Integration of Apoptosis Signal-Regulating Kinase 1-Mediated Stress Signaling with the Akt/Protein Kinase B-IκB Kinase Cascade

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Cellular processes are tightly controlled through well-coordinated signaling networks that respond to conflicting cues, such as reactive oxygen species (ROS), endoplasmic reticulum (ER) stress signals, and survival factors to ensure proper cell function. We report here a direct interaction between inhibitor of κB kinase (IKK) and apoptosis signal-regulating kinase 1 (ASK1), unveiling a critical node at the junction of survival, inflammation, and stress signaling networks. IKK can be activated by growth factor stimulation or tumor necrosis factor alpha engagement. IKK forms a complex with and phosphorylates ASK1 at a sensor site, Ser967, leading to the recruitment of 14-3-3, counteracts stress signal-triggered ASK1 activation, and suppresses ASK1-mediated functions. An inhibitory role of IKK in JNK signaling has been previously reported to depend on NF-κB-mediated gene expression. Our data suggest that IKK has a dual role: a transcription-dependent and a transcription-independent action in controlling the ASK1-JNK axis, coupling IKK to ROS and ER stress response. Direct phosphorylation of ASK1 by IKK also defines a novel IKK phosphorylation motif. Because of the intimate involvement of ASK1 in diverse diseases, the IKK/ASK1 interface offers a promising target for therapeutic development.

Within the intracellular networks that control stress response, cell differentiation, and apoptosis, apoptosis signal regulating kinase 1 (ASK1) plays a pivotal role as a signaling hub (1). ASK1 senses, processes, and transmits various environmental cues to intracellular signaling machinery, impacting both physiological and pathophysiological processes. In response to stress signals, such as reactive oxygen species (ROS) or infectious agents, ASK1 initiates a mitogen-activated protein kinase (MAPK) signaling cascade that ultimately results in activation of MAPKs, jun N-terminal kinase (JNK) and p38, and their corresponding biological outputs. Importantly, pathological signals, including expanded poly-Q-induced endoplasmic reticulum (ER) stress in Huntington’s disease, as well as stress signals in other neurodegenerative diseases, engage ASK1 in the propagation of damage signals. Similarly, a number of other pathological signals, such as ROS, evoke sustained ASK1 activation, which triggers cellular damage in diseases such as cardiac hypertrophy and diabetes. However, how ASK1 activity is neutralized in cells under survival conditions remains to be fully elucidated. ASK1 appears to be regulated by two mechanisms: protein-protein interactions and posttranslational modifications. For example, stress signals, such as ROS, impact ASK1 by triggering reversible binding of thioredoxin and phosphorylation-induced association with 14-3-3 proteins. Thioredoxin, in its reduced form, can bind ASK1, keeping it in an inactive conformation. However, elevated ROS levels lead to oxidized cysteines in thioredoxin, inducing the release of ASK1, recruitment of TRAF2/6 to the kinase, and facilitating ASK1 activation (2). Increased ROS also triggers dissociation of 14-3-3 proteins from ASK1, relieving ASK1 inhibition (3). ASK1 binding to 14-3-3 is mediated by phosphorylated Ser967, which serves as a molecular sensor for signal integration (4). When bound to 14-3-3, ASK1 activity is inhibited, suppressing ASK1-mediated apoptosis. Stress signals reduce this phosphorylation and, subsequently, 14-3-3 binding (3, 4). Similarly, the protein phosphatase calcineurin activates ASK1 through the dephosphorylation of Ser967 (5). Conversely, increased ASK1/14-3-3 binding is correlated with decreasing ASK1 activity and increased cell survival (6, 7). By controlling the phosphorylation status of Ser967, an upstream protein kinase cascade(s) may be able to integrate diverse signaling pathways with ASK1-mediated stress responses.

Here, we report a central node at the junction of survival, inflammation, and stress signaling networks through a direct interaction between ASK1 and the inhibitor of κB kinase (IKK), which reveals a critical mechanism by which IKK neutralizes stress and apoptotic signaling by a transcription-independent mechanism. An inhibitory role of IKK in JNK signaling was previously attributed to the NF-κB induced XIAP and GADD45 in a transcription-dependent manner (8). Discovery of the IKK/ASK1 complex as a novel signaling integration machinery may offer unique opportunities to precisely manipulate disease-evoked stress response through this newly uncovered molecular interaction interface for future therapeutic interventions.

MATERIALS AND METHODS
Reagents. H2O2, epidermal growth factor (EGF), insulin-like growth factor 1 (IGF-1), wortmannin, and PS1145 (all from Sigma), Akt inhibitor,
phosphatidylinositol ether analog, and recombinant Akt1 (all from Calbiochem), LY294002 (Alomone Labs), tumor necrosis factor alpha (TNF-α; BD Pharmingen), recombinant MEK (Cell Signaling), recombinant IKKα (Upstate Cell Signaling Solutions), IKKβ (Invitrogen), Akt (Invitrogen), histone 2B (Sigma), IκB (Abcam), and [γ-32P]ATP (PerkinElmer) were used in supplied solution or reconstituted according to the manufacturer’s instructions.

**Kinase assays.** For Akt kinase assays, recombinant Akt1 (20 ng), immunoprecipitated was added to purified ASK1 C-terminal fragment (0.5 µg) or recombinant histone 2B (0.5 µg) in a kinase buffer (20 mM HEPES, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM diithiothreitol [DTT], 25 mM MgCl). Reactions were started with an ATP mixture consisting of 20 mM cold ATP and 3 µCi of [γ-32P]ATP and carried out for 2 h at 30°C, after which reactions were terminated by boiling in 6X sodium dodecyl sulfate (SDS) sample buffer. Two kinase assay formats were used for IKK: an antibody-based assay to specifically monitor phosphorylated S967 and a radioisotope incorporation assay. (i) For antibody-based IKK kinase assays, active recombinant IKKβ (0.5 µg) was added to immunoprecipitated ASK1 in a kinase buffer (8 mM mopholinepropanesulfonic acid, 200 mM EDTA, 15 mM MgCl) with 100 µM ATP. Kinase reactions were performed at 37°C for 10 to 30 min. All kinase reactions were terminated by boiling in SDS sample buffer. Ser967 phosphorylation was determined by immunoblotting. (ii) For the direct radiolabeling in vitro IKK kinase assay, a purified ASK1 C-terminal fragment (0.5 µg) or recombinant IκB (0.5 µg) was incubated with recombinant IKKβ (5 to 40 ng) in a kinase buffer. Reactions were started with an ATP mixture consisting of 20 mM cold ATP and 3 µCi of [γ-32P]ATP and carried out for 10 min to 2 h, after which reactions were terminated by boiling them in 6X SDS sample buffer. For in vitro kinase assays, samples were run on an SDS-PAGE gel, and [γ-32P]ATP incorporation into substrates was measured by exposure on film and scintillation counting. The total protein levels were assessed by Coomassie blue staining.

**Endogenous ASK1 immunoprecipitation.** COS7 or murine embryonic fibroblast (MEF) cells were grown on 10-cm plates to confluence and harvested with 0.2% NP-40 lysis buffer. Lysates were cleared with 30 µl of 50% slurry of protein G-Sepharose. ASK1 was immunoprecipitated with 5 µl of an ASK1-specific antibody (F-9; Santa Cruz) and 25 µl of a 50% slurry of protein G-Sepharose at 4°C for 4 to 6 h, rotating slowly. Samples were washed 500X of lysis buffer, and beads were isolated by quick centrifugation. The supernatant was discarded, and the process was repeated three times. The protein levels were assessed by immunoblotting.

**Purification of ASK1 C terminus from Escherichia coli.** One colony of bacteria harboring an expression plasmid for gluthathione S-transferase (GST)–ASK1 C-terminal fragment (ASK1K939-T1374) was grown in 500 ml of ZYP-5052 autoinduction medium [1% N-Z-amime AS, 0.5% yeast extract, 50 mM Na₂HPO₄, 50 mM KH₂PO₄, 25 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.2× metals, 0.5% glycerol, 0.05% glucose, 0.2% α-lactose] overnight at 37°C with shaking. The sample was spun down at 14,000 rpm for 15 min, and the pellet was resuspended in 10 ml of 1× phosphate-buffered saline (PBS; 1 M DTT, 100 mM phenylmethylsulfonyl fluoride [PMSF], 5 mM NaCl) and sonicated six times for 30 min each time at 4°C on ice. The sample was centrifuged for 10 min at 14,000 rpm at 6°C. The supernatant was mixed with 2 ml of a 50% slurry of glutathione-Sepharose (GE Healthcare) in 1× PBS and rotated slowly at 4°C for 2 h. Samples were eluted with elution buffer (20 mM reduced glutathione, 0.25 mM PMSF, 5 mM DTT, 0.1% Triton X-100). Samples were concentrated by centrifugation and dialyzed into storage buffer (10 mM HEPES, 150 mM NaCl, 10% glycerol).

**Apoptosis assays.** Caspase activity was evaluated using a CaspACE assay (Promega) according to the manufacturer’s protocol. In brief, DEVD-pNA substrate was used to test the effect of cell lysates on the liberation of p-nitroaniline from the substrate in a colorimetric assay. The caspase-3 specific activity was determined by normalizing each sample to the protein concentration. An annexin V assay was evaluated according to the manufacturer’s protocol (BD Pharmingen). The percentages of annexins V- and 7-aminoactinomycin D (7-AAD)-positive cells were determined by using a Guava flow cytometer using Guava Nexin software.

**Time-resolved FRET.** Cell lysates containing overexpressed proteins were mixed on 384-well black plates. Lysates were serially diluted in a reaction buffer (20 mM Tris-HCl [pH 7.0], 50 mM NaCl, 0.01% NP-40). GST-Terbiurn (HTRF)-conjugated antibody was used to couple GST-IKKβ as a FRET donor. Venus-ASK1 served as a fluorescence resonance energy transfer (FRET) acceptor. Samples were incubated at room temperature for 1 h, and the FRET signal was measured on the Envision spectrophotometer (excitation wavelength, 337 nm; emission wavelength, 320 nm).

**PC12 neurite outgrowth assay.** To induce ASK1ΔN expression, PC12 cells were cultured in Dulbecco modified Eagle medium (DMEM) with 1% horse serum. All cell treatments were carried out for 3 days, after which cells were fixed with 2% paraformaldehyde for 30 min and solubilized with 0.1% Triton X-100 for 20 min. Fixed and solubilized cells were incubated with Alexa Fluor 488-phallloidin (Invitrogen) in 5% bovine serum albumin (BSA) at a final concentration of 1:1,000 overnight. Nuclei were counterstained with 5 µM of Hoechst dye (Invitrogen)/ml. Cells were washed three times with 1× PBS between each step. Fluorescence was measured with an ImageExpress spectrophotometer (Molecular Devices), and neurite outgrowth was quantified by using MetaExpress software (Molecular Devices).

**Statistical analysis.** The data in bar graphs in the figures are presented as means with the standard deviations represented by error bars. Additional information is given in the figure legends.

**RESULTS**

Diverse growth factor-initiated pathways impinge on ASK1 at Ser967. Reversible phosphorylation of ASK1 at Ser967 serves as a sensing mechanism, integrating diverse environmental cues to illicit critical biological responses through ASK1 (3, 6). Stress signals often induce dephosphorylation of Ser967 and promote cell death and, indeed, Ser967 was dephosphorylated upon serum withdrawal (Fig. 1a) and by exposure to ROS, stimuli known to increase ASK1 kinase activity (3, 9, 10). Consistent with this notion, serum withdrawal enhanced ASK1-mediated apoptosis (Fig. 1b).

To reveal pathways that control this molecular sensing system, we examined factors that might enhance phosphorylation of ASK1 Ser967. Reintroduction of serum after serum starvation restored ASK1 Ser967 phosphorylation, leading to the hypothesis that there may be a prosurvival kinase-activating component(s) within serum, resulting in the phosphorylation of Ser967 and, subsequently, 14-3-3 binding and ASK1 inhibition. Correspondingly, serum blocked apoptosis induced by wild-type ASK1 but not an unphosphorylatable ASK1 mutant (S967A) (Fig. 1b), indicating that serum’s prosurvival function occurs at least in part by controlling the phosphorylation status of ASK1 Ser967. In order to identify this kinase component, well-characterized growth factors were tested: EGF, IGF-1, platelet-derived growth factor (PDGF), and transforming growth factor β (TGF-β). Treatment of cells with EGF, IGF-1, and PDGF, but not TGF-β, resulted in marked phosphorylation of Ser967 (Fig. 1c), suggesting that these growth factors play a key role in regulating this event. Further kinetics studies revealed that IGF-1 promoted Ser967 phosphorylation in both a time and dose dependent manner (Fig. 1d and e). Notably, IGF-1 was also able to block ROS-induced dephosphorylation of ASK1 (Fig. 1g), confirming that our findings were relevant under physiological conditions.
Akt/PKB is an upstream kinase for Ser967 of ASK1. IGF-1 is known to induce the activation of Akt/protein kinase B (PKB). Akt has a major prosurvival role in many settings and downregulates ASK1 activity through phosphorylation of Ser83 (9). Similar to Ser83, the region surrounding Ser967 of ASK1 fits nicely within the defined phosphorylation motif of Akt (RXRXXSXX), suggesting the site could potentially be modified by Akt. In order to investigate whether Akt mediates IGF-1 induced Ser967 phosphorylation, constitutively active Akt (Akt\textsuperscript{H9004 PH}) was utilized. Expression of wild-type or Akt\textsuperscript{H9004 PH} resulted in dramatic increase in Ser967 phosphorylation, which is correlated with decreased ASK1 activity as shown with reduced phosphorylation at Thr838 (Fig. 2a).

To complement the results obtained by Akt overexpression, we used a pharmacological approach to examine the role of Akt in Ser967 phosphorylation. The PI3K inhibitors, wortmannin and LY294002, or the specific Akt inhibitor 124005 were used to manipulate Akt activity. Both inhibition of PI3K signaling and direct inhibition of Akt reduced phosphorylation levels of Ser967 (Fig. 2b), confirming a role for Akt in the phosphorylation of this site.

To further examine whether Akt mediates growth factor-evoked Ser967 phosphorylation, cells overexpressing various Akt proteins (wild-type Akt, Akt\textsuperscript{ΔPH}, or Akt KM) were treated with IGF-1. Akt KM is a catalytically inactive Akt mutant (K179M). Although active Akt enhanced phosphorylation of ASK1 Ser967, overexpression of the dominant-negative Akt blunted IGF-1’s ability to increase ASK1 phosphorylation (Fig. 2c). These results suggest a critical role of Akt in bridging the growth factor effects with suppression of ASK1 activity.

Given the evidence that Akt directly phosphorylates BAD and Forkhead transcription factors within 14-3-3 consensus-binding motifs (11–13), it seemed likely that Akt also phosphorylates ASK1 directly. We tested this notion with an in vitro kinase assay. Active, recombinant Akt was utilized to phosphorylate ASK1 as a substrate. Surprisingly, no transfer of phosphate from ATP to Ser967 ASK1 was detected (Fig. 2d). After confirming the activity of recombinant Akt by its ability to phosphorylate the known substrate histone 2B, we came to the conclusion that Akt does not likely phosphorylate ASK1 directly at Ser967.

IKK mediates growth factor and Akt-induced phosphorylation of ASK1. After discovering that Akt is likely to be upstream of the kinase responsible for phosphorylation of ASK1 at Ser967, we...
inhibited Ser967 phosphorylation induced by the constitutively active Akt ΔPH, dominant-negative Akt KM failed to block active IKK-catalyzed Ser967 phosphorylation (Fig. 3g and h), placing Akt upstream of IKK. As shown in Fig. 1, IGF-1 treatment restored Ser967 phosphorylation after serum starvation through activation of a Ser967 kinase. Importantly, inhibition of Akt or IKK reduced the effect of IGF-1 on Ser967 phosphorylation (Fig. 3h). However, active IKK overcame the effect of phosphatidylinositol 3-kinase and Akt inhibitors, restoring phosphorylation of ASK1 at Ser967 (Fig. 3h). Further, IGF-1 treatment showed increased levels of p-ASK1, a marker of IKK activity, indicating that IKK is indeed activated in response to growth factors (data not shown). Together, these data establish a novel signaling axis by which IKK acts downstream of Akt to phosphorylate ASK1 at Ser967.

IKK directly interacts with ASK1. To provide further evidence of cross talk between IKK and ASK1, we examined the possibility of a direct interaction between these two kinases. With a GST-affinity pulldown assay, ASK1 is found in the IKK protein complex (Fig. 3i). Then, we utilized time-resolved fluorescence resonance energy transfer (TR-FRET) technology, which can detect protein interactions within the distance of 100 Å. Indeed, incubation of IKK with ASK1 led to an increase in FRET signal (Fig. 3i). These results indicate that ASK1 and IKK likely interact directly.

The IGF-1/Akt/IKK signaling antagonizes H2O2-induced ASK1 activation. If IKK is directly coupled to ASK1-mediated signaling, stimulation of IKK by survival signals and upstream regulators is expected to counteract ASK1 activation induced by stress signals. To test this model, we examined the role of IKK in the cross talk between IGF-1 and ROS induced-ASK1 pathways. As shown in Fig. 1, H2O2 triggered dephosphorylation of ASK1 at Ser967, which can be reversed by IGF-1. Like IGF-1, the expression of Akt ΔPH, as well as IKK, effectively blocked dephosphorylation of Ser967 by H2O2, supporting a role for Akt and IKK in mediating the IGF-1 effect (Fig. 4a and see also Fig. 3h). Consistent with our model, IGF-1 addition is associated with IKK activation (p-IKK) and inhibition of ASK1 as shown with reduced pThr838 and the downstream pJNK. Similarly, serum starvation reduced pSer967 of ASK1 (Fig. 4b). Because pSer967 mediates 14-3-3 binding, reduced pSer967 should impact 14-3-3 recruitment. Indeed, serum starvation of cells was correlated with the decreased 14-3-3 association with ASK1 (Fig. 4b). Significantly, the overexpression of IKK reversed Ser967 phosphorylation as well as 14-3-3 association with ASK1 (Fig. 4b), keeping ASK1 in an inhibited state as seen with reduced pJNK. Thus, the IGF-1/Akt/IKK kinase cascade supports cell survival in part by suppressing ASK1 activity through enhanced phosphorylation of Ser967, counteracting the action of prodeath and stress signals such as ROS and serum starvation.

IKK suppresses ASK1-mediated neurite outgrowth. To probe the effect of IKK activation on the biological function of ASK1, we utilized an established ASK1 functional assay in PC12 cells. In this PC12 cell system, ASK1ΔN, a constitutively active ASK1 mutant, is induced through the removal of tetracycline to promote neurite outgrowth (18) (Fig. 5a). ASK1-induced neurite outgrowth serves as a biological readout for ASK1 function (Fig. 5a). For our purpose, PC12 cells were treated with TNF-α to activate IKK upon ASK1 induction, and the IKK activity was monitored by NF-κB translocation. IKK activation significantly decreased ASK1-induced neurite outgrowth (Fig. 5a and b). Furthermore, treatment of cells with the IKK inhibitor PS1145 restored ASK1-induced neurite outgrowth to control levels. These
data together suggest that activation of IKK can suppress ASK1-mediated function.

**IKK inhibits ASK1-mediated apoptosis in a Ser967-dependent manner.** ASK1 is a critical mediator of apoptosis signaling and, by inducing phosphorylation at Ser967, IKK would inhibit ASK1-mediated apoptosis. To test this hypothesis, COS7 cells were transfected with ASK1 to induce apoptosis with or without IKK. Caspase-3 protease activity was monitored as the readout for apoptotic cell death, and phospho-Ser967 levels in cell lysates were monitored by Western blotting. Consistent with a prosurvival role of IKK, expression of IKK inhibited ASK1-in-
The Ser/Thr protein kinases, IKK, are critical mediators of the NF-κB signaling axis (17, 23, 24). Diverse environmental signals can trigger the activation of IKK through various transmembrane receptors such as TNF-α receptors, Toll-like receptors, and the IGF-1 receptor (17). Although TNFR1 is ubiquitously expressed, TNFR2 expression is more limited (21). Previous research has shown, however, that TNF-α can promote cell survival in PC12 cells through TNFR2 (22). In our model, TNF-α may be preferentially activating TNFR2, leading to the activation of IKK and subsequent ASK1 inhibition. Previous studies have also indicated that TNF-α signaling through TNFR2 can lead to JNK inhibition. The mechanism by which this occurs, however, is poorly understood. Our data suggest that the ASK1/IKK signaling node may be responsible for this, highlighting a novel pathway by which IKK promotes cell survival.

Considering the critical roles of both ASK1 and IKK in a range of diseases, the functional interaction between IKK and ASK1 has significant mechanistic implications and offer promising therapeutic potentials. For example, the enhanced generation of ROS that activates ASK1 has been associated with the toxic action of amyloid-β in Alzheimer’s disease and angiotensin II-induced cardiac hypertrophy and injury (25, 26). Our demonstration that IKK-mediated signaling blunts ROS-induced ASK1 activation suggests a possible neuroprotective mechanism of the IKK-ASK1 interaction. In a similar vein, IKK-ASK1 association may counteract the action of ER stress-induced, ASK1-mediated neuronal cell death, as in poly-Q diseases, including Huntington’s disease and spinocerebellar ataxias (27). Blocking ASK1 activity by IKK activation may alleviate the effect of poly-Q-induced ER stress. On the other hand, ASK1 also mediates apoptotic cell death triggered by a number of stimuli, including cytokines, ROS, ER stressors, infectious agents, and cancer chemotherapeutics (1). Due to its physiological importance, ASK1’s activity is tightly controlled. One critical control mechanism is through the reversible phosphorylation of Ser967, which senses the activation state of upstream protein kinases and dictates the docking of the 14-3-3 effector protein for ASK1 inhibition. Our results revealed IKK as an upstream kinase that controls phosphorylation of Ser967, constituting a new node in this complex of signaling networks. Even though we only presented the evidence for the importance of the IGF-1/Akt/IKK cascade in the regulation of ASK1, it is envisioned that other signals that can lead to IKK activation may be able to impact the ASK1-mediated JNK and p38 signaling in a wide range of biological cellular context. Thus, the IKK/ASK1 association may serve as a central integration mechanism for diverse, opposing signaling pathways, such as functional interactions between IKK-transmitted growth factor, cytokine, and pathogenic signals and ASK1-transmitted stress signals, including ROS and ER stress.

Although ASK1 and IKK can be activated by TNF-α, ASK1 is primarily activated through TNFR1 signaling (19), whereas IKK can become activated in response to TNFR1 and TNFR2 signaling (20). Although TNFR1 is ubiquitously expressed, TNFR2 expression is more limited (21). Previous research has shown, however, that TNF-α can promote cell survival in PC12 cells through TNFR2 (22). In our model, TNF-α may be preferentially activating TNFR2, leading to the activation of IKK and subsequent ASK1 inhibition. Previous studies have also indicated that TNF-α signaling through TNFR2 can lead to JNK inhibition. The mechanism by which this occurs, however, is poorly understood. Our data suggest that the ASK1/IKK signaling node may be responsible for this, highlighting a novel pathway by which IKK promotes cell survival.
have direct impact on tumor response to therapeutic agents. Indeed, tumor resistance to a number of anticancer agents, such as cisplatin and taxol, has been associated with the upregulated Akt and IKK function, which may be in part due to the enhanced IKK-ASK1 interaction and the reduced ASK1 response. Thus, the IKK-ASK1 interaction may provide a molecular target for potential therapeutic interventions. It is envisioned that lowering the IKK inhibitory effect on ASK1 by an IKK-ASK1 protein-protein interaction inhibitor may sensitize tumor cells to therapeutic agent-induced ASK1 activation and cell death to enhance therapeutic efficacy. Such a strategy may be particularly promising in many solid and hematological malignancies where the IKK complex is dysregulated (33).

Although only a few targets of IKKα and IKKβ have been confirmed outside the NF-κB signaling pathway, such as DOK1, FOXO3a, and TSC1, these IKK substrates indeed play pivotal roles in the functional effects of this kinase family (32–36). Our discovery of ASK1 as an IKK substrate, through a novel phosphorylation motif around Ser967, immediately widens the reach of IKK to the critical regulation of the cell’s response to diverse stress signals, such as ROS and ER stress. In this way, the IKK/ASK1 signaling node may serve as a critical integration point that mechanistically dictates the flow of conflicting pathways within stress response networks, allowing growth factor-activated IKK to counter ROS-triggered ASK1 activation and apoptosis.

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