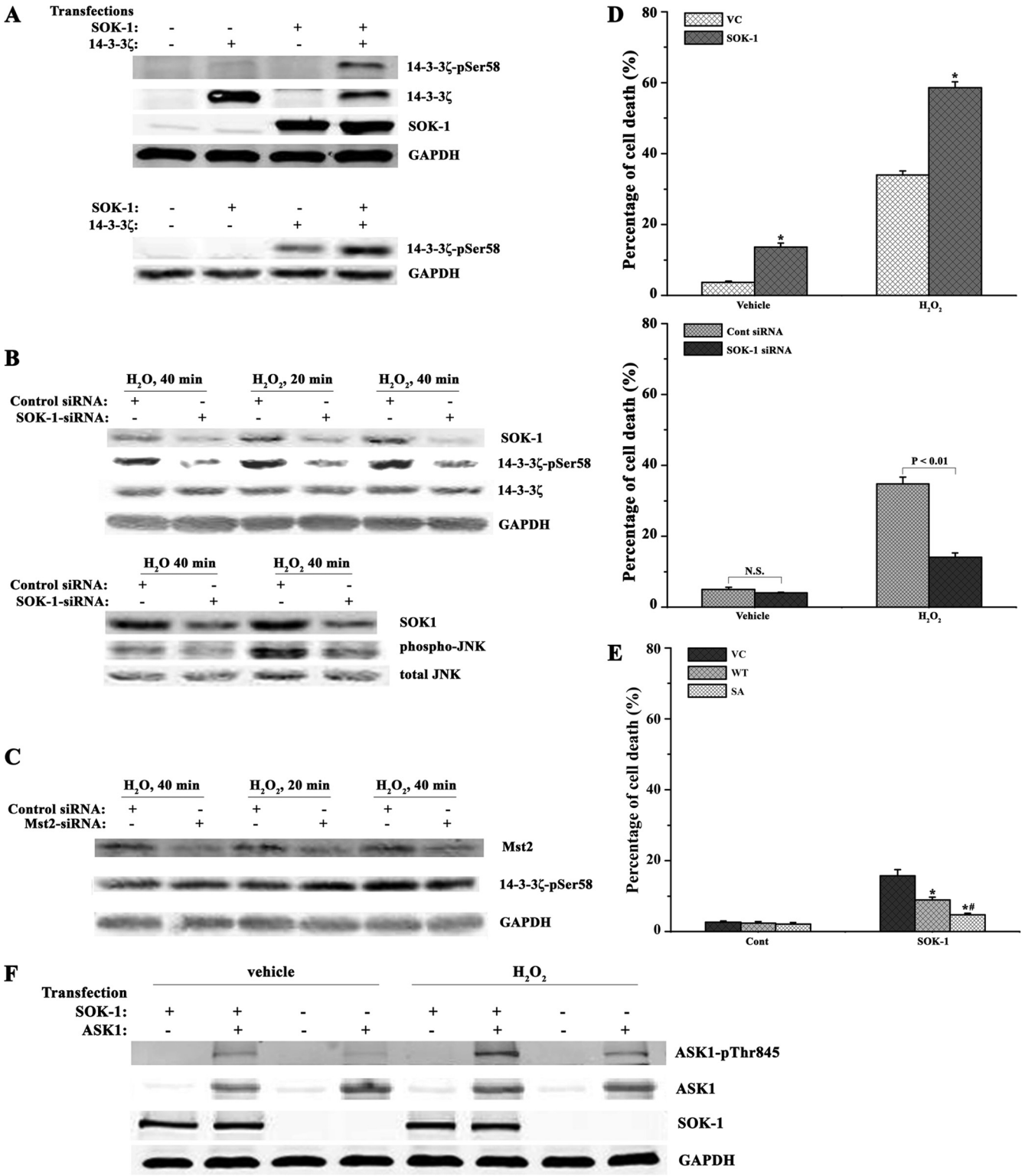


FIG. 3. SOK-1 is a Ser 58 kinase that regulates oxidant stress-induced cell death. (A) Expression of SOK-1 increases phosphorylation of Ser 58 of 14-3-3ζ. (Top) Cells were transfected with SOK-1 (+) or vehicle control (VC) (−) and either WT 14-3-3ζ (+) or VC (−). Twenty-four hours later, cell lysates were prepared and blotted with the anti-phospho-Ser 58 antibody. (Bottom) Cells were transfected as described above. Twenty-four hours later, the cells were exposed to H_2O_2 for 40 min. The lysates were immunoblotted for 14-3-3ζ S58 phosphorylation or GAPDH. (B) SOK-1 is necessary for Ser 58 phosphorylation. (Top) Cells were transfected with an siRNA to SOK-1 or a control siRNA for 48 h and then were treated with H_2O_2 or vehicle. At the times shown, lysates were prepared and immunoblotted for SOK-1, phospho-Ser 58 14-3-3ζ, total 14-3-3ζ, or GAPDH. (Bottom) Cells were transfected with the second SOK-1 siRNA versus control siRNA as described above and then stimulated with H_2O or H_2O_2 for 40 min. The lysates were immunoblotted for SOK-1, phospho-JNK, or total JNK. (C) Mst2 is not necessary for Ser 58

2C). Functionally, the S58 phosphorylation status not only regulates 14-3-3ζ/ASK1 binding, but also regulates ASK1 activity, since expression of SA 14-3-3ζ decreased oxidant stress-mediated phosphorylation of T845 within the activation loop of ASK1, whereas expression of SD 14-3-3ζ increased phosphorylation of T845 (Fig. 2D).

To further examine the biological consequences of S58 phosphorylation, we coexpressed ASK1 with WT, SA, or SD 14-3-3ζ and found that H_2O_2-induced cell death was significantly reduced by expression of SA 14-3-3ζ and was increased by expression of SD 14-3-3ζ (Fig. 2E). The increase in cell death with expression of SD 14-3-3ζ, which was a consistent trend throughout these studies, is consistent with SD acting as not only a loss of function mutation but as a dominant negative, similar to findings by Xing et al. with the R56A/R60A mutant (38).

Many factors bind to 14-3-3ζ, and any of them could be regulated by the S58 phosphorylation status and could account for the regulation of oxidant stress-induced cell death. We therefore examined whether ASK1 was the specific mediator of cell death that was regulated by the S58 phosphorylation status. Consistent with our findings in NIH 3T3 fibroblasts, expression of SA 14-3-3ζ in WT MEFs protected the cells from oxidant stress-induced cell death, whereas expression of SD 14-3-3ζ tended to enhance injury (Fig. 2F, top). In contrast, $ASK1^{-/-}$ MEFs were largely protected against oxidant injury, and expression of SA or SD 14-3-3ζ had little effect on cell death in these cells (Fig. 2F, middle). When ASK1 was transfected into $ASK1^{-/-}$ MEFs, SA 14-3-3ζ was again protective, whereas SD 14-3-3ζ enhanced injury (Fig. 2F, bottom). Thus, regulation of cell death by the phosphorylation status of S58 of 14-3-3ζ appears to be mediated in large part via effects on ASK1.

We next reasoned that if the effect of the Ser 58 phosphorylation status on oxidant stress-induced cell death was mediated by ASK1, then the downstream pathways regulating death might be the JNK family of stress-activated protein kinases, since they play key roles in mediating the proapoptotic effects of ASK1. We found that oxidant stress-induced activation of JNKs was significantly reduced in cells expressing WT 14-3-3ζ and was even more reduced by expression of SA 14-3-3ζ (Fig. 2G). In contrast, JNK activation was not reduced in cells expressing SD 14-3-3ζ (Fig. 2G). Furthermore, a peptide antagonist of JNKs, based on the JNK binding domain of JIP1, significantly decreased death in cells expressing SD 14-3-3ζ (Fig. 2H). Similar results were seen for p38-MAPK, since the small molecule inhibitor SB239063 also significantly protected cells expressing SD 14-3-3ζ (Fig. 2H). These data are consistent with a central role for stress-activated protein kinase signaling downstream of the ASK1/14-3-3 module in oxidant stress-induced cell death.

We then explored kinases that might mediate phosphorylation of S58 in the setting of oxidant stress. As noted, the p38 target, MK2, and protein kinase C δ had been reported to be S58 kinases. However, neither SB239063 nor either of two protein kinase C δ inhibitors, bisindolylmaleimide (Gö6850) and Gö6983, altered oxidant stress-induced phosphorylation of S58 (data not shown).

We then turned to the Mst family of protein kinases, since as noted, one member, SOK-1, was initially identified as a kinase that was activated by oxidant stress and was later reported to phosphorylate S58 of 14-3-3ζ. Consistent with the prior report (26), expression of SOK-1 induced phosphorylation of S58 (Fig. 3A, top). Thus, SOK-1 is sufficient to induce S58 phosphorylation. Furthermore, expression of SOK-1 increased oxidant stress-induced S58 phosphorylation (Fig. 3A, bottom). In addition, siRNA-mediated knockdown of SOK-1 reduced basal phosphorylation of Ser 58 and largely prevented any increase in phosphorylation following H_2O_2 treatment (Fig. 3B, top). SOK-1 knockdown also significantly reduced oxidant stress-induced phosphorylation of JNK, the downstream target of the pathway (Fig. 3B, bottom). In contrast, although Mst2, another Mst family member that is activated by oxidant stress, phosphorylated S58 when overexpressed (data not shown), siRNA-mediated knockdown of Mst2 had no effect on S58 phosphorylation (Fig. 3C). Furthermore, the closely related Mst4 does not phosphorylate S58 of 14-3-3ζ (26), and MstI is expressed at extremely low to undetectable levels in NIH 3T3 cells (2, 3). Thus, of the Mst family members, SOK-1 appeared to be an important S58 kinase in these cells.

Consistent with the SOK-1/14-3-3ζ/ASK1 module being a central mechanism mediating oxidant stress-induced cell death, expression of SOK-1 amplified oxidant stress-induced cell death (Fig. 3D, top) and knockdown of SOK-1 via siRNA significantly reduced H_2O_2-induced cell death (Fig. 3D, bottom). The latter finding was confirmed with a second siRNA to SOK-1 (see Fig. S1 in the supplemental material). That SOK-1 indeed acts via S58 phosphorylation to induce cell death is suggested by the finding that cell death induced by expression of SOK-1 was significantly reduced by coexpression of SA 14-3-3ζ and was partially inhibited by expression of WT 14-3-3ζ (Fig. 3E).

phosphorylation in 3T3 cells. Cells were transfected with Mst2 siRNA or control siRNA and then treated with H_2O_2 or vehicle. The lysates were immunoblotted for Mst2 or phospho-Ser 58 14-3-3ζ. Despite successful knockdown of Mst2, phosphorylation of 14-3-3ζ at S58 was not affected. (D) SOK-1 regulates oxidant stress-induced cell death. (Top) Cells were transfected with SOK-1 or VC and then were exposed to H_2O_2 versus vehicle prior to determination of the percentage of TUNEL-positive cells. Expression of SOK-1 induced cell death and enhanced oxidant stress-induced death. *, $P < 0.01$ versus VC. (Bottom) Cells were transfected with siRNA to SOK-1 versus control siRNA for 48 h, and then were exposed to H_2O_2 versus vehicle. The TUNEL-positive cells were then quantified. Knockdown of SOK-1 reduced oxidant stress-induced cell death. *, $P < 0.01$ versus VC; #, $P < 0.01$ versus control siRNA treated with H_2O_2; N.S., not significant. The error bars indicate SEM. (E) SOK-1-induced cell death is inhibited by 14-3-3ζ. Cells were transfected with SOK-1 versus VC and either WT or SA 14-3-3ζ or VC for 24 h prior to determining the percentage of TUNEL-positive cells. *, $P < 0.01$ versus VC; # $P < 0.05$ versus WT. (F) SOK-1 enhances activation of ASK1. Cells were transfected with SOK-1 (+) versus vector (−) and either ASK1 (+) or vector (−). The cells were then exposed to vehicle versus H_2O_2 for 1 h. The lysates were immunoblotted with anti-phospho-ASK1 (Thr 845). SOK-1 enhanced the oxidant stress-induced phosphorylation of Thr 845 (compare lanes 6 and 8 from left).

To further confirm SOK-1 as a regulator of the 14-3-3/ASK1 module, we cotransfected ASK1 and SOK-1 and then examined the effects of this on ASK1 and JNK activation. We found that SOK-1 expression modestly increased phosphorylation of T845 of ASK1 but more markedly enhanced oxidant stress-induced phosphorylation of T845 (Fig. 3F).

DISCUSSION

ASK1 is a central regulator of oxidant stress-induced cell death. Critical to this is the release of ASK1 from 14-3-3 proteins (38, 43). Although the mechanism driving ASK1 binding to 14-3-3 proteins via S966 phosphorylation was known, signaling mechanisms regulating the release of ASK1 from 14-3-3 proteins were not, despite intense interest in this kinase (8). In this paper, we identify a novel mechanism that regulates oxidant stress-induced cell death. We show that following oxidant stress, the Mst family member SOK-1/YSK1 phosphorylates 14-3-3ζ at S58, thereby disrupting ASK1 binding to 14-3-3ζ, allowing ASK1 activation and inducing cell death.

As noted above, S58 had been known to be phosphorylated in situ for some time, but the mechanisms regulating this and the biological consequences of that phosphorylation were not known until the effects on Golgi apparatus reorganization and cell motility were reported (26). Our studies indicate that a major biological role of S58 phosphorylation is triggering oxidant stress-induced cell death. This is the first direct demonstration of a proapoptotic role and regulation of a proapoptotic factor (ASK1) by S58 phosphorylation.

A large number of proteins bind to 14-3-3 proteins. In fact 14-3-3ζ alone has been demonstrated to interact with at least 20 partners (25, 35). Thus, S58 phosphorylation could disrupt the binding of a number of factors, any of which might be responsible for the cell death seen in the cells expressing SD 14-3-3ζ. Thus, the studies of ASK1$^{-/-}$ MEFs that specifically implicate ASK1 as a key mediator are critical. However, the enhancement of cell death by expression of SD 14-3-3ζ certainly raises the possibility that binding of proapoptotic factors in addition to ASK1 may be regulated by S58 phosphorylation and/or that S58-phosphorylated 14-3-3ζ is not only incompetent to bind ASK1, but cannot effectively dimerize with other 14-3-3ζ molecules, leading to release of ASK1 and other factors, thereby enhancing injury.

Ser 58 of 14-3-3ζ and the sequence surrounding it is broadly conserved across species from *Drosophila* to humans. Furthermore, five of seven 14-3-3 isoforms in mouse, rat, dog, and human have S58 conserved, suggesting that S58 phosphorylation likely plays a role in regulating the interactions of all of these isoforms with client proteins (7). This makes it difficult, if not impossible, to examine the role of S58 phosphorylation of specific 14-3-3 isoforms by employing knockdown strategies, since compensation by other isoforms would be expected unless one isoform is dominant in a particular cell type. Intriguingly, S58 is not conserved in sigma and tau/theta. Rather, these isoforms have an alanine residue in this position. Our findings would predict that these isoforms might constitutively sequester ASK1 and might be particularly protective against oxidant stress. Consistent with this hypothesis, mice with heterozygous deletion of 14-3-3τ, which is normally highly expressed in the heart, were much more susceptible to oxidant stress-induced or ischemia-induced injury, the latter being a setting in which oxidant stress plays a major role in injury (11). In the hearts of 14-3-3τ$^{(+/-)}$ mice, ASK1, JNK, and p38-MAPK activities were increased. We believe our studies now provide a mechanism for these findings.

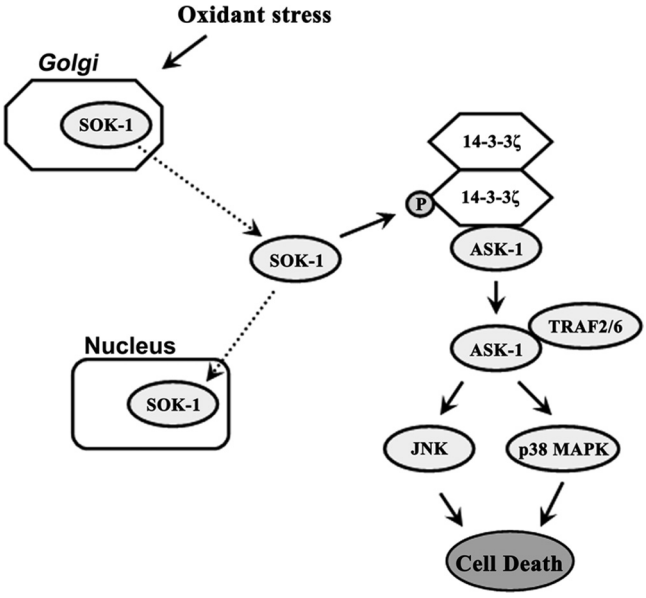


FIG. 4. Schematic of the SOK-1/14-3-3ζ/ASK1 pathway. Following oxidant stress, SOK-1 exits the Golgi apparatus and interacts with the 14-3-3ζ/ASK1 complex, phosphorylating Ser 58 of 14-3-3ζ. This leads to dissolution of the 14-3-3ζ/ASK1 complex, allowing the ASK1 signalosome to form (30), which includes TRAF2 and TRAF6. ASK1 then activates JNKs and p38-MAPKs, culminating in cell death. Also shown is SOK-1 translocation to the nucleus, which has been shown to play a role in chemical-anoxia-induced cell death (19).

The mechanism of oxidant stress-induced cell death that we have identified may be complementary to the MstI/FOXO pathway that has recently been shown to regulate oxidant injury in rat cerebellar neurons (12). In that case, MstI phosphorylated FOXO3a, resulting in the release of FOXO3a from 14-3-3 proteins and induction of the expression of cell death genes. It is very unlikely that the MstI/FOXO mechanism could have played a significant role in our experiments since, as noted, MstI is expressed at very low or undetectable levels in NIH 3T3 fibroblasts (2, 3). That said, the expression patterns of Mst proteins differ, with Mst2, for example, being expressed at very low levels in the heart, lung, liver, and brain, whereas SOK-1 is highly expressed in lung and brain, tissues of the gastrointestinal tract, and, to a somewhat lesser extent, the heart and liver. Thus, since MstI and Mst2 are capable of phosphorylating Ser 58 when overexpressed (data not shown), it is possible that MstI and/or Mst2 could also function as an S58 kinase in cell types in which they are more highly expressed.

How do such different biological responses as Golgi apparatus reorganization and cell death result from activation of the same kinase, phosphorylating the same substrate at the same residue? In nonstressed cells, SOK-1 appears to exist in two pools—an inactive cytosolic pool and an active Golgi pool

(Fig. 4). As noted above, we have reported that exposure of cells to chemical anoxia, a cell culture model of ischemia, leads to exit of SOK-1 from the Golgi apparatus, and this plays an important role in chemical-anoxia-induced cell death (19). We also found that SOK-1 exits the Golgi apparatus in cells exposed to oxidant stress (data not shown). We suggest that the very different biological responses resulting from SOK-1 activation and S58 phosphorylation are critically dependent on the subcellular localization of activated SOK-1 and its target, 14-3-3ζ. Since ASK1 (and the ASK1/14-3-3 complex) is predominantly cytosolic, it appears that SOK-1 may need to exit the Golgi apparatus in order to induce maximal release of ASK1 and cell death. Indeed, regulation of subcellular localization in stressed versus nonstressed cells may be a central mechanism regulating SOK-1 biological functions.

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