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Mary Puckett Date

Protein-Protein Interactions Regulating ASK1 Function

By

Mary Puckett
Doctor of Philosophy

Graduate Division of Biological and Biomedical Science
Molecular and Systems Pharmacology

_____

Haian Fu, Ph.D.
Advisor

_____

Kathy Griendling, Ph.D.
Committee Member

_____

John Hepler, Ph.D.
Committee Member

_____

Fadlo Khuri, M.D.
Committee Member

_____

Rita Nahta, Ph.D.
Committee Member

Accepted:

_____

Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____

Date

Protein-Protein Interactions Regulating ASK1 Function

By

Mary Puckett
B.S., Georgia Institute of Technology, 2006

Advisor: Haian Fu, Ph.D.

An abstract of a dissertation submitted to the Faculty of the
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Abstract

Protein-Protein Interactions Regulating ASK1 Function

By Mary Puckett

Cancer is characterized in large part by aberrant cell survival. Other diseases, such as Parkinson's disease, Huntington's disease, and other neurodegenerative disorders, are characterized by excessive cell death. Regulation of the balance between cell survival and cell death is critical for the proper functioning of every cell in the body. Many proteins function together to maintain this balance, including the apoptosis signal-regulating kinase 1 (ASK1) and its binding partners. Here we describe two distinct mechanisms by which ASK1 function is regulated. ASK1 interacts with ASK2 via distinct regions in the C-termini of both proteins and disruption of this interaction leads to decreased ASK1-mediated signaling. Additionally, ASK2 can act to inhibit ASK1 by promoting ASK1 interaction with 14-3-3 proteins. Upon inhibitory phosphorylation, ASK2 can facilitate ASK1/14-3-3 interaction, and knockdown of ASK2 reduces ASK1 binding to 14-3-3.

ASK1 is also regulated by Inhibitor of κB kinase (IKK) via a unique region of its NEMO binding domain. Overexpression or activation of IKK reduces ASK1 mediated signaling, and disruption of ASK1/IKK interaction, as well as inhibition of ASK1 phosphorylation by IKK, increases ASK1-mediated apoptosis. Taken together, these results shed light on the intricate regulation of cell death and survival signaling mediated by ASK1. Understanding these protein-protein interactions may serve as a starting point for the development of ASK1-directed therapeutics.

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List of Abbreviations

ABIN-1	A20 binding inhibitor of NF-κB 1
AIP-1	ASK1 Interacting Protein 1
APP	Amyloid Precursor Protein
ALS	Amyotrophic Lateral Sclerosis
ASK	Apoptosis Signal-regulating Kinase
BAFF	B-cell Activating Factor
CA	Constitutively Active
CBP	CREB Binding Protein
CC	Coiled Coil
CYLD	Cylindromatosis protein
DD	Death Domain
Dok1	Docking protein 1
EAE	Experimental Autoimmune Encephalomyelitis
EDA-ID	Ectodermal Dysplasia - Immunodeficiency
EGF	Epidermal Growth Factor
ER	Endoplasmic Reticulum
ERK	Extracellular-signal Regulated Kinase
GADD45	Growth Arrest and DNA Damage 45
GPCR	G-Protein Coupled Receptor
GST	Glutathione S Transferase
HLH	Helix Loop Helix
IGF-1	Insulin-like Growth Factor 1

IκB	Inhibitor of κB
IKK	IκB Kinase
IL	Interleukin
IP	Immunoprecipitation
IR	Ischemia-Reperfusion
IRAK-1	IL-1 Receptor Associated Kinase 1
IRE-1	Inositol Requiring Enzyme 1
IRF	Interferon Regulatory Factor
IRS	Insulin Receptor Substrate
JAK2	Janus Kinase 2
JNK	Jun N-terminal Kinase
KD	Kinase Dead
LPS	Lipopolysacchride
LTβR	Lymphotoxin β Receptor
LZ	Leucine Zipper
MAPK	Mitogen Activated Protein Kinase
MAP2K	MAPK kinase
MAP3K	MAP2K kinase
MAP4K	MAP3K Kinase
MCRS2	Microspherule Protein 2
MDM2	Mouse Double Minute 2
MEK	MAPK/ERK Kinase
MEKK	MEK Kinase

MLK	Mixed Lineage Kinase
MNK	MAPK interacting kinase
MPK38	Murine Protein serine/threonine Kinase 38
MS	Multiple Sclerosis
MSK	Mitogen and Stress-activated Kinase
mTOR	Mammalian Target of Rapamycin
NBD	NEMO Binding Domain
NEMO	NF-kB Essential Modulator
NF-κB	Nuclear Factor κB
NIK	NF-κB Inducing Kinase
OA	Osteoarthritis
PAMP	Pathogen Associated Molecular Pattern
PCA	Protein Complementation Assay
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PDK1	3-Phosphoinositide-Dependent protein Kinase 1
PERK	Proline-rich Extensin-like Receptor Kinase
PI3K	Phosphatidylinositide 3-Kinase
PKB	Protein Kinase B
PP	Protein Phosphatase
PPI	Protein-Protein Interaction
RA	Rheumatoid Arthritis
RHD	Rel Homology Domain

RIP1	Receptor Interacting Protein
ROS	Reactive Oxygen Species
RSK	Ribosomal S6 Kinase
SAPK	Stress Activated Protein Kinase
SDD	Scaffold/Dimerization Domain
SHP2	Src Homology-2 domain-containing Phosphatase 2
SNAP-23	Synaptosomal-Associated Protein 23
SOD1	Superoxide Dismutase 1
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrlyamide Gel Electrophoresis
STRAP	Serine-Threonine Kinase Receptor-Associated Protein
TAK1	TGFβ Activated Kinase 1
TBK1	TANK Binding Kinase 1
TGFß	Transforming Growth Factor β
TLR	Toll-Like Receptor
TNFα	Tumor Necrosis Factor α
TNFR	TNF Receptor
TRADD	TNF Receptor Associated Death Domain protein
TRAF	TNF Receptor-Associated Factor
TR-FRET	Time Resolved-Fluorescence Resonance Energy Transfer
TSC1	Tuberous Sclerosis 1
ULD	Ubiquitin Like Domain
UPR	Unfolded Protein Response
XIAP	X-linked inhibitor of Apoptosis Protei

Chapter 1: Introduction

1.1. Protein-protein interactions

Protein-protein interactions (PPIs) are at the heart of all cellular processes. Both intracellular and extracellular signaling pathways regulate these interactions in order to ensure proper cell function. Protein-protein interactions can consist of enzyme-substrate, receptor-ligand, and many other interactions that modulate protein activity (1). Proteins can form both homologous and heterologous protein complexes, both of which play important roles in cell function (2). Understanding the interactions of proteins can help to unravel the complicated signaling networks that regulate cell function (3).

Because of their importance in cell function, proteins are the major target for therapeutics. While most drugs targets are receptors due to their exposure on the cell surface, receptor signaling is not the only method proteins have for manipulating cell function, and many intracellular protein-protein interactions are becoming of interest as drug targets (4). Though protein-protein interactions have typically been thought of as seemingly impossible drug targets due to their large interaction interfaces and the flatness of these interactions, which makes disruption of interactions with a small molecule difficult, strides are being made in the development of protein-protein interaction modulators (4; 5). While some protein-protein interactions have been successfully inhibited with antibodies (6; 7) or small peptides (8), these types of drugs are typically costly to produce and administer to patients. Some successful small molecule inhibitors of protein-protein interactions, however, have been developed, suggesting that targeting these interactions may be a viable method for developing a wide array of therapeutics (4).

Identifying new protein-protein interactions and understanding these protein interfaces, as well as the signaling mechanisms that regulate them, could lead to the development of novel therapies for a variety of diseases.

The apoptosis signal regulating kinase 1 (ASK1), a mitogen-activated protein kinase kinase kinase (MAP3K), serves as a critical regulator of both intracellular and extracellular stress signals. ASK1 function is tightly regulated by protein-protein interactions. By better understanding how ASK1 is controlled by such PPIs, we may shed light on its role in both normal physiology and disease. My research focuses on two protein-protein interactions of ASK1 that regulate its function: ASK2 and inhibitor of κB kinase (IKK).

1.2. Mitogen-activated protein kinase signaling

Cells are constantly bombarded with a variety of extracellular and intracellular signals and must properly interpret these signals in order to propagate a desired cellular result. One way in which cells interpret and propagate these signals is through Mitogen Activated Protein Kinase (MAPK) signaling cascades. These signaling cascades consist of a terminal MAPK as well as upstream kinases, referred to as MAPK Kinases (MAP2Ks) and a MAP2K Kinases (MAP3Ks) that, in response to particular cues, activate a specific signaling cascade leading to the activation of a specific MAPK: Extracellular-signal Regulated Kinase (ERK), Jun N-terminal Kinase (JNK), or p38 (9). Several MAP3K kinases (MAP4Ks) have also been identified, though the precise role they play in MAPK signaling is less understood than that of downstream kinase components (10).

The MAPK signaling cascades sense and integrate a wide variety of extracellular stimuli to elicit an appropriate response. The three MAPK families ERK, JNK, and p38 each consist of multiple members, which help fine tune cellular responses (11). The ERK family consists of ERK1 and ERK2, which share many overlapping functions. ERK is activated by a Ras-Raf-MAPK/ERK Kinase (MEK) signaling cascade in response to growth factors, as well as cytokines and G-protein coupled receptor (GPCR) ligands. Activation of ERK can lead to the phosphorylation of multiple substrates including transcription factors such as Elk1, c-Fos, and c-Myc, as well as other proteins such as MAPK interacting kinase (MNK), mitogen and stress activated kinase (MSK), and ribosomal S6 kinase (RSK). Due to its wide array of substrates, activation of ERK leads to a variety of responses, including proliferation, cell survival, differentiation, and even cell motility (12).

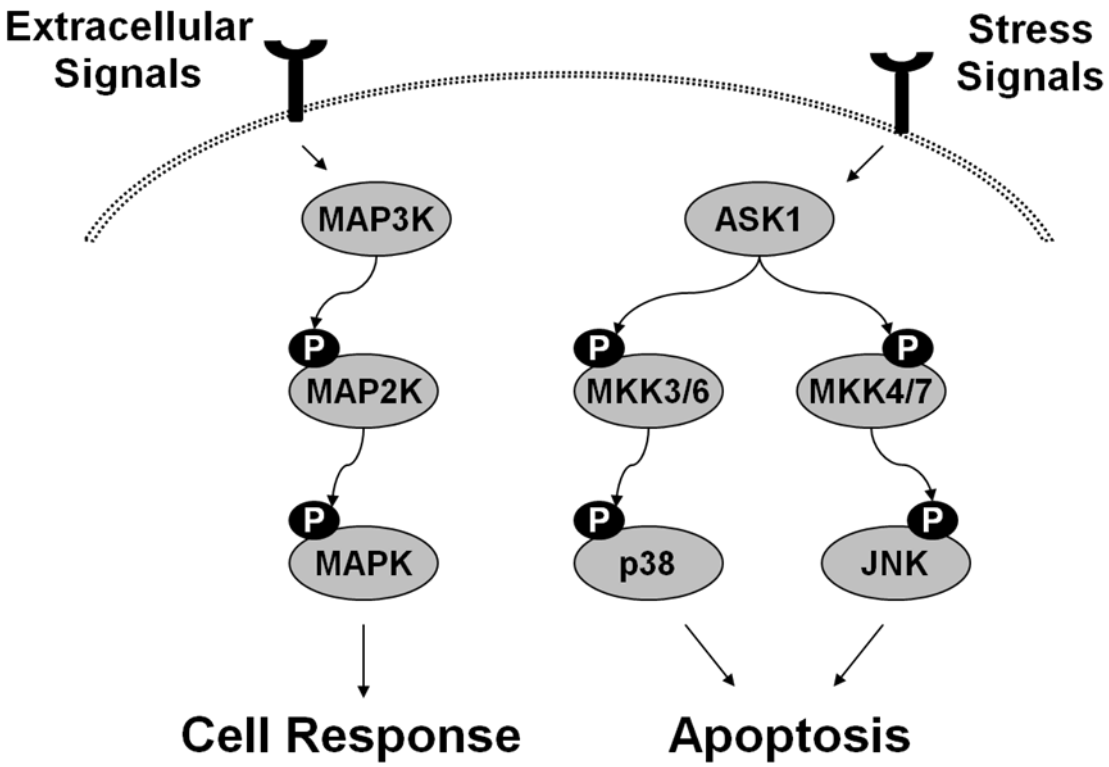
While ERK is predominantly activated by cell survival signaling, the MAPK JNK responds to a wide variety of stress signals. Three isoforms of JNK exist: JNK1, JNK2, and JNK3, all of which can exist in short and long splice variants and have differential expression in various tissue types. JNK, also referred to as the stress activated protein kinase (SAPK), is activated by oxidative stress, ultraviolet radiation, and pro-inflammatory cytokines, among other stimuli. JNK is directly activated by the MAP2Ks MKK4 and MKK7 in response to multiple MAP3Ks including MEKK, mixed lineage kinase (MLK), and ASK family kinases. Once activated, JNK can also phosphorylate multiple substrates including transcription factors such as c-Jun and JunB, as well as Bcl2 family members, leading to apoptosis, cell differentiation, or inflammation and other cellular responses (13).

Much like JNK, the four isoforms of the p38 family (α, β, γ, δ) predominantly respond to stress signals such as pro-inflammatory cytokines, ultraviolet radiation, and hypoxia. p38 is activated directly by the MAP2Ks MKK3 and MKK6 and can phosphorylate a number of substrates including transcription factors such as ATF1/2 and p53. p38 can also indirectly regulate transcription factor phosphorylation by activating kinases like MSK1/2, which can in turn itself phosphorylate transcription factors such as CREB, ATF1, and p65. Because p38 can activate a wide variety of substrates, cellular responses to p38 activation are quite varied and range from inflammation and differentiation to tissue homeostasis and immune response (14). My current dissertation research focuses on an upstream regulator of both JNK and p38 in response to stress signals, ASK1.

1.3 ASK1 function and regulation

Considering only three MAPK families have been identified, the specific MAP3K/MAP2K/MAPK interactions are critical in the proper transduction of signals. In particular, ASK1 functions as a MAP3K, which activates the MAP2Ks MKK4/7 or MKK3/6, leading to the activation of the MAPKs JNK and p38, respectively (Figure 1-1). Structurally, ASK1 is a 1374 amino acid protein in humans consisting of a kinase domain (residues 670-938) flanked by N- and C-terminal regulatory domains (1-669 and 939-1374, respectively). ASK1 orthologs have been identified in both Caenorhabditis elegans and Drosophila melonagaster (15), highlighting its importance throughout evolution.

Figure 1.1. General MAPK/ASK1 signaling. In response to stimuli, a MAP3K phosphorylates a MAP2K which in turn phosphorylates the corresponding MAPK(s), leading to a cellular response. In response to stress signals such as TNFα, ROS or ER stress, ASK1 phosphorylates MKK4/7 or MKK3/6 which in turn phosphorylate JNK or p38, respectively. Activation of JNK or p38 by ASK1 can lead to a variety of cellular responses, including inflammation, senescence, differentiation, and, most notably, apoptosis.


Extracellular Signals
Stress Signals
MAP3K
ASK1
P
MAP2K
P
MKK3/6
P
MKK4/7
P
MAPK
P
p38
P
JNK
Cell Response
Apoptosis

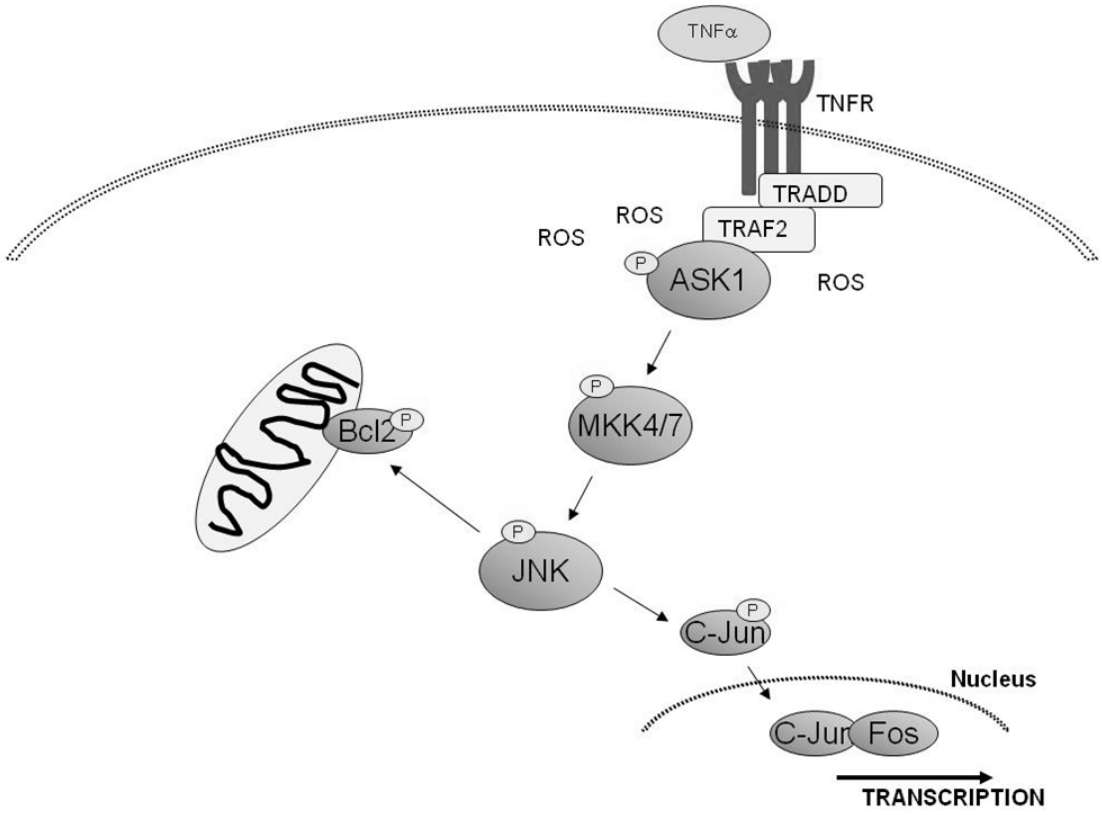
1.3.1 ASK1-mediated apoptosis

Upon the initial discovery of ASK1, it was determined that ASK1 preferentially activates the MAPKs JNK and p38 but not ERK and that this activation leads to apoptosis (16). These ASK1 signaling cascades are activated in response to stress signals, including pro-inflammatory cytokines such as tumor necrosis factor α (TNFα), reactive oxygen species (ROS), endoplasmic reticulum (ER) stress, lipopolysacchride (LPS), and calcium influx (17).

While the cytokine TNFα both positively and negatively regulates cell survival, it can initiate apoptotic signaling through the activation of ASK1 (Figure 1.2). When TNFα binds the TNF Receptor (TNFR), the receptor can then trimerize with other TNFRs. This oligomerization brings the death domains (DDs) of the receptors close enough together to facilitate binding of the TNF receptor I-associated death domain protein (TRADD) (18). TRADD, in turn, promotes binding of TNF receptor associated factors (TRAFs), which interact with ASK1 and facilitate an active conformation of the kinase, leading to activation of downstream kinases and the initiation of apoptosis (19).

In addition to signaling by TNFα, ASK1 can be activated in response to endoplasmic reticulum (ER) stress. The ER normally functions to promote calcium homeostasis, lipid biosynthesis, and protein secretion (20). When misfolded proteins are present in the ER, the cell will initiate a process termed the unfolded protein response (UPR) in an attempt to restore proper ER function and promote cell survival (21; 22). To accomplish this, ER protein synthesis is decreased, and transcription of genes that promote proper protein folding and degradation of misfolded proteins is increased, and permanently misfolded proteins are shuttled to the cytoplasm for degradation by the

Figure 1.2. Activation of ASK1 by TNFα. In response to binding of TNFR by TNFα, ASK is recruited to the receptor and activated by TRAF2 binding. ASK1 then phosphorylates MKK4/7, which in turn can phosphorylate and activate JNK. JNK can then phosphorylate a variety of substrates, including Bcl2 family members and the transcription factor c-Jun to regulate cellular response.


TNFα
TNFR
TRADD
ROS
ROS
TRAF2
ROS
P
ASK1
P
MKK4/7
Bcl2
P
P
JNK
P
C-Jun
Nucleus
C-Jun
Fos
TRANSCRIPTION

proteosome. If this process fails however, these protein aggregates can overwhelm ER folding and degradation machinery, leading to further ER stress.

When the ER cannot properly degrade the misfolded proteins, an apoptotic cascade that relies on ASK1 is initiated (21). ASK1 is activated through an indirect interaction with the ER transmembrane protein, the inositol requiring enzyme 1 (IRE-1). TRAF2, a facilitator of ASK1 activation, is recruited to IRE-1, which in turn leads to ASK1 recruitment and activation (23). This, in turn, leads to activation of JNK, which promotes activation of pro-apoptotic Bcl-2 family members and inhibition of anti-apoptotic members (24; 25).

Another well studied activator of ASK1 is ROS. ROS are produced as part of normal physiological processes. While excess ROS in the cell are usually neutralized by native antioxidants, ROS can overwhelm the cell under stress conditions (26). ASK1 is kept in an inactive state in part by interaction with thioredoxin through oxidizable cysteine bonds. Elevated ROS levels can oxidize these bonds, removing thioredoxin's inhibition of ASK1. When this occurs, ASK1 is activated and initiates apoptosis through the intrinsic, mitochondrial dependent apoptotic pathway.

Interestingly, ROS appear to be a secondary mediator of ASK1 activation in response to a variety of primary inputs. LPS induced activation of ASK1 requires ROS production (27). Likewise, TNFα induced activation of ASK1 appears to be dependent on the production of ROS, indicating ROS may be the predominant factor in activating ASK1 (28). Whether ROS are required for ER stress induced activation is less clear (29). Nevertheless, knockout of ASK1 renders cells insensitive to TNFα, ROS, and ER stress induced cell death, and overexpression of ASK1 mutants with impaired negative

regulation have been shown to increase levels of apoptosis, indicating ASK1 plays a critical role in stress induced apoptosis (30).

1.3.2 Non-apoptotic functions of ASK1

While ASK1 is most known for its role in promoting apoptosis, activation of the kinase can have additional functions, depending on cell context. In addition to apoptosis, ASK1 can promote senescence or cell differentiation and has been shown to play a significant role in the innate immune response (31). In relation to ASK1's role in senescence, expression of a constitutively active form of ASK1 lacking its N-terminal regulatory domain promoted endothelial cell senescence in the presence of high glucose (32). Additionally, ASK1 induced differentiation has been noted in multiple studies. In particular, angiotensin II has been shown to induce cardiac hypertrophy by generating ROS to activate ASK1 (33), and transient activation of JNK and p38 by ASK1 has also been shown to promote differentiation of erythroid cells (34). In addition, ASK1 may play a role in the differentiation of keratinocytes in response to stress signals (35).

Furthermore, ASK1 has a significant role in the innate immune response. Certain pathogen associated molecular patterns (PAMPs), including LPS and peptidoglycan, can lead to ASK1 activation. When ASK1 is knocked out, the production of pro-inflammatory cytokines in response to PAMPs, particularly through TLR4, is reduced. ASK1 knockout mice were shown to be resistant to septic shock from LPS exposure (27), suggesting ASK1 activation is important for immune response to these stimuli.

In addition to eliciting multiple cellular responses through the activation of MAPK signaling, recent studies have revealed ASK1 may have substrates outside of this pathway, including 3-Phosphoinositide-Dependent protein Kinase 1 (PDK1) and Serine-

Threonine Kinase Receptor-Associated Protein (STRAP), both of which reciprocally regulate ASK1 activity by phosphorylation (36; 37). ASK1 has also been shown to phosphorylate $p21^{Cip1}$, a protein that normally functions to regulate the cell cycle, both *in vitro* and *in vivo*, which may, in turn, regulate ASK1 activity (38). As research into ASK1 signaling progresses, it is likely more ASK1 substrates and the consequences of phosphorylating those substrates will be identified.

1.3.3 ASK1 regulation by protein-protein interactions

Because ASK1 is such a critical mediator of apoptosis and other cellular events, its activity it tightly controlled, in large part by protein-protein interactions. Since its identification, ASK1 has been known to exist in a high molecular weight complex, called the ASK1 signalosome (39). While not all components of the ASK1 signalosome have been determined, several proteins have been identified to directly interact with ASK1 to regulate its activity. Even in an inactive state, ASK1 homodimerizes or heterodimerizes with ASK2 through its coiled coil (CC) domains located in its C-terminus (40; 41). Under nonoxidizing conditions, ASK1 is also bound to thioredoxin through reduced cysteines in its N-terminal CC domain, which keeps the kinase in an inactive conformation (42). When these cysteine residues are oxidized, ASK1 is released from thioredoxin and can bind TRAF family members, which facilitate an activate confirmation of ASK1 (43).

As the literature on ASK1 grows, additional interacting partners have been identified, which both positively and negatively regulate ASK1 activity. The microspherule protein 2 (MCRS2) has been shown to bind ASK1 and inhibit stress induced kinase activation (44). Likewise, DJ-1 has been shown to directly interact with ASK1, preventing its dimerization and impairing ROS induced activation (45). The

Serine-threonine kinase receptor-associated protein (STRAP) has also been shown to interact with ASK1 and inhibit its activity by stabilizing its interaction with other known inhibitors, including 14-3-3 and thioredoxin, by unknown mechanisms (37). Additionally, 14-3-3 family members interact with ASK1 and impair its kinase activity and are discussed in further detail below.

1.3.4 ASK1 regulation by phosphorylation

In addition to protein-protein interactions, reversible phosphorylation provides another mechanism that regulates the activity of many proteins. ASK1, in particular, is regulated by a multitude of activating and inhibitory phosphorylation events (Table 1.1). Most notably, ASK1 is activated through phosphorylation at Thr838, which occurs by both auto-phosphorylation and phosphorylation by another ASK1 family member, ASK2 (40; 41). Evidence exists suggesting that other kinases may activate ASK1 by phosphorylating this site. At least *in vitro*, the murine protein serine/threonine kinase 38 (MPK38) has been shown to phosphorylate Thr838 (46). Conversely, PP5 can inhibit ASK1 by dephosphorylating this site, as can PP2C (47; 48).

ASK1 activity is inhibited by phosphorylation at a number of sites. Phosphorylation of Ser83 by Akt or Pim1 inhibits ASK1 activity by an unknown mechanism (49; 50), and Ser967 phosphorylation promotes ASK1 interaction with 14-3-3 family members and inhibits kinase activity, which is discussed in detail below (51). Ser1034 phosphorylation also negatively regulates ASK1 activity, though the kinase responsible for phosphorylation at this site has not been identified (52), and phosphorylation of ASK1 at Tyr718 by Janus Kinase 2 (JAK2) promotes its degradation.

Table 1.1. ASK1 phosphorylation sites.

Site	Kinase	Function	Reference
Ser83	Akt, Pim1	Inhibition	(49; 50)
Tyr718	JAK2	Inhibition (Degradation)	(53)
Thr838	ASK1, ASK2, MPK38 (*in vitro*)	Activation	(40; 41; 46)
Ser967	PDK1, IKK	Inhibition (14-3-3 binding)	(36)
Ser1034	Unknown	Inhibition	(52)

List of phosphorylation sites of ASK1 including kinases identified for each site.

The Src Homology-2 (SH2) domain-containing Phosphatase 2 (SHP2) also serves as a phosphatase for this site (53; 54). Interestingly, the dual specificity phosphatase 13A has been shown to enhance ASK1 activity not through its inherent phosphatase activity but through competition with Akt for ASK1 binding, which prevents Akt itself from phosphorylating ASK1 (55). Structural studies of ASK1 have also suggested the existence of additional phosphorylation sites. The function of these phosphorylation events, however, has not yet been determined (56).

1.3.5 ASK1/ASK2 interaction

One key regulator of ASK1 activity is another ASK family member, ASK2. Though neither kinase has been fully crystallized, ASK2 consists of 1288 amino acids with similar domain architecture to ASK1. It was initially identified in a yeast two hybrid screen as an interacting partner of ASK1, and much like ASK1 homodimerization, ASK1 heterodimerizes with ASK2 via its C-terminal coiled coil domains. Likewise, our data presented here suggests that the C-terminus of ASK2 mediates its interaction with ASK1, suggesting symmetry in the interaction of these two proteins. This heterodimerization allows ASK2 to directly phosphorylate ASK1 at Thr838, promoting its activity (40). Conversely, ASK1can also regulate ASK2 function. In the absence of ASK1, ASK2 is unstable, and overexpression of the C-terminal domain of ASK1 is sufficient to prevent ASK2 degradation (40), indicating it is ASK1 interaction rather than its kinase activity that promotes ASK2 stability.

Interestingly, both overexpression and knockdown of ASK2 have been shown to promote apoptosis (57), indicating a fine tuned interaction of these two proteins is required for proper signaling. Our evidence also supports a unique role for ASK2 in both

activating and inhibiting ASK1, with both disruption of ASK1/ASK2 interaction decreasing downstream signaling and knockdown of ASK2 decreasing ASK1 mediated inhibition by other binding partners.

Recently, a third member of the ASK family, ASK3, has been reported. ASK3 was identified by sequence homology to ASK1 and ASK2, and knockdown of ASK3 reduced apoptosis in response to well characterized activators of ASK1, including TNFα and hydrogen peroxide (58). Its precise relationship to ASK1 and ASK2, however, remains to be determined.

1.3.6 ASK1 regulation by Ser967 phosphorylation and 14-3-3 binding

Of the many protein-protein interaction and phosphorylation events that regulate ASK1, its regulation by phosphorylation of Ser967 and subsequent interaction with 14-3-3 proteins is particularly important (51). Ser967 phosphorylation is regulated by pro-growth and stress signaling. Exposure to stress signals, such as ROS, has been shown to decrease ASK1 phosphorylation at the site and reduce 14-3-3 interaction, both of which correspond to increased kinase activity (59). Conversely, calcineurin and PP2A, with the help of ASK1-interacting protein 1 (AIP1), have both been shown to dephosphorylate Ser967 (60; 61). Evidence from our studies indicates that IKK is a kinase for ASK1 Ser967. Others, however, have also identified PDK1 as a potential ASK1 Ser967 kinase (36), indicating that phosphorylation at this site may be regulated by multiple upstream signaling pathways.

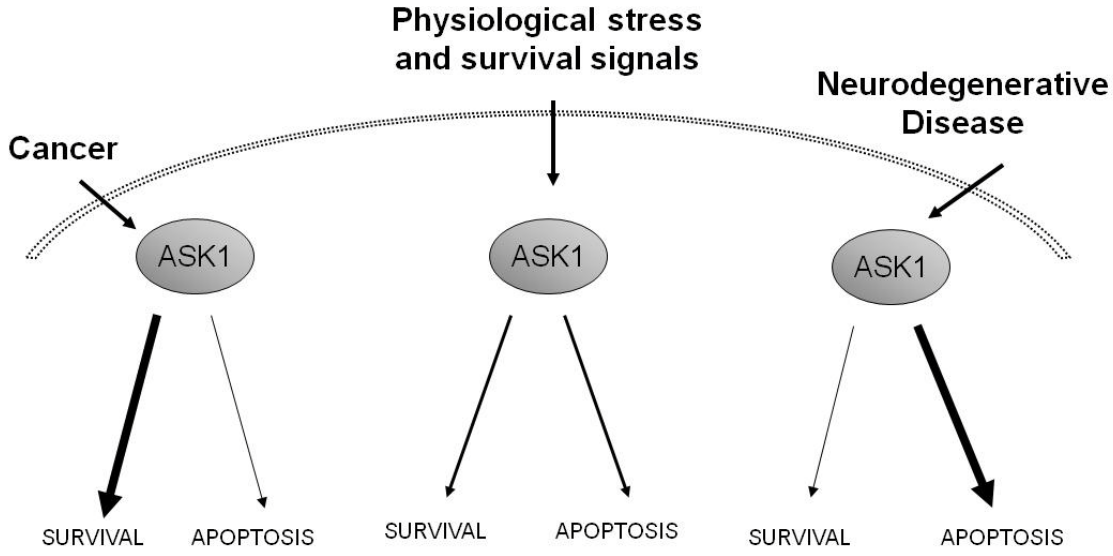
Phosphorylation of Ser967 inhibits ASK1 activity by promoting interaction with 14-3-3 family members (59). The 14-3-3 family of proteins consists of seven isoforms (β, γ, ε, ζ, η, σ, τ) which bind to other proteins via phosphorylated serine/threonine motifs

(RSxpSxP) in order to regulate protein function. 14-3-3s bind to a variety of proteins that regulate cell survival, cell cycle control, and apoptosis (62). In the case of ASK1, interaction with 14-3-3 proteins keeps the kinase in an inactive state. ASK1 interacts with all seven isoforms of 14-3-3, though to differing degrees. Overexpression of 14-3-3, however, has been shown to reduce ASK1-mediated apoptosis, and expression of an ASK1 mutant unable to bind 14-3-3 dramatically increases ASK1 mediated apoptosis, indicating 14-3-3 mediated inhibition is a important mode of ASK1 regulation under physiologic conditions (51). Stress signals like ROS decrease ASK1/14-3-3 interaction in addition to promoting dephosphorylation of Ser967 (59), and other proteins have been shown to activate ASK1 at least in part by decreasing ASK1/14-3-3 interaction (37; 63). Alternatively, our evidence suggests that, in addition to activating ASK1, ASK2 may impair ASK1 activity by promoting 14-3-3 interaction after phosphorylation. Additionally, benzodiazapinones have been shown to inhibit ER stress induced cell death by promoting ASK1/14-3-3 interaction (64), indicating modulation of ASK1/14-3-3 binding may have therapeutic potential.

1.4 Disease relevance to ASK1

$ASK1^{-/-}$ mice show no significant differences when compared to wild type controls under normal conditions. When stressed, however, these mice and their derived cell lines do show insensitivity to apoptotic stimuli, indicating that ASK1 function is critical for the proper regulation of these signals. Notably, ASK1 function has been implicated in several disease states, including neurodegenerative diseases, cardiovascular disease, diabetes, and cancer (30) (Figure 1.3).

Figure 1.3. Dysregulation of ASK1 in disease. Under physiological conditions, ASK1 regulates the balance of cell survival and cell death in response to stress and survival signals. This balance can become dysregulated in disease. In neurodegenerative disease, excessive activation of ASK1 can lead to increased apoptosis, and, in cancer, suppression of ASK1 can lead to increased cell survival.


Physiological stress
and survival signals
Neurodegenerative
Disease
Cancer
ASK1
ASK1
ASK1
SURVIVAL
APOPTOSIS
SURVIVAL
APOPTOSIS
SURVIVAL
APOPTOSIS

1.4.1 ASK1 in neurodegenerative disease

Several neurodegenerative diseases have been specifically linked to ER stress. While the UPR may be initiated in an attempt to promote cell survival under many of these conditions, prolonged stress can ultimately lead to the activation of apoptosis and the loss of affected neurons. In Huntington's disease and other "PolyQ" diseases with expansions of protein regions with repeating glutamine residues, lead to protein misfolding and aggregation (65). This can trigger ER stress, leading to ASK1 mediated cell death (21). Interestingly, sequence variations in the ASK1 gene have also been linked to a delayed age of onset of symptoms in patients with Huntington's disease (66), suggesting reduced ASK1 activity may delay neuronal cell death.

Similarly, ER stress induced, ASK1-mediated cell death has been linked to the pathogenesis of Amyotrophic Lateral Sclerosis (ALS). Approximately 20% of patients with familial ALS have mutations in the superoxide dismutase 1 (SOD1) gene, which encodes for copper/zinc superoxide dismutase (67), and SOD1 inclusion bodies have recently been linked to sporadic cases of ALS (68). SOD1 mutation has been shown to cause motor neuron death by initiating ER stress and activating cell death machinery through ASK1 (69). Interestingly, it is not the loss of function that contributes to disease but rather the toxicity of the mutant protein itself (70). When ASK1 is knocked down, cells are resistant to death induced by mutations in the SOD1 gene, and mice expressing mutant SOD1 exhibit an increased survival time when ASK1 is knocked out (69).

In Alzheimer's disease, overactivation of proline-rich extensin-like receptor kinase (PERK), a protein activated as part of the UPR, has been seen upon autopsy, and has been linked to activation of ASK1 (71). Mutation of presenilin 1, a condition

associated with AD, has also been shown to increase ER stress, which can in turn activate ASK1 (72). Similarly, expression of the protein parkin, which is often mutated in Parkinson's disease, is induced by ER stress. While overexpression of wild type parkin can reduce ER stress induced death, mutant versions cannot, and their expression can sensitize cells to death through this pathway (73).

In addition to ER stress related neurodegeneration, protein-protein interactions and responsiveness to ROS are thought to contribute to Alzheimer's disease related cell death. Alzheimer's disease is characterized by plaques containing amyloid β and tangles containing tau (74), and amyloid precursor protein (APP), from which amyloid β is cleaved, has been shown to interactive with and activate ASK1, a phenomenon which also leads to an increase in phosphorylated tau protein (75). Additionally, buildup of amyloid β increases ROS in cells, which in turn activates ASK1. Treatment with antioxidants can block both ROS production and ASK1 induced cell death from amyloid β (76).

Further, ASK1 may promote apoptosis in Parkinson's disease by similar mechanisms (77). Parkinson's disease, which is characterized by the loss of dopamine neurons, has been linked to the dysfunction of several proteins, including DJ-1. Under normal conditions, DJ-1 promotes neuron survival by antagonizing ROS signaling. Under oxidizing conditions, DJ-1 binds to ASK1, inhibiting its ability to promote apoptosis (45). DJ-1 also negatively regulates Daxx, another activator of ASK1, by keeping the protein sequestered in the nucleus away from ASK1 (78). Without proper DJ-1 function, ASK1 activity is increased, which can drive cell death. Mutations in the cytoprotective protein DJ-1 have also been linked to autosomal-recessive cases of Parkinson's disease,

and DJ-1 is modified in sporadic PD. Specifically, the DJ-1 mutation M261I seen in Parkinson's disease leads to a decrease in the DJ-1/ASK1 association, leading to activation of the kinase (79).

1.4.2 ASK1 in cardiovascular disease, diabetes, and inflammatory diseases

ASK1 function has been linked to cardiovascular disease in multiple studies but most notably in ischemia-reperfusion (IR) injury (80-82). Ischemia occurs when blood flow is cut off to a tissue, such as during a myocardial infarction or stroke. When blood re-enters this area (reperfusion), ROS are overwhelmingly generated, which can lead to the activation of ASK1 (83). When ASK1 is knocked down, cell death due to IR is reduced (84), and pharmacological inhibition of ASK1 revealed a similar effect (85).

In addition to its role in IR injury, ASK1 has been implicated in other facets of cardiovascular disease. Knockout of ASK1 has also been shown to decrease cardiovascular inflammation and fibrosis in response to a high salt diet in mice (86), and, in a separate study with $ASK1^{-/-}$ mice, ASK1 activity was also linked to endothelial dysfunction and cardiovascular remodeling (87). ASK1 may also promote cardiac hypertrophy in response to angiotensin II (33). Conflicting studies, however, have indicated that ASK1 overexpression cannot increase cardiac hypertrophy alone, indicating this pathway may rely on more than ASK1 activation (82).

In addition to cardiovascular disease, ASK1 function has also been implicated in diabetes. Improper sensitivity to insulin is linked to development of diabetes. Insulin receptor substrate (IRS) proteins are important to this sensitivity, and IRS proteins can be negatively regulated by JNK (88). In response to TNFα, ASK1 becomes activated, which leads to JNK activation and phosphorylation of IRS-1 (89). Once phosphorylated, IRS-1

can no longer interact with the insulin receptor, decreasing responsiveness to insulin. Furthermore, knockout of JNK, a downstream mediator of ASK1 activity, has been shown to increase insulin sensitivity, indicating this pathway may play a significant role in the development of diabetes (90).

Because ASK1 is a sensor of pro-inflammatory signals, it is not surprising that its function has also been implicated in inflammatory diseases, including rheumatoid arthritis (RA) and multiple sclerosis (MS) (77). RA involves aberrant production of pro-inflammatory cytokines such as TNFα, one of the main activators of ASK1, and, p38, a downstream mediator of ASK1 signaling, has been implicated in the pathogenesis of RA (91). More recently, ASK1 has been directly implicated in RA through knockout studies. $ASK1^{-/-}$ mice were completely resistant to the induction of arthritis in an experimental model of RA (92). Similarly, ASK1 knockout or pharmacological inhibition has been shown to reduce apoptosis seen in Experimental Autoimmune Encephalomyelitis (EAE), one experimental model for MS (93), indicating ASK1 function may be linked to both the inflammation and cell death seen in inflammatory diseases.

1.4.3 ASK1 in cancer

Notably, though ASK family members are most known for their role in apoptosis, both pro-tumorigenic and anti-tumorigenic effects of these kinases have been observed. By promoting inflammation, ASK1 has been shown to increase tumorigenesis in a mouse model of skin cancer (94). Additionally, ASK1 has been shown to promote gastric tumorigenesis through regulation of cyclin D1 (95), and treatment with an ASK1 inhibitor was sufficient to prevent the proliferation of gastric cancer cells (96).

However, ASK1 has also been shown to promote apoptosis in cancer cells in a number of studies. Resveratrol has been shown to promote ASK1 mediated apoptosis in leukemia cells (97), and anti-microtubule chemotherapies have been shown to promote apoptosis by activating ASK1/JNK signaling (98). Treatment with other compounds can also promote apoptosis through ASK1 in breast, colon, and prostate cancer cells (99-101). Similar to ASK1's anti-tumorigenic effects, ASK2 has been shown to promote apoptosis in a model of skin cancer (94). These conflicting roles for ASK proteins point to the complexity of this signaling node.

1.5 Small molecule inhibitors of ASK1

Identification of a successful small molecule inhibitor of ASK1 could have therapeutic implications for a wide variety of diseases, and, in recent years, the development of potential small molecule inhibitors of ASK1 has become a key area of study. A group of compounds, Imidazo[1,2-a]pyridines, derived from benzothiazole, have shown ASK1 inhibition in the nM range (102), and a separate compound, K8111, has been shown to inhibit ASK1 mediated inflammation and proliferation in gastric cancer cells (96). Additionally, a separate small molecule inhibitor of ASK1 reduced apoptosis in response to ischemia/reperfusion in a rat model (85). A virtual screen of ASK1 inhibitors also revealed 3H-Naphtho[1,2,3-de]quinoline-2,7-diones as a potential class of ASK1 inhibitors (103), and additional virtual screens for ASK1 inhibitors have been performed (104). With the combination of virtual screens and physiological experiments, the possibility of finding a suitable small molecule inhibitor of ASK1 is becoming more likely.

1.6 IKK signaling

While MAPK signaling cascades regulate a wide range of cellular processes, many other signaling networks exist to keep cells functioning properly. One such network involves nuclear factor-κB (NF-κB) and the inhibitor of κB kinases (IKKs). The IKK family consists of several proteins, including two catalytically active kinases, IKKα and IKKβ, which were first discovered as regulators of the transcription factor NF-κB. IKKα was identified first, as a kinase capable of phosphorylating inhibitor of κB (IκB) proteins, which normally inhibit NF-κB activity, in response to TNFα treatment (105). In a separate study, IKKβ, along with IKKα, was identified as a kinase capable of phosphorylating IκBs, and mutation of IKKβ lead to impaired NF-κB signaling (106). IKKα and IKKβ have both overlapping and non-redundant functions and share 52% sequence identity with a predicted N-terminal kinase domain and predicted C-terminal leucine zipper (LZ) and helix-loop-helix (HLH) domains and (107). IKKβ, however, has been recently crystallized, revealing a slightly different architecture than expected, consisting of just three domains: a kinase domain, a ubiquitin like domain (ULD), and a scaffold/dimerization domain (SDD) (108). Other family members include IKKγ, or the NF-κB Essential Modulator (NEMO), which has no kinase activity, and IKKε/TBK, which do have kinase activity, and will be discussed later.

1.6.1 NF-κB signaling

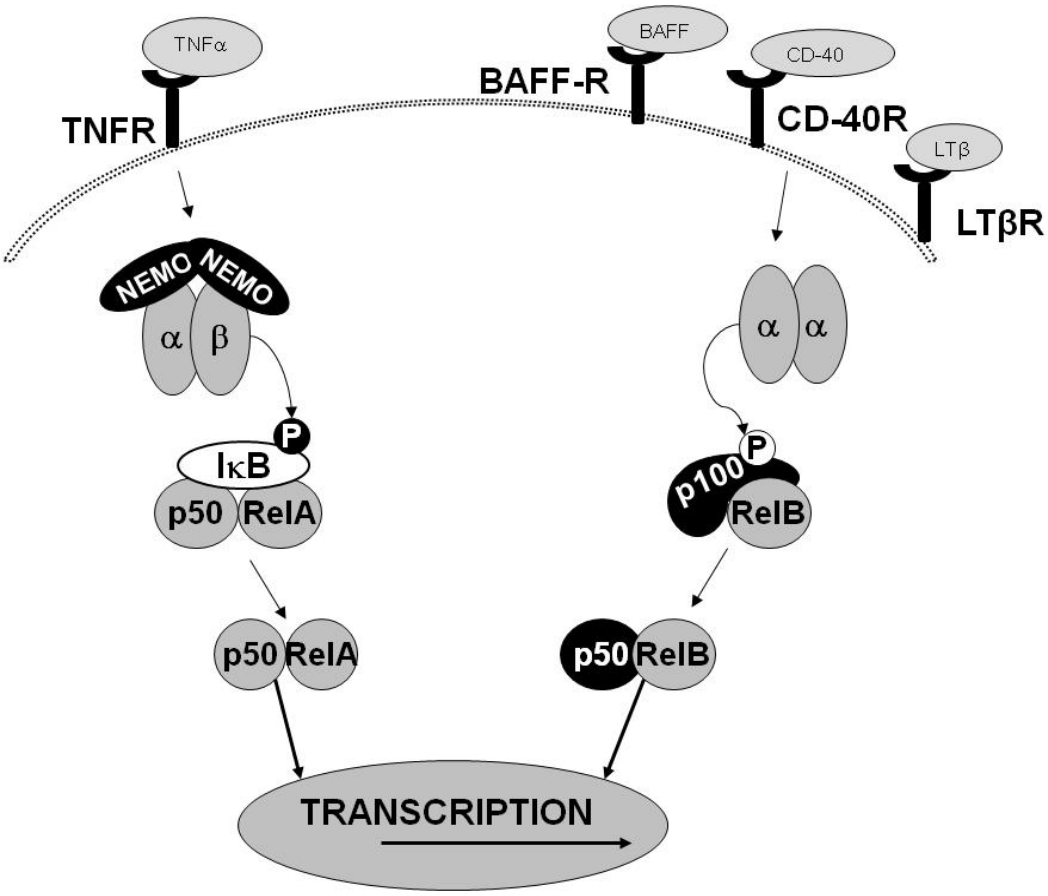
NF- κB regulates the transcription of a wide array of genes, including cytokines, growth factors, and others (109). NF-κB consists of family of proteins, including p65 (RelA), p50 (RelB), and c-Rel, which can dimerize with one another and are held inactive

in the cytoplasm by IκB proteins, and p50/p105 and p52/p100, which can be cleaved into active protein fragments (110). All family members share Rel homology domains (RHDs) that regulate their interactions DNA. IκB proteins bind to the RHDs in p65, p50, and c-Rel through ankyrin repeat domains, and the internal RHDs of p105 and p100 are masked when in an inactive state (111). When IKK is activated, it phosphorylates IκB proteins, leading to their ubiquitination and degradation. Once degraded, the nuclear localization sequence of NF- κB is completely revealed, allowing it to drive transcription (109). p105 and p100 contain ankyrin repeat domains similar to those found in IκBs, and phosphorylation by IKK leads to ubiquitination and degradation of the c-terminal portion of the protein, leading to its activation (112).

NF-κB can be activated by two pathways termed the canonical and non-canonical pathways, both of which require IKK for activation (Figure 1.2). In the canonical, or classical, signaling pathway, NF-κB is activated by pro-inflammatory cytokines such as TNFα and IL-1 or by pathogen associated molecular patterns (PAMPs). These molecules interact with TNFR or Toll Like Receptors (TRLs) to activate IKK (110).

For complete activation, the IKK complex, which is evolutionarily conserved all the way to Drosophila Melonagaster, is formed. This complex consists of an IKKα/IKKβ heterodimer or IKKβ homodimer bound by at least two subunits of NEMO (IKKγ) (113). While most studies have pointed to IKKβ as the catalytic subunit of the IKK complex, one study revealed that some signaling by the canonical pathway still occurs even in $IKK\beta^{-/-}$ cells, indicating IKKα may play a larger role in this signaling pathway than is currently understood (114). Others have also shown that while IKKα is not required for

Figure 1.4. IKK mediated NF-κB signaling. IKK mediates NF-κB signaling via two pathways, the canonical signaling pathway and the non-canonical signaling pathway. In response to TNF signaling, the IKK complex consisting IKKα, IKKβ, and at least two subunits of NEMO, along with other proteins, is formed. Once activated, IKKβ phosphorylates IκB proteins, which normally bind to NF-κB proteins such as p50/RelA dimers keeping them sequestered in the cytoplasm. Phosphorylation of IκBs leads to degradation, which allows NF-κB to translocation to the nucleus and alter transcription. In response to BAFF, CD-40, or LTβR signaling, an alternative pathway is initiated in which IKKα homodimers can phosphorylate p100, leading to partial degradation of the protein to p52. p52/RelB dimers can then translocate to the nucleus to alter transcription.


TNFα
BAFF
CD-40
TNFR
BAFF-R
CD-40R
LTβ
LTβR
NEMO
NEMO
α
β
α
α
P
IκB
p50
RelA
p100
P
RelB
p50
RelA
p50
RelB
TRANSCRIPTION

IκB degradation in response to TNFα signaling, cells lacking IKKα are deficient in activation of NF-κB mediated transcription (115).

The IKK complex is activated by phosphorylation of specific serine residues of IKK, Ser176 and Ser180 of IKKα and Ser177 and Ser181 of IKKβ (106). Several kinases have been hypothesized to phosphorylate these sites, including the possibility of IKK auto-phosphorylation (116). Specifically, TGFβ Activated Kinase 1 (TAK1) and MEKK3 have been shown to directly phosphorylate IKK (117; 118). Once activated, this complex phosphorylates IκB proteins at two specific serine residues, which leads to their ubiquitination and degradation by the proteosome. NF-κB is then free to travel to the nucleus and drive gene transcription.

The activation of the canonical NF-κB signaling pathway plays a critical role in the regulation of the innate immune response. Activation of NF-κB by this pathway through TLRs leads to increased transcription of a variety of proteins related to the innate immune response, including pro-inflammatory cytokines and chemokines, and cellular adhesion molecules as well as other inflammatory mediators and pro-survival genes (119). These newly transcribed genes play vital roles in the cellular response to invading microorganisms. It is interesting to note that even though distinct TLRs are activated in response to different stimuli, activation of all these receptors converges on the activation of NF-κB to regulate the transcription of genes involved in innate immunity (110).

In addition to canonical signaling by IKK, a second signaling pathway for NF-κB activation has been identified. In this non-canonical, or alternative, signaling pathway, signaling from receptors such as Lymphotoxin β Receptor (LTβR), B-cell activating factor (BAFF), and CD40 ligand receptor activate the NF-κB Inducing Kinase (NIK),

which, in turn, activates IKKα by preferentially phosphorylating it over IKKβ (120). Homodimers of IKKα, without NEMO, can then phosphorylate the C-terminal of p100 (121). Once phosphorylated, the C-terminal portion of p100 is ubiquitinated and degraded by the proteosome in a similar manner to IκBs. The remaining N-terminal protein fragment, RelA, can associate with other Rel family members and translocate to the nucleus to alter gene transcription (112). Activation of this pathway also plays a role in the innate immune response in the spleen, with knockout of IKKα impairing immune response (121).

Loss of IKK can have deleterious effects with respect to NF-κB function both inside and outside the innate immune response. Knockout of IKKβ is embryonic lethal due to severe liver degeneration, similar to the effect seen by knockout of p65 itself. Interestingly, this phenotype can be rescued if the TNF Receptor (TNFR) is also knocked out, indicating that IKKβ is critical in the prevention of TNFα mediated apoptosis during development (122). Even when IKKβ knockout-mediated apoptosis is attenuated by additional TNFR knockout, mice still show an increased susceptibility to infection (123; 124), indicating IKKβ is important for cell function throughout the lifespan. IKKβ is also required for the proliferation and survival of B cells, with $IKK\beta^{-/-}$ mice showing reduced numbers of B cells (125).

While knockout of IKKα had no effect on signaling downstream of TNFα, it did lead to abnormal bone and skin development in mice, as well as defects in the development of the gastrointestinal tract, and was ultimately lethal (126). Furthermore, knockout of IKKα or its upstream activating kinase NIK has been shown to lead to impaired lymphorganogenesis, resulting in impaired immune response (127), and has

been linked to the prevention of B cell maturation (121). Knockout of both IKKβ and IKKα, however, leads to a complete insensitivity to NF-κB activators and exhibited neural tube defects and liver degeneration leading to embryonic lethality (128), indicating these kinases are essential for proper development and cell function.

1.6.2 Regulation of IKK by NEMO

In addition to the roles played by IKKα and IKKβ in activating NF-κB, IKKγ, or NEMO, is also required for activation of NF-κB by the canonical signaling pathway, and loss of NEMO leads to decreased sensitivity to stimulation by TNFα, LPS, and other pro-inflammatory cytokines (129; 130). Both IKKα and IKKβ interact with NEMO via a specific stretch of amino acids in their N-termini, which has been termed the NEMO Binding Domain (NBD). Expression of a peptide containing this region is sufficient to compete with IKK for NEMO binding and inhibit cytokine induced NF-κB activation (131). While its role in the activation of the IKK complex has been well documented, it is currently unclear how many subunits of NEMO are present in the IKK complex. Some studies have suggested that NEMO exists as a tetramer, or more specifically a dimer of dimers (132), while others suggest NEMO may be present in a trimer (133). Proper assembly and function of NEMO, however, are important to IKK complex activity. Mutations in NEMO that impair its ability to oligomerize have negative effects on IKK activity (134), and knockout of NEMO yields severe liver degeneration and embryonic lethality in mice, a phenotype also seen with IKKβ knockout (135).

While NEMO has no catalytic activity itself, it is indispensible for canonical NF-κB signaling, and it may have roles in many other situations as well. NEMO has been shown to interact with other proteins, which both positively and negatively regulate IKK

activity. Because ubiquitination of NEMO appears to be important for activation of the IKK complex (136), several proteins involved in ubiquitination and deubiquitination have been identified as interactors of NEMO, including CARD-MAGUK protein 1 (CARMA1), the cellular inhibitor of apoptosis protein 1 (cIAP-1), and parkin, which promote its ubiquitination, and A20, the A20 binding inhibitor of NF-κB 1(ABIN-1), and cylindromatosis protein (CYLD) which promote its deubiquitination (137; 138). NEMO has also been shown to interact with a variety of proteins required for IKK activation by certain stimuli, including receptor interacting protein (RIP1), which is needed for activation by TNFα, and Interleukin-1-receptor-associated kinase-1 (IRAK-1), which is required for activation by IL-1 (138). Mutations in NEMO have been linked to disease, including the skin disorder incontinentia pigmenti, which is characterized by skin and teeth deformities, and ectodermal dysplasia with immunodeficiency (EDA-ID), which is an auto-immune disorder leading to increased susceptibility to infection (139), highlighting NEMO's importance in normal cell function.

1.6.3 IKKε/TBK1

In addition to NEMO, two other IKK family members, IKKε and TBK1, have been identified (140). Overexpression of IKKε has been shown to increase NF-κB signaling, and endogenous IKKε has been shown to respond to activators of non-canonical NF-κB signaling, indicating there may be redundancy in this pathway to ensure NF-κB activation in the presence of stimuli (141; 142). Likewise, TBK1 has been shown to indirectly activate NF-κB (143). Both kinases may also indirectly activate NF-κB through activation of IKKα or IKKß (140). In addition to activating NF-κB, IKKε and TBK1 play a role in the response of interferon to viral infection by phosphorylating

interferon regulatory factors (144), suggesting all IKK family members regulate cellular responses inside and outside NF-κB signaling.

1.6.4 IKK signaling outside NF-κB and disease relevance

While there is no question that IKK is indispensible for NF-κB signaling, roles for IKK outside of NF-κB signaling are also becoming clear (Table 1.2). As promoters of survival, IKKs have been shown to play a role in the promotion of tumorigenesis. Similarly, there is also a strong link between inflammation and tumorigenesis, and increased IKK activity has been associated with a variety of cancer types (157). Additionally, IKKβ phosphorylates a number of tumor suppressor genes, leading to their degradation. It has been shown to inhibit p53 in this manner, independent of Mouse Double Minute 2 (MDM2) (150). Interestingly, however, IKKα and IKKβ can both phosphorylate β-catenin, which plays a role in metastasis (158), with opposing outcomes. Phosphorylation by IKKβ suppresses its transcriptional activity while phosphorylation by IKKα activates it (152).

Similarly, IKKα and IKKβ may promote tumorigenesis by regulating the stability of other substrates. IKKα can regulate the cell cycle by phosphorylating cyclin D1, leading to its degradation (153). IKKβ has also been shown to phosphorylate tuberous sclerosis 1 (TSC1), which, through its degradation, leads to increased activation of the mammalian target of rapamycin (mTOR) and promotes angiogenesis in tumors (145). Similarly, IKKβ has been shown to promote tumorigenesis by phosphorylating and increasing degradation of FOXO3a, which would otherwise drive transcription of a variety of genes regulating cell proliferation and survival (146). Likewise, IKKβ may

Table 1.2. IKK substrates outside of NF-κB signaling

Kinase	Substrate	Function	Reference
IKKβ	TSC1	Degradation (mTOR activation)	(145)
	FOXO3a	Degradation (tumorigenesis)	(146)
	Dok1	Activation (increased cell motility)	(147)
	SNAP-23	Activation (vesicle exocytosis)	(148)
	IRS-1	Inhibition (insulin resistance)	(149)
	p53	Degradation	(150)
	Aurora Kinase A	Inhibition	(151)
	β-catenin	Inhibition	(152)
IKKα	β-catenin	Activation	(152)
	cyclin D1	Degradation	(153)
	Histone H3	Acetylation	(115)
	CBP	Increased NF- κB binding	(154)
	IRF-7	Activation	(155)
	IRF-5	Inhibition	(156)

List of IKKα and IKKβ substrates outside of the IκBs.

contribute to cell invasion and metastasis by phosphorylating Docking protein 1 (Dok1) in response to pro-inflammatory cytokines, an event that allows Dok1 to increase cell motility (147). Conversely, IKK has also been shown to phosphorylate Aurora kinase A, promoting proper regulation of the cell cycle. Knockout of IKKβ and the subsequent loss of Aurora kinase A phosphorylation and degradation contributes to spindle abnormalities and promotes cancer formation (151), indicating IKK's role in cancer may not be so straight forward.

IKK may also influence immune response without directly activating NF-κB. IKKβ promotes the degranulation of mast cells, which leads to the release of histamine and other inflammatory molecules. IKK does so by phosphorylating the Synaptosomal-Associated Protein 23 (SNAP-23), which plays a role in the exocytosis of vesicles (148). Furthermore, IKKα may indirectly promote NF-κB activity by phosphorylating histone H3, which facilitates NF-κB mediated transcription (115). IKKα also both positively and negatively regulates interferon production by phosphorylating interferon regulatory factor 7 (IRF-7) and IRF-5, respectively (155; 156) and can phosphorylate the transcriptional co-activation CREB Binding Protein (CBP), which increases its preference for NF-κB as a binding partner (154).

In addition to its roles in tumorigenesis and immune response, the inflammation promoting activity of IKK may contribute to other diseases, such as diabetes and arthritis (116). IKKβ been shown to directly phosphorylate IRS-1, leading to decreased signaling from the insulin receptor and insulin insensitivity (149). Similarly, IKK has been implicated in promoting insulin resistance and inflammation in response to obesity in an animal model (159). IKKε, one of the non-canonical members of the IKK family, was

also seen in a mouse model to be required for high-fat diet induced obesity, with knockout of IKKε preventing obesity, chronic inflammation, and insulin resistance in mice (160). IKK mediated inflammation also contributes to the pathogenesis of osteoarthritis (OA) and RA. In arthritis, IKK activates NF-κB, which in turn contributes to the inflammation, swelling, and hyperplasia seen in OA and RA (161). In particular, overexpression of IKKβ was able to increase synovial inflammation and arthritis in a rat model of RA, whereas overexpression of dominant negative mutant of IKKβ was sufficient to reduce synovial inflammation in response to outside stimuli (162). Similarly, introduction of a NEMO binding domain (NBD) peptide, which impairs IKK signaling, decreased inflammation in rodent models of RA (163).

IKKβ activity has also been linked to a variety of neurodegenerative diseases, including Huntington's Disease, Alzheimer's Disease, and Parkinson's disease by promoting inflammation (164). Increased activation of NF-κB, which requires IKK, has been seen in patients with PD, and treatment with an NBD peptide, was sufficient to reduce inflammation associated with a mouse model of PD, indicating IKK may play a considerable role in inflammation associated with this disease (165). Interestingly, IKK may also play a protective role in HD by promoting the degradation of huntingtin, which could otherwise aggregate in cells and lead to ER stress induced apoptosis (166).

1.7 Crosstalk between ASK1 and IKK pathways

While no direct link to ASK1 signaling has previously been reported, crosstalk between IKKß and general MAPK signaling has been previously identified. LPS induced activation of IKK leads to activation of ERK(167), and LPS signaling can also activate

ASK1 (31). Additionally, ASK1 and IKK are both activated by TNFα, leading to very different outcomes (168). Likewise, TRAF family members, which are important in keeping ASK1 in an active conformation (43), also play a role in the activation of IKK (169), presenting a possible bridge for feedback from one signaling pathway to another. Because both of these kinases are activated in response to similar stimuli, mechanisms must be in place that allow for each signaling pathway to be differentially activated.

Significant crosstalk between NF-κB signaling and JNK signaling has also been noted (170). NF-κB mediated inhibition of JNK has previously been attributed to transcription of GADD45 and XIAP, which can block JNK activation (171; 172). Additionally, NF-κB signaling has been shown to decrease ROS accumulation, which normally activates JNK signaling (173). Knockout of IKKβ has also been shown to promote prolonged activation of JNK in a manner independent of GADD45 and XIAP expression (174), indicating that ASK1 and IKK mediated signaling pathways may converge with one another. However, the molecular basis for the cross-talk between IKK and ASK1 remains to be determined, which is the focus of my research.

1.8 Scope of dissertation

The scope of this dissertation aims to describe two protein-protein interactions that regulate ASK1 function. First, we describe the interaction of ASK1 with ASK2, which can both positively and negatively regulate ASK1 activity. We first provide evidence for novel 14-3-3 mediated inhibition of ASK1 by ASK2, and we identify the binding interface of ASK1 and ASK2 and provide evidence suggesting disruption of ASK1/ASK2 interaction can reduce ASK1-mediated signaling. Second, we provide

evidence for IKK regulation of ASK1, where IKK directly interacts with ASK1 via the NEMO binding domain of IKK and mediates ASK1 inhibition in response to growth factor signaling by regulating ASK1 phosphorylation at Ser967. Understanding interactions of ASK1 with regulatory partners such as ASK2 and IKK will not only provide a better understanding of complex cellular responses to stress and survival signals, but may reveal novel drug targets with relevance to a wide variety of diseases, from neurodegenerative diseases to cancer.

Chapter 2: ASK2 promotes ASK1 inhibition or activation through specific protein-protein interactions

Chapter 2.1: Dual engagement of 14-3-3 proteins controls signal relay from ASK2 to the ASK1 signalosome.

Cockrell LM*, Puckett MC*, Goldman EH, Khuri FR, Fu H.

*Co-first authors

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Abstract

Faithful and efficient transmission of biological signals through mitogen-activated protein kinase (MAPK) pathways requires engagement of highly regulated cellular machinery in response to diverse environmental cues. Here, we report a novel mechanism controlling signal relay between two MAP3Ks, apoptosis signal-regulating kinase (ASK) 1 and ASK2. We show that ASK2 specifically interacts with 14-3-3 proteins through phosphorylated S964. Although a 14-3-3-binding defective mutant of ASK1 (S967A) has no effect on the ASK2/14-3-3 interaction, both overexpression of the analogous ASK2 (S964A) mutant and knockdown of ASK2 dramatically reduced the amount of ASK1 complexed with 14-3-3. These data suggest a dominant role of ASK2 in 14-3-3 control of ASK1 function. Indeed, ASK2 S964A-induced dissociation of 14-3-3 from ASK1 correlated with enhanced phosphorylation of ASK1 at T838 and increased c-Jun N-terminal kinase phosphorylation, the two biological readouts of ASK1 activation. Our results suggest a model in which upstream signals couple ASK2 S964 phosphorylation to the ASK1 signalosome through dual engagement of 14-3-3.

Introduction

The evolutionarily conserved mitogen-activated protein kinase (MAPK) cascades consist of tiered protein kinases that undergo sequential phosphorylation and activation, allowing specific signal amplification to elicit a corresponding cellular response (175; 176). Each cascade module consists of an MAPK, an MAPK kinase (MAP2K), and an MAPK kinase kinase (MAP3K). MAPK cascades are activated by diverse stimuli to mediate multiple signaling pathways, resulting in a crucial impact on cell fate processes such as cell growth, differentiation and death. Among the MAPK cascades, those triggered by the MAP3K apoptosis signal-regulating kinase 1 (ASK1) are critical determinants of apoptosis (16; 31; 177) . ASK1 activation leads to phosphorylation and activation of MAP2K4/7 or MAP2K3/6 and the resulting MAPKs c-Jun N-terminal kinase (JNK) and p38 activation, respectively. A variety of intrinsic and extrinsic cellular stress stimuli induce ASK1 activation. For example, ASK1 relays signals from death receptors, such as those activated by tumor necrosis factor α (78). ASK1 is also activated by unfolded protein response-induced endoplasmic reticulum stress (21). Recently, important physiological and pathological roles of ASK1 have emerged to include the regulation of innate immunity, cellular differentiation, and various human diseases such as cardiac hypertrophy and remodeling, insulin resistance, neurodegeneration and tumorigenesis (27; 33; 89; 94; 178). Because of its importance as a central mediator of diverse developmental and stress signals, ASK1 activation is tightly controlled. The newly defined ASK1 signalosome reflects the numerous phosphorylation and protein interaction events critical to maintain ASK1 regulation (40).

One important ASK1 regulatory protein is 14-3-3, a phosphoserine/threonine-recognition protein (62; 179-184). 14-3-3 is a multifunctional regulatory protein that is important in maintaining a multitude of cellular processes, including cell cycle control, cell proliferation and inhibition of apoptosis. Seven mammalian isoforms of 14-3-3 have been identified and are denoted by Greek lettering (γ, τ, ζ, σ, β, ε, η). 14-3-3 binds to the majority of its cellular ligands through a phosphorylated motif. The canonical 14-3-3 recognition motif has been identified as RSXpSXP, in which phosphorylation of the second serine is critical for 14-3-3 recognition (185; 186). Binding of 14-3-3 to ASK1 through a phosphorylated S967 motif (RSIS967LP) has been shown to decrease ASK1 kinase activity and inhibit ASK1-induced apoptosis (59; 187; 188).

Recently, another MAP3K, ASK2, was found to bind with ASK1 (189). ASK2 has been shown to function as a tumor suppressor in combination with ASK1, and ASK2 levels are reduced in human gastrointestinal cancers (94). The heteromeric complex of ASK1 and ASK2 is thought to have reciprocal functionality, which means that ASK1 and ASK2 function to activate each other (178). ASK2 activation of ASK1 is mediated by phosphorylation of T838 within the activation loop of the human ASK1 kinase domain, whereas ASK1 activation of ASK2 seems to occur through a phosphorylation-independent mechanism. Thus, dissection of ASK1-associated protein interactions may offer opportunities to gain further understanding of the general mechanisms controlling signal transmission through MAP3Ks.

Here we describe a novel mechanism by which ASK2 suppresses ASK1 function through the induced recruitment of 14-3-3 proteins. Our work establishes a new signaling

complex consisting of ASK2, ASK1 and 14-3-3, suggests an inhibitory function of ASK2 for ASK1, and reveals a dynamic signal-relay function for the family of 14-3-3 proteins.

Methods

Cell culture, DNA transfection and plasmids

COS7 and HeLa cells were maintained in Dulbecco's modified Eagle's medium and plasmid transfections were performed using Fugene 6 or Fugene HD (Roche, Boulder, CO, USA) as previously described (16; 51; 187). ASK2–KC and full-length ASK2 were amplified by PCR and cloned into the pDEST27 GST mammalian expression vector (Invitrogen, Carlsbad, CA, USA).

ASK2 knockdown

Plasmids encoding ASK2 shRNAmir constructs (RH54430-98513431 and RH54430-98903615) were purchased from Open Biosystems (Huntsville, AL, USA). HeLa cells were transfected with shRNAmir plasmid using Fugene HD (Roche) (16; 51; 187). Transfected cells were selected with puromycin (2 mg/ml) for 3 days.

Protein interaction assays

Affinity pulldown assay. Cells were lysed in 200 µl of pulldown lysis buffer (59). HexaHis-tag fusion proteins in the clarified cell lysates were isolated with Ni^{2+}-charged resin (Novagen, Madison, WI, USA) as described (51).

Co-IP assay. Cells were lysed in either 200 µl (COS7) or 1000 µl (HeLa) of co-IP lysis buffer (59). Cleared cell lysates were incubated with Protein A or G conjugated sepharose (GE Healthcare, Piscataway, NJ, USA) and the appropriate antibody for 2 hr to overnight at 4°C. Following incubation, the resin was washed three times with lysis buffer and protein samples were eluted by boiling in 6xSDS sample buffer for western blot analysis.

Phosphatase assay

Cleared lysates of COS7 cells were divided into two samples, a 'plus inhibitors' sample with added phosphatase inhibitors ($Na_4P_2O_7$, NaF, Na_3VO_4), and a 'minus inhibitors' control sample. Time-course experiments were carried out as indicated. Lysates were then subjected to affinity pulldown as described above.

Western blotting

Proteins were separated on 8% or 12.5% SDS–PAGE gels and transferred to a nitrocellulose membrane, which were blocked with 5% milk. Membranes were probed overnight with anti-GST, anti-14-3-3 K19, anti-6xHis, anti-Raf anti-ASK1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Flag M2 (Sigma, St Louis, MO, USA), anti-ASK2 (Abnova, Taipei, Taiwan), or anti-phospho Ser967 ASK1 (Cell Signaling Technologies, Boston, MA, USA) antibodies, diluted in 5% milk. Corresponding horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) were used against each primary antibody. Proteins were detected using West-Pico or West-Dura enhanced chemiluminescent detection reagents (Pierce, Rockford, IL, USA)

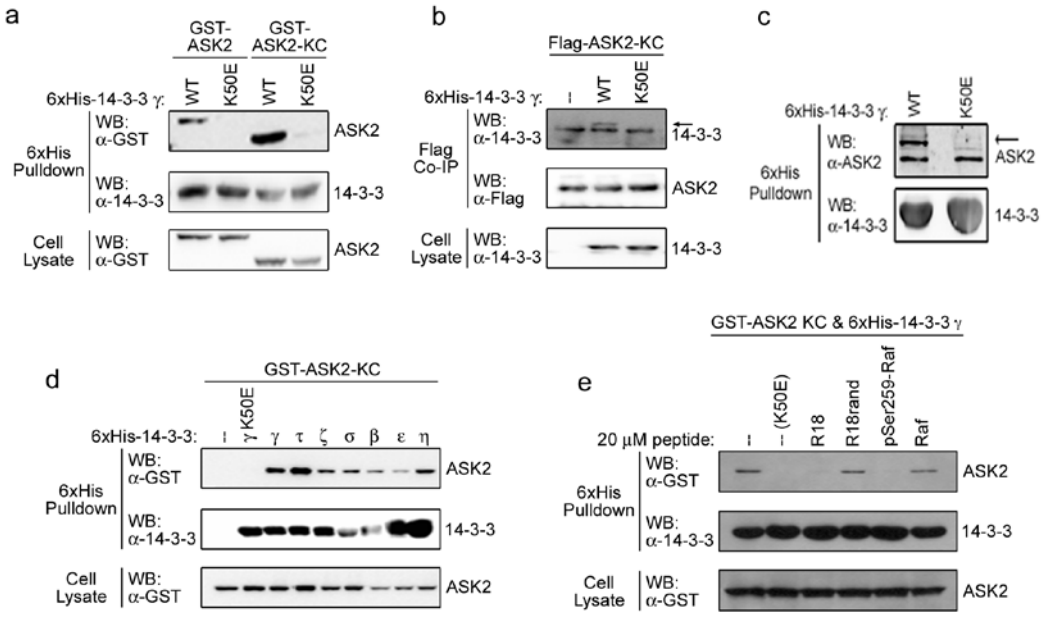
and a Kodak imaging system. Densitometry was performed with Kodak 1D imaging software, and statistical analysis was carried out using the Student's t-test.

Results

ASK2 specifically interacts with 14-3-3 proteins

In our mechanistic study of the ASK1 signalosome, we noticed the presence of a putative 14-3-3 recognition motif surrounding S916 (RSPS916SP) in the C-terminus of ASK2. This motif fits well with the defined consensus 14-3-3 binding motif, RSXpSXP, and raises the possibility of ASK2 as a new 14-3-3 target protein (183). To test whether ASK2 interacts with 14-3-3, we carried out a series of affinity pulldown assays using Ni2+-charged resin to isolate hexahistidine (6xHis) tagged 14-3-3γ protein complexes from COS7 cell lysates. 14-3-3γ K50E, a ligand-binding defective mutant, was used as a control (190; 191). As revealed by western blot, glutathione S-transferase (GST)-ASK2 was found within the 14-3-3γ wild-type (WT) complex, but was absent in the K50E complex (Figure 2.1-1A), suggesting a specific interaction of ASK2 with 14-3-3γ. Consistent with a potential role of ASK2 S916 in 14-3-3 binding, a truncated ASK2 lacking the sequence N-terminal to the kinase domain (ASK2–KC: 638–1288 aa) was still capable of binding with 14-3-3γ (Figure 2.1-1A). To confirm this interaction, a reciprocal experiment was performed. When Flag-ASK2–KC was isolated from COS7 cell lysates using co-immunoprecipitation (co-IP), 14-3-3γ WT, but not K50E, was found in the resulting ASK2 protein complex (Figure 2.1-1B). In further support of these

Figure 2.1-1. Interaction of apoptosis signal-regulating kinase 2 (ASK2) with 14-3-3 proteins. (A) ASK2 is in complex with 14-3-3γ. COS7 cells were co-transfected with either glutathione *S*-transferase (GST)-ASK2 or GST-ASK2–KC and hexahistidine (6xHis)-14-3-3γ wild type (WT) or K50E. The 14-3-3 protein complex was isolated 40 h after transfection with Ni^{2}+-charged resin and bound ASK2 was visualized by western blot. (B) 14-3-3 binds to ASK2 in a reverse co-immunoprecipitation (co-IP) assay. COS7 cell lysates over-expressing Flag-ASK2–KC and 6xHis-14-3-3γ WT or K50E were used to isolate the ASK2–KC complex with an anti-Flag antibody. The resulting ASK2 protein complex was used for the detection of 14-3-3 by western blot. (C) 14-3-3 specifically binds endogenous ASK2. COS7 cells were transfected with 6xHis-14-3-3γ (WT or K50E). The 14-3-3 γ complex was isolated with Ni_2+-charged resin, and endogenous ASK2 was detected by western blot. (D) Interaction of ASK2 with multiple 14-3-3 isoforms. The isolation of the 14-3-3 isoform complex and the detection of ASK2 in each 14-3-3 complex were determined as in (A). (E) Competitive binding of ASK2 to 14-3-3 with defined 14-3-3 peptide antagonists. The 14-3-3/ASK2 complexes were isolated with an affinity pulldown assay as in (A), either in the presence or in the absence of antagonistic peptides (pSer259Raf and R18). Non-phosphorylated Raf peptide (Raf) or a randomized R18 peptide (R18rand) was used as control.


a
GST-ASK2
GST-ASK2-KC
6xHis-14-3-3 γ:
WT
K50E
WT
K50E
6xHis Pulldown
WB: α-GST
ASK2
WB: α-14-3-3
14-3-3
Cell Lysate
WB: α-GST
ASK2
b
Flag-ASK2-KC
6xHis-14-3-3 γ:
–
WT
K50E
Flag Co-IP
WB: α-14-3-3
14-3-3
WB: α-Flag
ASK2
Cell Lysate
WB: α-14-3-3
14-3-3
c
6xHis-14-3-3 γ:
WT
K50E
6xHis Pulldown
WB: α-ASK2
ASK2
WB: α-14-3-3
14-3-3
d
GST-ASK2-KC
6xHis-14-3-3:
–
γ K50E
γ
τ
ζ
σ
β
ε
η
6xHis Pulldown
WB: α-GST
ASK2
WB: α-14-3-3
14-3-3
Cell Lysate
WB: α-GST
ASK2
e
GST-ASK2 KC & 6xHis-14-3-3 γ
20 μM peptide:
–
– (K50E)
R18
R18rand
pSer259-Raf
Raf
6xHis Pulldown
WB: α-GST
ASK2
WB: α-14-3-3
14-3-3
Cell Lysate
WB: α-GST
ASK2

results, 14-3-3γ was also able to pull down endogenous ASK2 (Figure 2.1-1C). Together, the above data suggest that ASK2 specifically interacts with 14-3-3γ through a binding site mapped to the KC fragment of the ASK2 protein. Therefore, ASK2–KC was used to further characterize binding with 14-3-3 in subsequent experiments.

There are seven 14-3-3 isoforms in mammalian cells (γ, τ, ζ, σ, β, ε, η). 14-3-3 interaction with ASK2 is not unique to the γ isoform, as ASK2 was found in complex with all seven 14-3-3 isoforms (Figure 2.1-1D). These data imply that ASK2 may use a common binding site within 14-3-3 proteins, such as the conserved amphipathic groove within the 14-3-3 structure (183). To test this possibility, we used two approaches: peptide competition assay and mutational analysis. Indeed, two well-defined 14-3-3 groove-binding peptides, a phosphorylated Raf peptide (pSer259-Raf) and an unphosphorylated antagonist peptide (R18), effectively competed away ASK2's interaction with 14-3-3γ (Figure 2.1-1E) (185; 192; 193). Conversely, inclusion of the respective peptide controls, unphosphorylated Raf peptide (Raf) or randomized R18 peptide (R18rand), showed no competitive effect. In further support of the involvement of the amphipathic groove in ASK2 binding, several charge-reversal mutations of 14-3-3ζ amino acid residues within the groove (K49E, R56E, R60E, V176D) greatly diminished interaction with ASK2 (data not shown; (189; 191; 194). These data show how the amphipathic groove of 14-3-3 proteins is used as an ASK2 docking site, allowing common and specific binding between 14-3-3 and ASK2.

ASK2 requires phosphorylation for 14-3-3 binding

We found that incubation of cell lysates at 37°C led to a decrease in ASK2's interaction

with 14-3-3γ (Figure 2.1-2A). This decrease is likely due to the increased action of an endogenous protein phosphatase(s), as the presence of a consensus motif (RSPS916SP) within ASK2 predicts a phosphorylation requirement for recognition by 14-3-3. In support of this notion, the inclusion of general phosphatase inhibitors during this incubation prevented the dissociation (Figure 2.1-2A). The phosphorylation dependence of the interaction was further confirmed in a separate assay, in which calf intestinal phosphatase was shown to accelerate the dissociation of ASK2 from 14-3-3 (data not shown). This phenomenon allowed the use of more specific phosphatase inhibitors to validate the requirement of phosphorylation for the interaction and to define the class of protein phosphatases involved. At lower concentrations, okadaic acid is more specific for inhibition of the PP1/PP2A phosphatase family, whereas cyclosporine A is specific for inhibition of PP2B, or calcinuerin (195). Treatment of cells with okadaic acid, but not cyclosporine A, led to a dramatic increase in the amount of the ASK2/14-3-3γ complex (Figure 2.1-2B). From these data, we conclude that ASK2's interaction with 14-3-3 is negatively regulated by the PP1/PP2A phosphatase family, supporting the importance of a regulated phosphorylation dictating the ASK2/14-3-3 interaction.

14-3-3 binds ASK2 through a novel S964-mediated motif

Both the phosphorylation dependence of the ASK2/14-3-3 interaction and the presence of a putative 14-3-3 binding motif within the ASK2 sequence (RSPS916SP) predict the importance of S916 as the critical phospho-acceptor mediating 14-3-3 interaction (Figure 2.1-3A). To test whether the S916-containing motif is indeed an essential determinant of 14-3-3 interactions, we performed a competitive binding assay using a phosphorylated

Figure 2.1-2. The apoptosis signal-regulating kinase 2 (ASK2)/14-3-3 interaction requires phosphorylation. (A) Phosphatase inhibition increased the amount of ASK2/14-3-3 interaction. Cell lysates with co-expressed glutathione *S*-transferase (GST)-ASK2–KC and hexahistidine (6xHis)-14-3-3γ were divided into two samples, and incubated at 37°C either in the presence or in the absence of 50 mm phosphatase inhibitors ($Na_4P_2O_7$, NaF, Na_3VO_4). The lysate was removed at the indicated times and subjected to affinity pulldown and western blot as in Figure 2.1-1a. (B) An inhibitor of the protein phosphatase 2A family blocks ASK2's dissociation from 14-3-3. Cells co-expressing GST-ASK2–KC and 6xHis-14-3-3γ were treated with increasing concentrations of either okadaic acid or cyclosporine A (Sigma), or vehicle (ethanol or methanol, respectively), for 1 hr. Cell lysates were used in an affinity pulldown assay as described in Figure 2.1-1A.

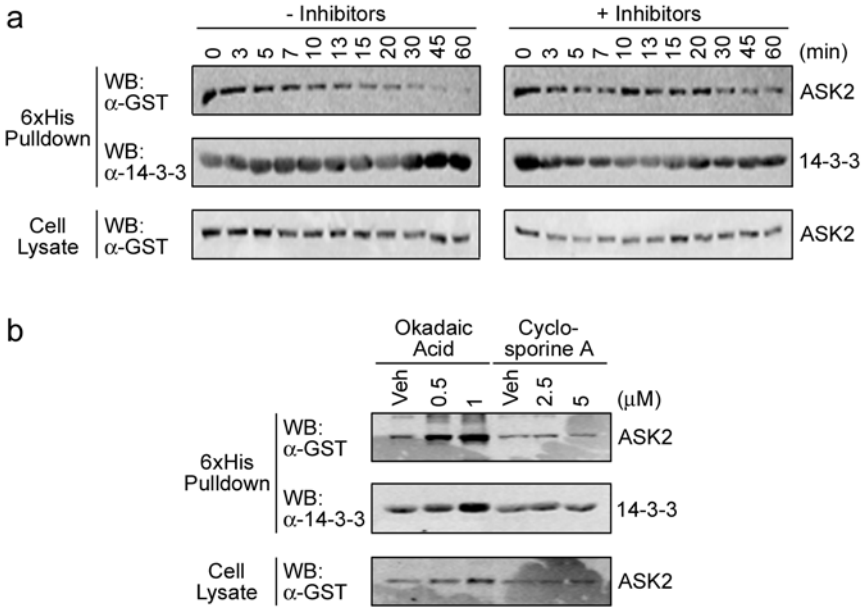
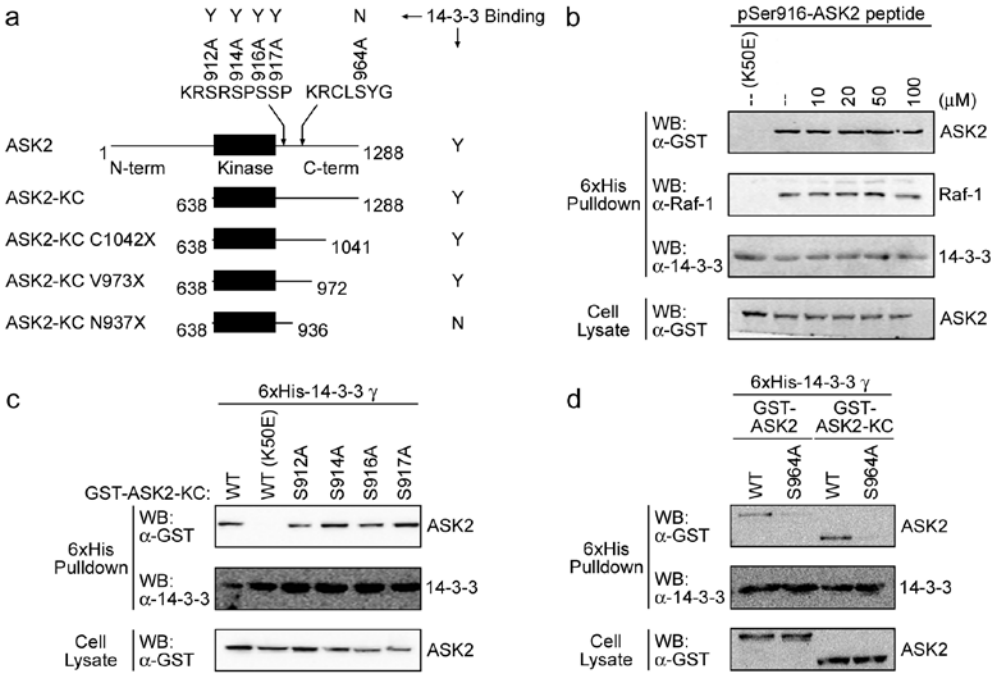

a
- Inhibitors
+ Inhibitors
0 3 5 7 10 13 15 20 30 45 60
(min)
6xHis Pulldown
WB: α-GST
WB: α-14-3-3
Cell Lysate
WB: α-GST
ASK2
14-3-3
ASK2
b
Okadaic Acid
Cyclo-sporine A
Veh 0.5 1 Veh 2.5 5
(μM)
6xHis Pulldown
WB: α-GST
WB: α-14-3-3
Cell Lysate
WB: α-GST
ASK2
14-3-3
ASK2

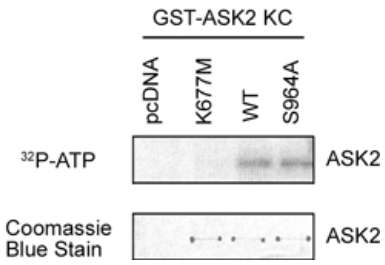
Figure 2.1-3. Determination of the 14-3-3 binding site within apoptosis signal-regulating kinase 2 (ASK2). (A) Schematic of the predicted 14-3-3 recognition motifs within ASK2. Amino acid sequences surrounding the tested 14-3-3 binding motifs within ASK2 are shown, along with truncations and Ser to Ala mutations used in (C) and (D). Binding results summarized as positive (Y) or negative (N). (B) Competition with a pSer916-ASK2 peptide (NH2-CQPGKRSRSPpSSPRH-COOH) has no effect on the interaction of ASK2 with 14-3-3. Experiments were performed as in Figure 2.1-1d, and the 14-3-3/Raf-1 interaction was used as a control. (C) Mutations in the S916 motif of ASK2 fail to disrupt 14-3-3 binding. Point mutations were generated as shown, and the presence of mutated ASK2 in the 14-3-3 complex was determined as in Figure 2.1-1A. (D) ASK2 S964A abolishes binding to 14-3-3. COS7 cells were co-transfected with 6xHis-14-3-3γ and glutathione *S*-transferase (GST)-ASK2 and GST-ASK2–KC wild-type (WT) or S964A mutants. After transfection, cells were lysed and used in the previously described 14-3-3 affinity pulldown assay.


a
Y Y Y Y
N
← 14-3-3 Binding
912A
914A
916A
917A
964A
KRSRSPSSP
KRCLSYG
ASK2
1
N-term
Kinase
C-term
1288
Y
ASK2-KC
638
1288
Y
ASK2-KC C1042X
638
1041
Y
ASK2-KC V973X
638
972
Y
ASK2-KC N937X
638
936
N
b
pSer916-ASK2 peptide
-- (K50E)
10
20
50
100
(μM)
WB: α-GST
ASK2
6xHis Pulldown
WB: α-Raf-1
Raf-1
WB: α-14-3-3
14-3-3
Cell Lysate
WB: α-GST
ASK2
c
6xHis-14-3-3 γ
GST-ASK2-KC:
WT
WT (K50E)
S912A
S914A
S916A
S917A
6xHis Pulldown
WB: α-GST
ASK2
WB: α-14-3-3
14-3-3
Cell Lysate
WB: α-GST
ASK2
d
6xHis-14-3-3 γ
GST-ASK2
GST-ASK2-KC
WT
S964A
WT
S964A
6xHis Pulldown
WB: α-GST
ASK2
WB: α-14-3-3
14-3-3
Cell Lysate
WB: α-GST
ASK2

peptide representing the residues surrounding S916 (pSer916-ASK2). Unexpectedly, this peptide failed to compete with ASK2 for 14-3-3 binding, even at concentrations up to 100μm (Figure 2.1-3B). Intriguingly, this peptide also did not disrupt the interaction of 14-3-3 with Raf-1, another well-defined 14-3-3 client protein. However, it remained possible that this peptide did not have the correct conformation for effective competition. To specifically test whether ASK2 S916 was necessary for 14-3-3 binding, we performed site-directed mutagenesis to generate a nonphosphorylatable GST-ASK2 S916A mutant. GST-ASK2 S916A was fully capable of binding to 14-3-3γ (Figure 2.1-3C). We also mutated the serines within and around this predicted 14-3-3 binding motif in ASK2, generating S912A, S914A and S917A. However, none of these mutations showed any effect on the ASK2/14-3-3 association (Figure 2.1-3C). The failure of these mutations to inhibit ASK2 binding with 14-3-3, together with the lack of peptide competition using the putative phosphorylated motif, suggests that 14-3-3 targets a structure other than the S916-mediated motif for binding.

In search of the 14-3-3 binding site, we generated a series of deletion mutants within GST-ASK2–KC to further narrow down the binding region (depicted in Figure 2.1-3A). Using these truncations, we localized the binding site within the ASK2 sequence between N937 and V973 (data not shown). Examination of the amino acid sequence within this region revealed that the most likely site for 14-3-3 recognition was a motif surrounding serine 964 (RCLS964YG; Figure 2.1-3A). We then mutated the S964 residue to alanine (S964A), and used this mutant in an affinity pulldown assay. Mutation of this site did not affect the autokinase activity of ASK2 (Supplementary Figure 2.1-S1).

Figure 2.1-S1. 14-3-3 interaction has no effect on ASK2 kinase activity. Overexpressed GST-ASK2-KC (WT, S964A, K677M) was isolated from COS7 cells with glutathione resin. Kinase activity of ASK2 was assayed by the incorporation of ^{32}P-ATP into ASK2 (30 min) and revealed by autoradiography. Inactive kinase K677M was used as a negative control. Lower panel shows equal amount of ASK2 proteins were used in the assay with a Coomassie Blue stained gel.


GST-ASK2 KC
pcDNA
K677M
WT
S964A
32P-ATP
ASK2
Coomassie Blue Stain
ASK2

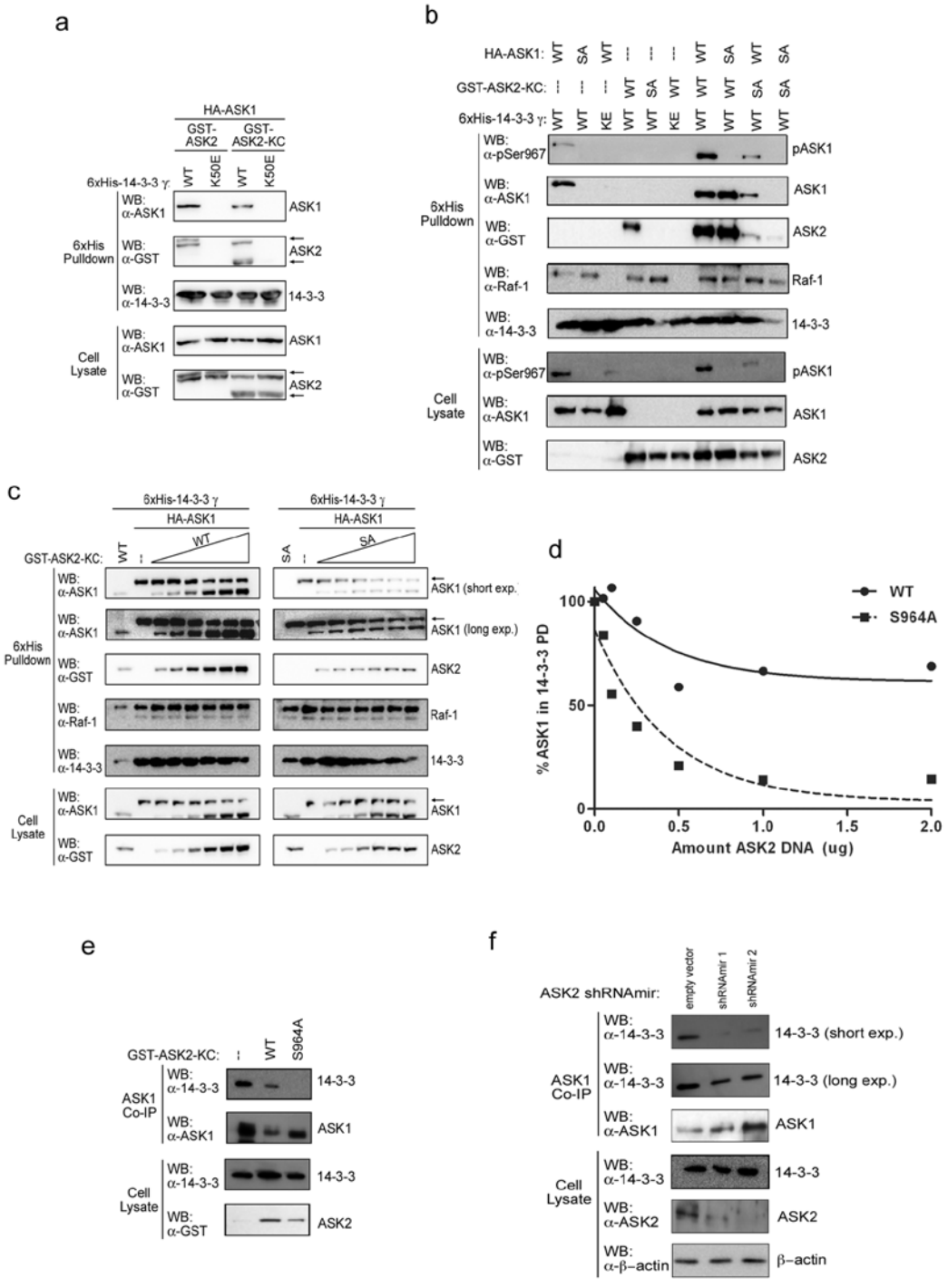
However, the single S964A point mutation alone was sufficient to diminish ASK2's interaction with 14-3-3 (Figure 2.1-3D), suggesting that phosphorylation at S964 has an essential function in 14-3-3 binding. Although minimal 14-3-3 binding was still seen to the S964A mutant, the significant loss of binding indicates that S964 is a critical 14-3-3 binding site. Thus, this study identifies S964 as the primary 14-3-3 recognition site within the ASK2 protein, and defines a novel, noncanonical 14-3-3 motif that may be present in other 14-3-3 client proteins.

14-3-3 is present in a ternary complex with ASK1 and ASK2

It is interesting to note that 14-3-3 interacts with both ASK2 and ASK1 (187) (Figure 2.1-1). This suggests the possibility of the formation of an ASK2/ASK1/14-3-3 ternary complex within the ASK1 signalosome. To test this model, affinity pulldown assays were performed with COS7 cell lysates transfected with HA-ASK1, GST-ASK2 and 6xHis-14-3-3γ. Indeed, both ASK1 and ASK2 were present in the resulting 14-3-3 protein complex (Figure 2.1-4A). This complex occurred independently of the expression of either the full-length or the KC versions of GST-ASK2. Further, this ternary complex was confirmed with reverse pulldown assays, which showed that 14-3-3 and ASK1 were found in the GST–ASK2 complex, and 14-3-3 and ASK2 were in the HA–ASK1 complex (data not shown). Neither ASK1 nor ASK2 was present in the 6xHis-14-3-3γ K50E pulldown, suggesting that the ternary complex was mediated specifically by the amphipathic groove of 14-3-3. However, the above results did not rule out the possibility that ASK1/14-3-3 and ASK2/14-3-3 may be present in separate binary protein

Figure 2.1-4. Association of apoptosis signal-regulating kinase 2 (ASK2) with ASK1 and 14-3-3 in a ternary complex. (A) ASK2, ASK1 and 14-3-3 exist in a multi-protein complex. HA-ASK1 and glutathione *S*-transferase (GST)-ASK2 were co-transfected into COS7 cells with either hexahistidine (6xHis)-14-3-3γ wild type (WT) or K50E. Cell lysates were used in a 14-3-3 affinity pulldown assay, and the presence of ASK1 and ASK2 was revealed by anti-HA and anti-GST antibodies in a western blot. (B) Phosphorylation status of ASK2 S964 controls the association of 14-3-3 with ASK1. COS7 cells were transfected with the expression plasmids for 6xHis-14-3-3γ (WT or K50E), HA-ASK1 (WT or S967A) and GST-ASK2–KC (WT or S964A). 14-3-3γ protein complexes were isolated by affinity pulldown and associated ASK1, ASK2 and Raf-1, and the phosphorylation status of ASK1 at S967 was detected using the respective antibodies by western blot. (C) Increased expression of ASK2 S964A is correlated with decreased association of ASK1 from 14-3-3. COS7 cells were co-transfected with HA-ASK1 WT and 6xHis-14-3-3γ, along with increasing amounts of expression vectors for either GST-ASK2–KC WT or S964A. The amount of ASK1 in the resulting 14-3-3 affinity pulldown complex from each sample was determined as in Figure 2.1-1a. (D) Quantification of data from (c). The relative percentage of ASK1 in each 6xHis-14-3-3γ pulldown sample is shown. Lysates containing GST-ASK2–KC WT and S964A are indicated by solid circles (•) and squares (▪), respectively. (E) ASK2 S964A mutation diminishes the association between endogenous ASK1 and 14-3-3. HeLa cells were transfected with pcDNA or GST-ASK2–KC (WT or S964A). Following transfection, endogenous ASK1 was immunoprecipitated from the cell lysates using a specific anti-ASK1 antibody. Endogenous 14-3-3 in the ASK1 co-immunoprecipitation (co-IP) was

detected with a pan anti-14-3-3 antibody by western blot. (F) ASK2 knockdown diminishes endogenous ASK1/14-3-3 binding. HeLa cells were transfected with shRNAmir constructs targeting ASK2 or pGIPz empty vector control. Cells were selected with puromycin, and endogenous ASK1 was immunoprecipitated from lysates using a specific anti-ASK1 antibody. Endogenous 14-3-3 in the ASK1 co-IP was detected with a pan anti-14-3-3 antibody by western blot.

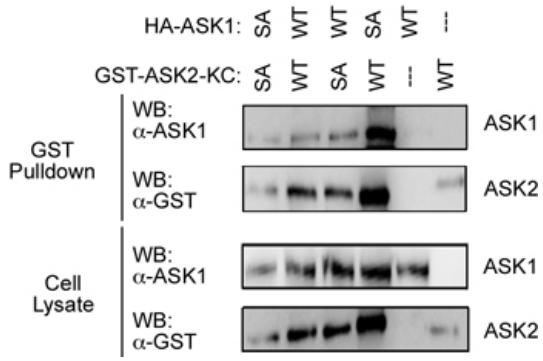

a
HA-ASK1
GST-ASK2
GST-ASK2-KC
WT
K50E
6xHis-14-3-3 γ
6xHis Pulldown
Cell Lysate
WB: α-ASK1
WB: α-GST
WB: α-14-3-3
ASK1
ASK2
14-3-3
b
HA-ASK1:
GST-ASK2-KC:
6xHis-14-3-3 γ:
WT
SA
KE
WB: α-pSer967
WB: α-ASK1
WB: α-GST
WB: α-Raf-1
WB: α-14-3-3
pASK1
ASK1
ASK2
Raf-1
14-3-3
c
6xHis-14-3-3 γ
HA-ASK1
GST-ASK2-KC:
WT
SA
ASK1 (short exp.)
ASK1 (long exp.)
ASK2
Raf-1
14-3-3
d
% ASK1 in 14-3-3 PD
Amount ASK2 DNA (ug)
WT
S964A
e
GST-ASK2-KC:
WT
S964A
ASK1 Co-IP
Cell Lysate
f
ASK2 shRNAmir:
empty vector
shRNAmir 1
shRNAmir 2
WB: α-14-3-3
WB: α-ASK1
WB: α-ASK2
WB: α-β-actin
14-3-3 (short exp.)
14-3-3 (long exp.)
ASK1
ASK2
β-actin

complexes. We therefore set out to test the specificity and functional significance of this ternary complex.

ASK2/14-3-3 interaction dictates ASK1/14-3-3 interaction

The interaction of 14-3-3 with both ASK2 and ASK1 is dynamically regulated by phosphorylation through S964 of ASK2 and S967 of ASK1 (187) (Figure 2.1-3D). Point mutation of these sites to alanine allows the generation of ASK1 or ASK2 proteins that are specifically defective in 14-3-3 binding, permitting the examination of 14-3-3's contribution to the ASK1/ASK2 complex. We used 14-3-3-binding defective mutants of ASK1 (S967A) and ASK2 (S964A) to establish the interplay among 14-3-3, ASK2 and ASK1 in the ternary protein complex. These point mutations did not affect the interaction between ASK1 and ASK2 (Supplementary Figure 2.1-S2). The ASK2/14-3-3 interaction seems to be independent of the ASK1/14-3-3 interaction, as overexpression of neither ASK1 WT nor S967A induced any detectable change in ASK2/14-3-3 binding (Figure 2.1-4B). However, the ability of ASK2 to bind 14-3-3 showed a dramatic impact on 14-3-3's interaction with ASK1. As previously shown, ASK1 WT is associated with 14-3-3, whereas ASK1 S967A has diminished 14-3-3 binding (187). Unexpectedly, when ASK2–KC WT was overexpressed along with ASK1 S967A and 14-3-3γ, ASK1 S967A was found in the 14-3-3γ complex (Figure 2.1-4B). One explanation for these results is that enhanced ASK2 WT expression increased the amount of ASK1 S967A in the ASK2/ASK1 protein complex, which was then pulled down with 14-3-3. However, overexpression of mutated ASK2 (S964A) dramatically decreased the amount of ASK1 WT in the 14-3-3 complex, lending strong support to a functional and regulated ternary protein association (Figure 2.1-4B). Importantly, the effect of ASK2 S964A could be

Figure 2.1-S2. 14-3-3 interaction is not required for ASK1/ASK2 binding. COS7 cells were transfected with HA-ASK1 (WT or S967A) and GST-ASK2-KC (WT or S964A). The ASK2-KC complex was isolated with glutathione conjugated resin. The presence of ASK1 in the GST-ASK2 complex was determined by Western blot.


HA-ASK1: SA WT WT SA WT --
GST-ASK2-KC: SA WT SA WT -- WT
GST Pulldown
WB: α-ASK1
ASK1
WB: α-GST
ASK2
Cell Lysate
WB: α-ASK1
ASK1
WB: α-GST
ASK2

recapitulated with endogenous 14-3-3/ASK1 interaction as well (Figure 2.1-4E). To further validate the ASK2 effect, dose–response experiments were carried out. Increasing amounts of ASK2 S964A, but not WT, led to a corresponding decrease in the amount of ASK1 appearing within the 14-3-3 complex (Figures 2.1-4C and D).

We further explored the specificity of the ASK2/ASK1/14-3-3 ternary complex formation by probing the effect of ASK2 or ASK1 on the interaction of 14-3-3 with Raf-1, another well-characterized MAP3K that binds 14-3-3. Interestingly, endogenous Raf-1 binding to 14-3-3 was unaffected by the overexpression of either WT or mutated ASK1 or ASK2 (Figures 2.1-4B and C). These data suggest that the ASK2/ASK1/14-3-3 ternary complex is distinct from the 14-3-3/Raf-1 complex.

To further support the hypothesis that ASK2 regulates the ASK1/14-3-3 interaction, we performed ASK2 silencing experiments and examined the effect of reducing ASK2 on endogenous ASK1/14-3-3 binding (Figure 2.1-4F). When ASK2 expression was knocked down, endogenous 14-3-3 binding to ASK1 was greatly reduced, indicating that ASK2 has a substantial role in regulating the interaction between these two proteins. Together, these results show that ASK2, ASK1 and 14-3-3 form a specific and unique ternary complex, in which the interaction between ASK2 and 14-3-3 determines the extent of ASK1 binding to 14-3-3.

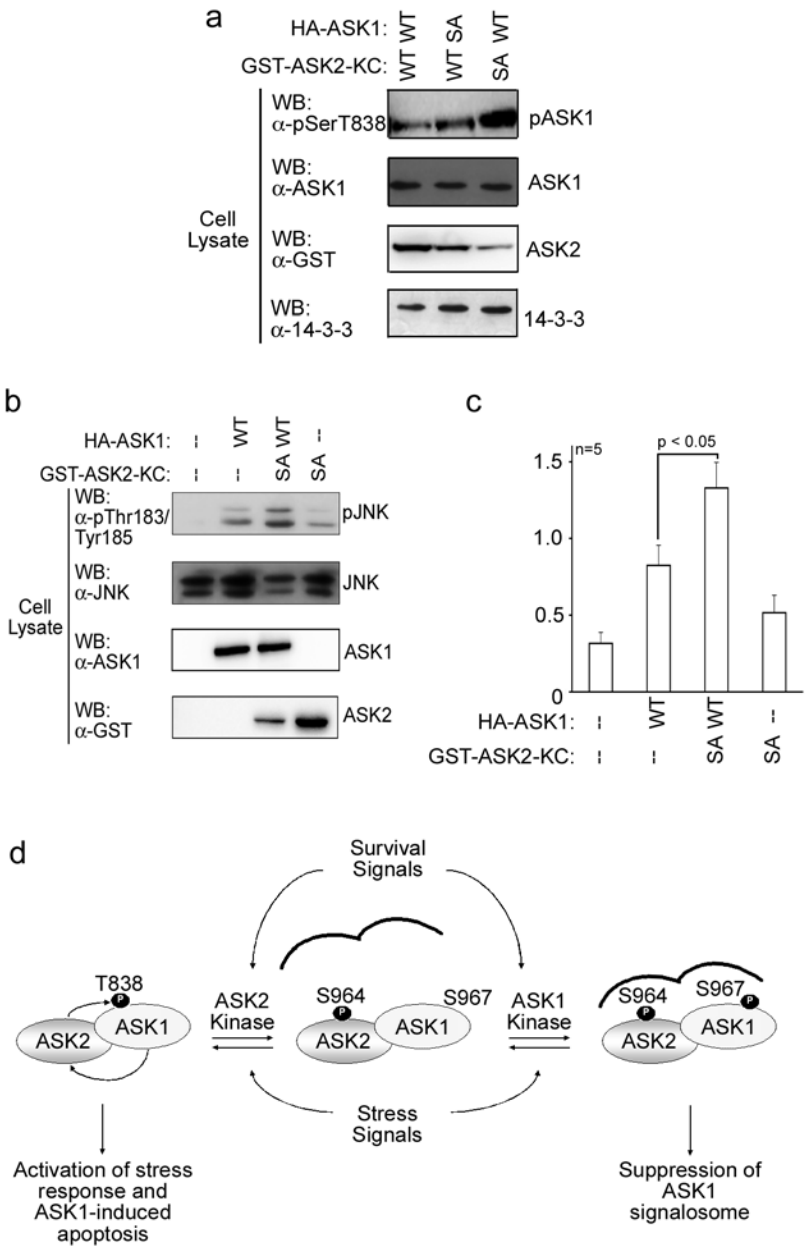
ASK2/14-3-3 interaction controls ASK1 function

Previous studies have shown that 14-3-3 binding suppresses ASK1 activity by maintaining S967 in a phosphorylated state, inhibiting ASK1-mediated JNK pathway activation (61; 187; 188). We reasoned that ASK2 might exert control over ASK1 by

dictating the action of 14-3-3 within this ternary complex. To probe the functional consequence of the dynamically regulated ASK2/14-3-3 interaction, we examined the effect of the 14-3-3-binding defective mutant of ASK2 (S964A) on the phosphorylation status of ASK1 at S967 and the activation state of ASK1. Indeed, co-expression of ASK2 S964A resulted in a decrease in ASK1 S967 phosphorylation, an effect not observed on ASK2 WT expression (Figure 2.1-4B). Although ASK1 S967A showed enhanced ASK1 activity (T838 phosphorylation) over ASK1 WT, the ASK1 WT activity was also stimulated on co-expression with ASK2 S964A, supporting an ASK2-mediated inhibitory role of 14-3-3 in the ternary complex (Figure 2.1-5A).

As an additional biological readout of ASK1 activity, we monitored JNK activation. As we have previously shown, dephosphorylation of ASK1 at S967 is correlated with an increase in ASK1 kinase activity and ASK1-induced apoptotic signaling (59; 187). After co-expression of ASK1 with ASK2 (WT or S964A), JNK activation was determined by western blot with an antibody directed against phosphorylated T183/Y185 of JNK (Figures 2.1-5B and C). Co-expression of ASK2–KC S964A showed a statistically significant increase in JNK activation ($p=0.028$) compared with ASK1 expression plus control pcDNA. No statistically significant increase in JNK activation was seen with expression of ASK2–KC S964A alone ($p=0.130$). These results indicate that ASK2's interaction with 14-3-3 is critical to allow 14-3-3 to engage and suppress ASK1, leading to a reduction in ASK1-mediated JNK signaling. Further evidence in support of a physiological role of an ASK2/14-3-3 connection in the regulation of the ASK1–JNK axis is provided by our peptide inhibitor study, in which disruption of the ASK2/ASK1 interaction was correlated with decreased ASK1/14-3-3

Figure 2.1-5. Effect of apoptosis signal-regulating kinase 2 (ASK2)/14-3-3 association on ASK1 function. (A) The phosphorylation state of ASK2 at S964 affects ASK1 activation. COS7 cells were co-transfected with HA-ASK1 (wild type (WT) or S967A) and glutathione S-transferase (GST)-ASK2–KC (WT or S964A). Activation of ASK1 was assessed by phosphorylation of T838 determined by western blot with a phospho-T838 specific antibody. (B) Expression of ASK2 S964A enhances ASK1–c-Jun N-terminal kinase (JNK) signaling. COS7 cells were transfected with HA-ASK1 and either GST-ASK2–KC S964A or a control pcDNA vector. JNK activation status in the resulting cell lysates was revealed by western blot with an anti-pT183/Y185 JNK specific antibody. (C) Quantification of pJNK to JNK ratio. Values given are mean±standard error (n=5). (D) Working model. Under stress conditions, ASK1 and ASK2 exist in a heteromeric complex, and are dephosphorylated at S967 and S964, respectively. In this complex, ASK2 facilitates ASK1 activation through phosphorylation of T838, whereas ASK1 exhibits positive feedback regulation on ASK2 through stabilization of the ASK2 protein (Osaka et al., 2007). These activities culminate in the activation of ASK1 downstream signals, leading to ASK1-induced apoptosis. Conversely, under conditions of cell survival, ASK2 S964 is phosphorylated through a pro-survival kinase-signaling cascade, generating a high-affinity 14-3-3 docking site. On phosphorylation at S964, ASK2 recruits 14-3-3 to the ASK2/ASK1 complex, leading to an interaction with ASK1 through phosphorylated S967 and subsequent suppression of the ASK1 signalosome.


a
HA-ASK1:
GST-ASK2-KC:
WT WT
WT SA
SA WT
WB: α-pSerT838
pASK1
WB: α-ASK1
ASK1
Cell Lysate
WB: α-GST
ASK2
WB: α-14-3-3
14-3-3
b
HA-ASK1:
GST-ASK2-KC:
WT
SA WT
SA
WB: α-pThr183/ Tyr185
pJNK
WB: α-JNK
JNK
Cell Lysate
WB: α-ASK1
ASK1
WB: α-GST
ASK2
c
n=5
p < 0.05
1.5
1.0
0.5
0
HA-ASK1:
GST-ASK2-KC:
WT
SA WT
SA
d
Survival Signals
T838
ASK2
ASK1
ASK2 Kinase
S964
S967
ASK2
ASK1
ASK1 Kinase
S964
S967
ASK2
ASK1
Stress Signals
Activation of stress response and ASK1-induced apoptosis
Suppression of ASK1 signalosome

interaction and H_2O_2-induced JNK activation (M Puckett, L Cockrell and H Fu, unpublished data).

Discussion

Our data suggest a novel mechanism of ASK1 signalosome regulation, through ASK2-mediated recruitment of 14-3-3 proteins under certain physiological conditions, such as cells receiving survival signals (Figure 2.1-5d). Our proposed model predicts an ASK2 kinase that phosphorylates S964 in response to a survival-promoting signal, which in turn induces association of ASK2 with 14-3-3 and recruits 14-3-3 to the ASK2/ASK1 complex. This allows 14-3-3 to bind to and suppress ASK1 through phosphorylated S967. Conversely, signals that promote dephosphorylation of ASK2 at S964 are expected to disengage 14-3-3 from ASK2, triggering 14-3-3's dissociation from the ASK1 complex. Once removed, the ASK1 signalosome is free of 14-3-3-imposed inhibition, allowing activation of downstream effector pathways, such as JNK stimulation. In this way, ASK2 functions as a dual regulator in the ASK1 signalosome: serving as an ASK1 activator under stress conditions (40) while acting as an ASK1 suppressor by recruiting 14-3-3 under other conditions such as cell survival. Thus, ASK2 provides a signal integration point by which external or internal environmental signals are faithfully transmitted to ASK1 through engaging the phospho-binding protein 14-3-3, leading to either activation or suppression of ASK1 signaling. Even though 14-3-3 also interacts with another MAP3K Raf-1, the interaction status of ASK2 with 14-3-3 showed no obvious effect on the 14-3-3/Raf-1 association, suggesting a functionally distinct ASK2/14-3-3/ASK1 signaling complex. Because 14-3-3 proteins bind most members of the MAP3K family, this may represent a general mechanism by which 14-3-3 regulates

signal relay among MAP3Ks to ensure signaling integrity in various physiological processes.

Chapter 2.2: Distinct Regions within ASK1 and ASK2 facilitate kinase interaction

Mary C. Puckett, Lisa M. Cockrell, Mia DeSimone, and Haian Fu

Abstract

Jun N-terminal kinases (JNKs) respond to stress signals and are involved in cellular processes such as apoptosis, proliferation, metabolism, and DNA repair. Dysregulated activation of JNK signaling has been implicated in numerous disease states, including neurodegenerative diseases, chronic inflammatory disease, ischemia, diabetes, and cancer. The apoptosis signal regulating kinase 1 (ASK1) plays an important role in JNK signaling. Recent evidence suggests the ASK1 binding partner ASK2 is intricately involved in JNK regulation, but the mechanism by which this occurs is unknown. We have identified the critical residues needed for ASK1/ASK2 interaction through deletion mapping and binding assays, and these residues comprise a regulatory element for JNK signaling. Expression of this regulatory element can reduce JNK phosphorylation in response to stress signals, including hydrogen peroxide, while ASK2 peptides without this regulatory element have no effect on JNK phosphorylation. These results indicate ASK2 plays a critical regulatory role in ASK1-mediated JNK activation and could serve as a basis for the development of a small molecule or peptide inhibitor of JNK signaling.

Introduction

Depending on the cellular context, ASK1 signaling can lead to apoptosis, cellular differentiation, or even cell survival. ASK1 functions in a large protein complex, termed the ASK1 signalosome. In this complex, many interacting partners regulate ASK1's function (31). Thioredoxin, in its reduced form, can bind ASK1, keeping it in an inactive conformation. In the presence of ROS, the cysteine residues on thioredoxin can be oxidized, and the protein can no longer bind ASK1. This allows for the recruitment of TRAF2/6 to the ASK1 signalosome, which facilitates formation of the active conformation of ASK1 (43) . As a mitogen activated protein kinase kinase kinase (MAP3K), active ASK1 can phosphorylate and activate MAP2Ks, which can in turn phosphorylate and activate MAPKs. Specifically, ASK1 can phosphorylate the MAP2Ks MKK3/6 and MKK4/7 leading to the activation of the MAPKs p38 and JNK, respectively. Additionally, like ROS, ER stress can activate ASK1. Under ER stress conditions, IRE1, a transmembrane protein located in the ER, can bind TRAF2, which recruits ASK1 (21). This process can activate the ASK1-JNK signaling pathway and lead to apoptosis.

ASK1 activity is also regulated by various kinases, phosphatases, and other binding proteins. One such protein is ASK2, a MAP3K with significant homology to ASK1. Interestingly, ASK2 seems to both activate and inhibit ASK1 signaling under various stress conditions. ASK2 can directly activate ASK1 through phosphorylation of Thr838 in its kinase domain. ASK1, however, cannot phosphorylate the equivalent threonine residue on ASK2, but it can stabilize ASK2 through physical interaction (40). Knockout of ASK2 renders cells insensitive to ROS induced cell death, indicating that

ASK2 is required for ASK1 activation in response to this stress signal (40). Similarly, in a two-step skin tumorigenesis model, knockout of ASK2 increased papilloma formation and decreased apoptosis (196). On its own, however, ASK2 can only weakly activate JNK in response to H_2O_2 treatment. Recent evidence from our lab indicates that ASK2 may also regulate ASK1/14-3-3 binding. When ASK1 is bound to 14-3-3, it is unable to promote apoptosis (187). Our evidence shows that decreased ASK2/14-3-3 interaction results in significantly reduced ASK1 bound to 14-3-3, indicating that ASK2/14-3-3 binding is needed for 14-3-3 inhibition of ASK1 (Chapter 2.1).

While these results suggest ASK2 is a crucial regulator of ASK1 signaling, many questions remain as to how this occurs. ASK1 and ASK2 are known to bind, and the C-terminus of ASK1 has been shown to be sufficient for ASK2 binding, but little is known about the details of the interface between these two proteins. Here we describe the binding interface of ASK1 and ASK2, as well as consequences of binding disruption. With a better understanding of how these two proteins interact, we may be able to identify potential sites for future therapeutic development.

Materials and Methods

Plasmids and reagents. HA-ASK1, HA-ASK2, and Flag-ASK2 were present in pcDNA3 vectors and have been previously described (187). GST-ASK1 and GST-ASK2 truncations and peptide fragments were inserted in pDEST27 mammalian expression vector using Gateway cloning (Invitrogen). DNA was purified from E. coli with the Qiagen Maxi Kit. H_2O_2 was obtained from Sigma.

Cell culture and transfections. COS7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, CellGro) with 10% fetal bovine serum (FBS, Atlanta Biologicals) and 1% penicillin/streptomycin (Life Technologies) at 37ºC with 5% CO_2 in a humidified incubator. DNA transfection was performed with FuGENE HD reagent (Promega) according to the manufacturer's instructions.

Cell lysis and immunoblotting. Cells were lysed 48 hr after transfection in 200 µL (GST pull-down and immunoprecipitations) or 60µL (TR-FRET) of 0.2% lysis buffer [0.2% Nonidet P-40 (NP-40); 10 mM HEPES, pH 7.4; 150 mM NaCl; 1:1000 dilution phosphatase inhibitor cocktail (Sigma), 1:1000 dilution protease inhibitor cocktail (Sigma)]. Proteins were resolved by SDS-PAGE and immuno-blotted with specific antibodies: GST (Z-9), ASK1 (F-9), and HA-probe antibodies were obtained from Santa Cruz. Secondary antibodies used were horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz) and horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz) for monoclonal and polyclonal primary antibodies respectively. Proteins were visualized using enhanced West Pico or West Dura (Thermo Scientific).

Time-resolved fluorescence resonance energy transfer. 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% NP40). GST-Terbium (HTRF) conjugated antibody was used couple GST-ASK2 as a FRET donor. Venus-ASK1 served as a FRET acceptor. Samples were incubated at room temperature for 1 hr and FRET signal was measured on the Envision spectrophotometer (Excitation 337 nm and Emission 520 nm).

GST pull-down and immunoprecipitation Assays. To perform GST pull-down assays, 160 μL of lysate was added to 25 μL of a 50% slurry of glutathione beads (GE Healthcare) in lysis buffer and samples were rotated slowly at 4ºC for 2-4 hr. For immunoprecipitations, 160 μL of lysate was added to 25 μL of a 50% slurry of Protein G beads (GE Healthcare) and samples were rotated slowly at 4ºC for 30 min to clear lysates. Supernatant was collected, added to 2 μL of anti-ASK1 antibody (F-9, Santa Cruz), and rotated slowly at 4ºC for 2 hr. 25 μL of 50% slurry of Protein G beads was added, and samples were rotated an additional two hours. Beads were washed three times with 200 μL of lysis buffer to decrease nonspecific binding. Beads were then boiled in 20 μL of 2X SDS sample buffer for 5 min to recover bound proteins for analysis by SDS-PAGE and Western blot.

Split-luciferase protein complementation assay. ASK1 fused to the C-terminal half of Renilla luciferase was overexpressed with ASK2 protein fragments fused to the N-terminal half of Renilla luciferase. 48 hr after transfection, cells were lysed and benzyl-coelenterazine was added to the lysates in a final concentration of 50 μM. Total luminescence was read with the Envision spectrophotometer.

Results

ASK1 and ASK2 interact via distinct regions within their C-termini

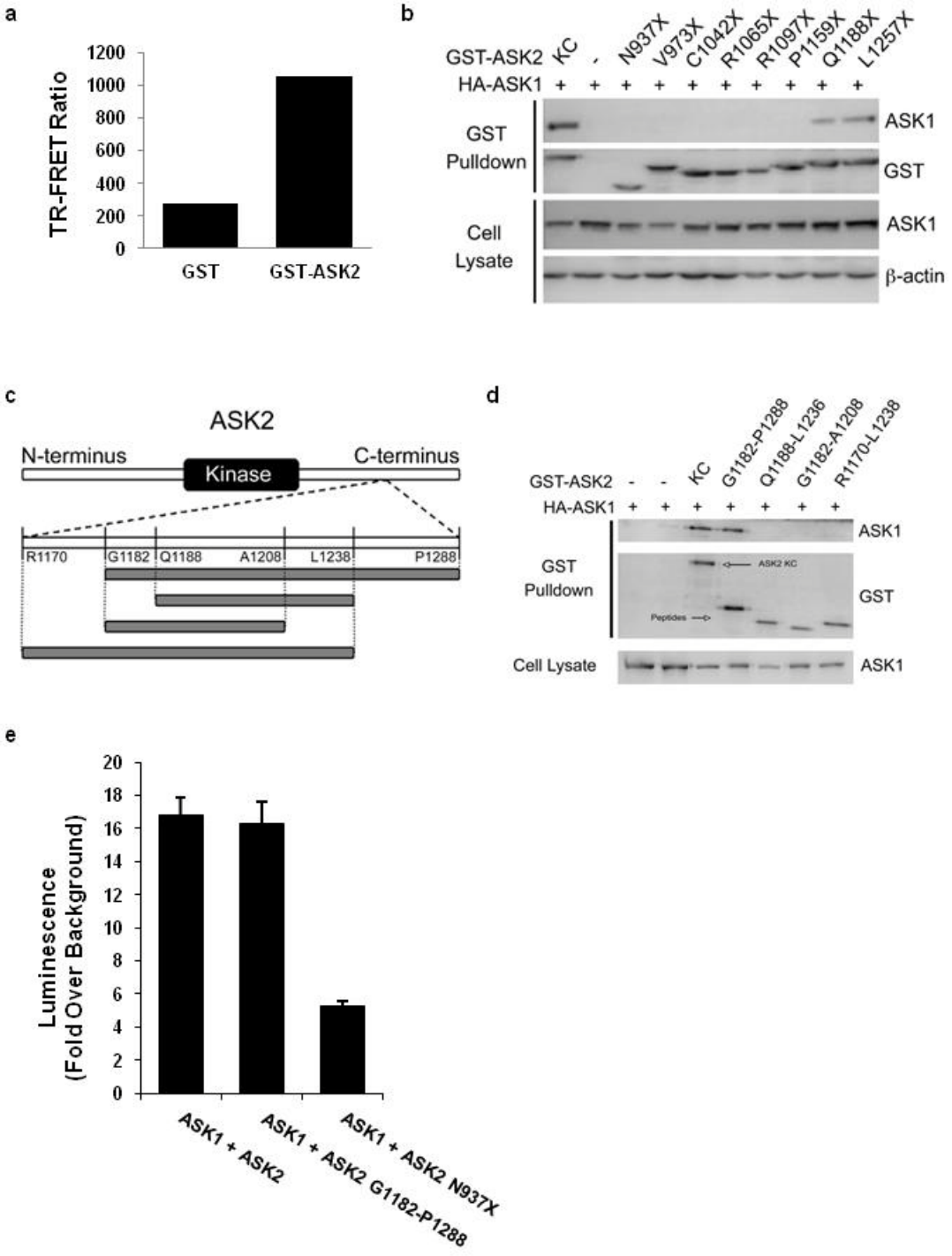
ASK2 has been previously shown to form a complex with ASK1 (40; 197), and this complex is important for activation of both kinases. To confirm the interaction

between ASK1 and ASK2, we used time resolved fluorescence resonance energy transfer (TR-FRET) to assess direct interaction between these two proteins. A positive TR-FRET signal can only be obtained when proteins are within 100 Angstroms of one another, a distance indicating direct interaction. We obtained a positive signal via TR-FRET when Venus-ASK1 was overexpressed with GST-ASK2 but not when overexpressed with GST alone, confirming previous reports that ASK1 and ASK2 directly interact (Figure 2.2-1A).

To better understand this interaction, we next used an iterative deletion approach to determine the regions of ASK1 and ASK2 that are important for protein-protein interaction. When truncated forms of ASK2 were co-expressed with ASK1, only truncations containing regions after amino acid P1159 were able to bind ASK1, indicating that this region was necessary for protein-protein interaction (Figure 2.2-1B). To further narrow down the specific region of ASK2 that mediates ASK1 binding, we constructed a series of protein fragments spanning the C-terminal region of ASK2 (Figure 2.2-1C). When these fragments were tested for ASK1 interaction, only fragments amino acids G1182-P1288 showed binding, indicating that this particular region within the C-terminus is important for ASK2/ASK1 interaction (Figure 2.2-1D).

To confirm this interaction, we used a protein complementation assay (PCA) as an additional approach. For this assay, we overexpressed ASK1 fused to the C-terminus of Renilla luciferase with ASK2 fragments fused to the N-terminus of Renilla luciferase. In this assay, binding of any ASK2 fragments with ASK1 brings the two halves of the Renilla luciferase in close enough proximity to reconstitute an active enzyme, after which luminescence can be measured with after the addition of an appropriate substrate such as

Figure 2.2-1. ASK2 binds ASK1 via a 100 amino acid region. (A) ASK1 and ASK2 directly interact. Cell lysates containing overexpressed Venus-ASK1 and GST-ASK2 or GST alone were mixed, and fluorescence was measured with the Envision spectrophotometer. (B) ASK1 interaction with ASK2 truncations. HA-ASK1 was overexpressed in COS7 cells with indicated GST-ASK2 truncations. ASK2 complexes were isolated by GST pull-down and bound ASK1 was assessed by Western blot. (C) Schematic of ASK2 peptides. (D-E) ASK1 interacts with G1182-P1288 of ASK2. (D) HA-ASK1 was overexpressed in COS7 cells with indicated GST-ASK2 peptides. ASK2 complexes were isolated by GST pull-down and bound ASK1 was assessed by Western blot. (E) ASK1 tagged with the C-terminal half of Renilla luciferase was overexpressed with ASK2 fragments tagged with the N-terminal half of Renilla luciferase.


a
TR-FRET Ratio
1200
1000
800
600
400
200
0
GST
GST-ASK2
b
GST-ASK2
KC
N937X
V973X
C1042X
R1065X
R1097X
P1159X
Q1188X
L1257X
HA-ASK1
GST Pulldown
ASK1
GST
Cell Lysate
ASK1
β-actin
c
ASK2
N-terminus
Kinase
C-terminus
R1170
G1182
Q1188
A1208
L1238
P1288
d
GST-ASK2
KC
G1182-P1288
Q1188-L1236
G1182-A1208
R1170-L1238
HA-ASK1
GST Pulldown
ASK1
ASK2 KC
Peptides
GST
Cell Lysate
ASK1
e
Luminescence (Fold Over Background)
20
18
16
14
12
10
8
6
4
2
0
ASK1 + ASK2
ASK1 + ASK2 G1182-P1288
ASK1 + ASK2 N937X

benzyl-coelenterazine. In this assay, the greatest interaction of ASK1 and ASK2 was also seen by G1182-P1288, supporting the importance of this region in ASK1/ASK2 binding (Figure 2.2-1E).

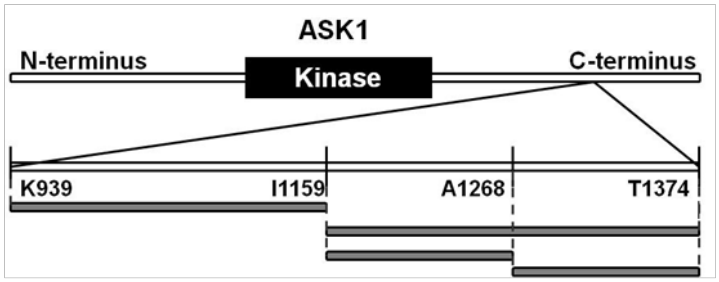
Conversely, the C-terminus of ASK1 has previously been identified as the interaction site for ASK2 (40). However, to better understand this region, we created protein fragments of the ASK1 C-terminus (Figure 2.2-2A) and assessed them for binding to ASK2. When GST-ASK1 fragments were overexpressed with HA-ASK2 and the ASK1 fragments were isolated by GST pull-down, ASK2 showed the strongest interaction with the second half of the c-terminus (I1159-T1374), indicating this region is important for ASK1/ASK2 interaction. Additionally, the fragment I1159-A1268 showed binding to ASK2 with A1268-T1374, though at significantly lower levels than the larger C-terminal fragment, suggesting the critical residues for ASK1/ASK2 interaction may lie within amino acids I1159-A1268 (Figure 2.2-2B).

Expression of an ASK2 peptide reduces ASK1 signaling

Because interaction of ASK1 and ASK2 has been shown to play important roles in the regulation of both kinases, we wondered if disruption of this interaction could be achieved and if this disruption would have any effect on downstream signaling. To test this, we overexpressed ASK1 and ASK2 peptides consisting of the minimal binding regions we identified, ASK1 I1159-A1268 and ASK2 G1182-P1288. The ASK1 peptide was capable of reducing ASK1/ASK2 interaction as seen by western blot (Figure 2.2-3A). Additionally, expression of the ASK2 peptide G1182-P1288 decreased phosphorylation of JNK, a downstream component of ASK1 signaling, in response to

Figure 2.2-2. ASK1 interacts with ASK2 via its C-terminus. (A) Schematic of ASK1 peptides. (B) ASK2 interactions with I1159-A1268 of ASK1. HA-ASK2 was overexpressed with indicated GST-ASK1 constructs. GST-ASK1 complexes were isolated by GST pull-down, and bound ASK2 levels were assessed by Western blot.

a


ASK1
N-terminus
Kinase
C-terminus
K939
I1159
A1268
T1374

b

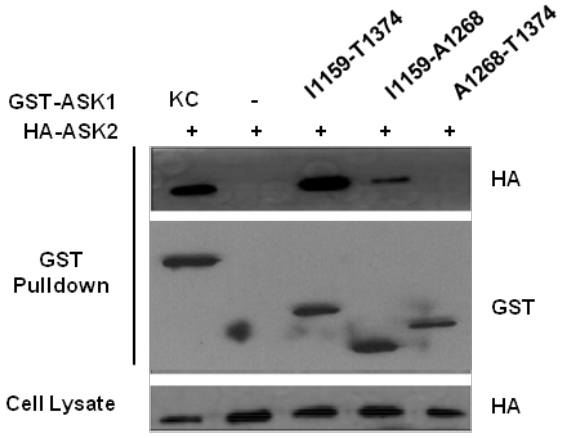
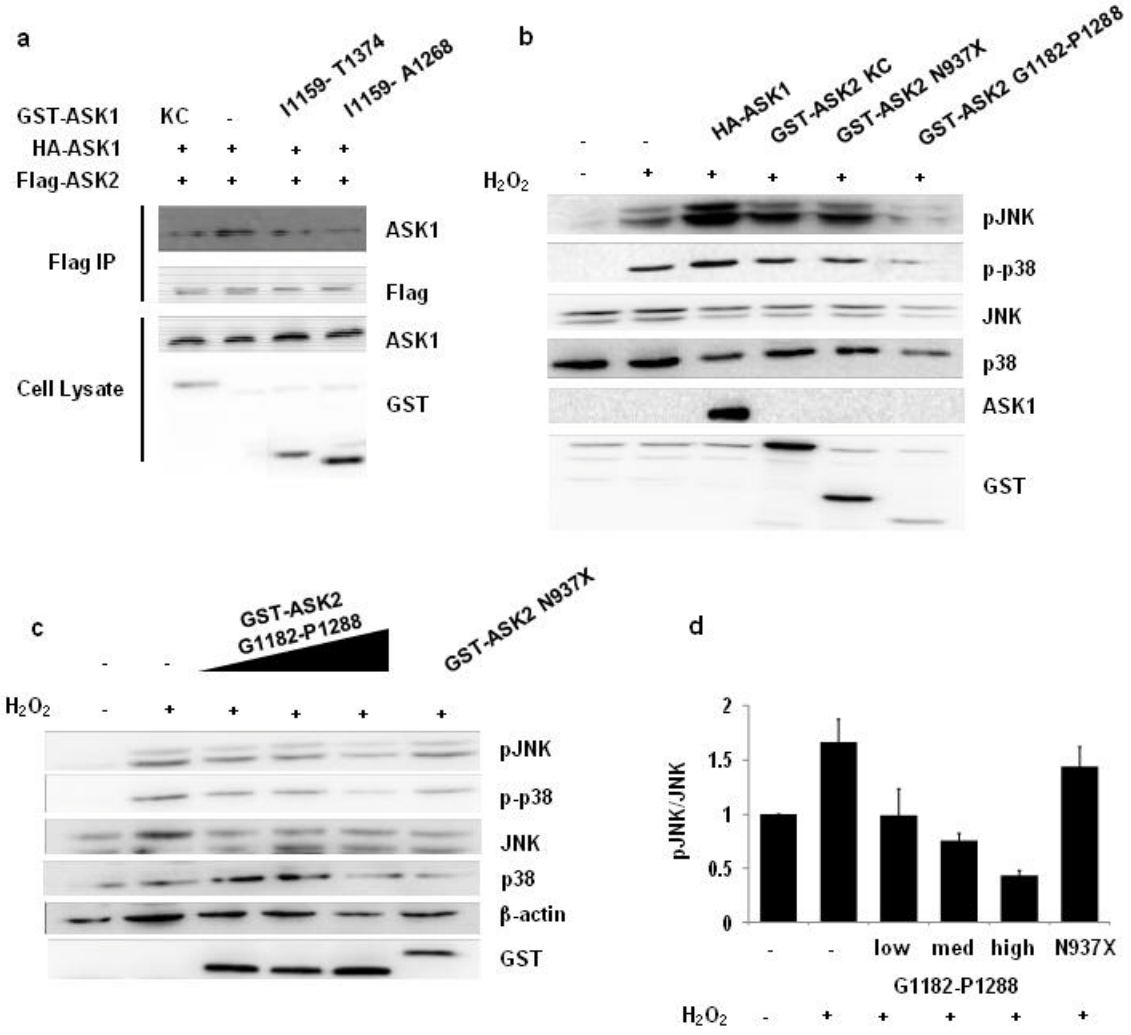

I1159-T1374
I1159-A1268
A1268-T1374
GST-ASK1
KC
-
HA-ASK2
+ + + + +
HA
GST
Pulldown
GST
Cell Lysate
HA

Figure 2.2-3. Inhibition of ASK1/ASK2 interaction decreases downstream signaling. (A) Expression of an ASK1 peptide decreases ASK1/ASK2 interaction. HA-ASK1 was overexpressed with Flag-ASK2 and indicated GST-ASK1 peptides. ASK2 complexes were isolated by Flag IP, and bound ASK1 was measured by western blot. (B) Expression of an ASK2 peptide decreases pJNK. COS7 cells were transfected with the indicated constructs. 40 hr after transfection, cells were treated with 500 μM H_2O_2 for 20 min. (C) Peptide expression decreases pJNK in a dose dependent manner. COS7 cells were transfected with the increasing amounts of GST-ASK2 G1182-P1288 and other indicated constructs. 40 hr after transfection, cells were treated with 500 μM H_2O_2 for 20 min. (D) Quantification of the relative levels of pJNK/JNK in (C). Value given is mean +/- standard error. n=3.


a
GST-ASK1
KC
-
I1159- T1374
I1159- A1268
HA-ASK1
Flag-ASK2
Flag IP
ASK1
Flag
Cell Lysate
ASK1
GST
b
HA-ASK1
GST-ASK2 KC
GST-ASK2 N937X
GST-ASK2 G1182-P1288
H2O2
pJNK
p-p38
JNK
p38
ASK1
GST
c
GST-ASK2
G1182-P1288
GST-ASK2 N937X
H2O2
pJNK
p-p38
JNK
p38
β-actin
GST
d
pJNK/JNK
2
1.5
1
0.5
0
low
med
high
N937X
G1182-P1288
H2O2

H_2O_2 treatment while a fragment of ASK2 that cannot bind ASK1 had no effect on downstream signaling (Figure 2.2-3B). Furthermore, expression of this ASK2 peptide decreased JNK phosphorylation in a dose dependent manner (Figure 2.2-3C-D), suggesting ASK1/ASK2 interaction may be important for stress signaling.

Discussion

ASK1 plays an essential role in the propagation of stress signals, and, as such, it is regulated by a variety of protein-protein interactions (31). One recently described binding partner of ASK1 is ASK2 (40). Here, we have identified the binding regions which regulate this interaction (Figure 2.2-1, 2.2-2). It has been previously reported that ASK1 and ASK2 interact, but here we show that an approximately 100 amino acid region within each protein is sufficient to support interaction. With the production of small ASK1 and ASK2 fragments, this region could likely be narrowed even further.

Additionally, we have shown that ASK1/ASK2 interaction can be impaired by the introduction of a small ASK1 peptide (Figure 2.2-3). While such peptides as the ones we have identified, ASK2 G1182-P1288 and ASK1 I1159-A1268, are likely too large to serve as therapeutics themselves, they do provide a useful tool for the identification of novel small molecules with similar or better effects. Small molecule inhibitors of ASK1 have gained attention recently, but most of them have focused on directly altering ASK1 kinase activity (77). Here we provide a new potential target for modulating ASK1 activity by altering the interaction between ASK1 and ASK2. Overexpression of our ASK2 peptide was sufficient to reduce endogenous ASK1- mediated JNK and p38 phosphorylation in response to H_2O_2 treatment (Figure 3.2-3), indicating this may be a

viable target in diseases, particularly in those where ROS-induced apoptosis has been noted.

ASK1 activation by ROS has been attributed to the cell death seen in ischemia-reperfusion injury (80; 81). ROS induced ASK1 signaling has also be attributed to neuronal cell death in both Parkinson's disease and Alzheimer's disease (76; 77). Similarly, ROS is thought to play a role in ASK1 activation by TNFα and ER stress (27-29), suggesting that ROS mediated ASK1 signaling may be important in variety of other inflammatory and neurodegenerative diseases that induce death by these pathways. Blocking ASK1/ASK2 interaction might relieve or reverse the apoptosis associated with these diseases. While protein-protein interactions have often been viewed as a less than ideal candidate for drug development (4), the more we understand about an interaction, the greater the opportunity to identify potential target sites. Understanding the ASK1/ASK2 interaction could lead to the development of therapies targeting this pathway.

Chapter 3: IKKβ inhibits ASK1 activity through phosphorylation and protein-protein interactions

Chapter 3.1: Integration of the Apoptosis signal-regulating kinase 1-mediated stress signaling with the Akt/PKB-IκB kinase cascade.

Mary C Puckett*, Erinn H Goldman*, Lisa M Cockrell*, Bei Huang, Andrea L Kasinski , Yuhong Du, Cun-Yu Wang, Anning Lin, Hidenori Ichijo, Fadlo R Khuri, Haian Fu.

*Co-first authors

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Abstract

Cellular processes are tightly controlled through well-coordinated signaling networks that respond to conflicting cues, such as reactive oxygen species (ROS), ER stress signals, and survival factors to ensure proper cell function. Here we report a direct interaction between inhibitor of kappa B kinase (IKK) and apoptosis signal-regulating kinase 1 (ASK1), unveiling a critical node at the junction of survival, inflammation, and stress signaling networks. IKK forms a complex with and phosphorylates ASK1 at a sensor site, leading to inhibition of ASK1-mediated signaling. 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. Because of the intimate involvement of ASK1 in diverse diseases, the IKK/ASK1 interface offers a promising target for therapeutic development.

Introduction

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 (17). ASK1 senses, processes, and transmits various environmental cues to intracellular signaling machinery, impacting both physiological and patho-physiological 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 polyQ-induced ER stress in Huntington's 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 (31). Increased ROS also triggers dissociation of 14-3-3 proteins from ASK1, relieving ASK1 inhibition (59). ASK1 binding to 14-3-3 is mediated by at phosphorylated Ser967, which serves as a

molecular sensor for signal integration (187). 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 (59; 187). Similarly, the protein phosphatase calcinuerin activates ASK1 through dephosphorylation of Ser967 (60). Conversely, increased ASK1/14-3-3 binding is correlated with decreasing ASK1 activity and increased cell survival (64; 197). By controlling the phosphorylation status of Ser967, 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 kappa B 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 Growth Arrest and DNA Damage 45 (GADD45) in a transcription dependent manner (172). 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. H_2O_2, EGF, IGF-1, wortmannin, and PS1145 (Sigma), Akt inhibitor, phosphatidylinositol ether analog, and recombinant Akt1 (Calbiochem), LY294002 (Alomone Labs), TNFα (BD), recombinant MAPK/ERK Kinase (MEK) (Cell Signaling),

recombinant IKKα and IKKß (Upstate Cell Signaling Solutions) were used in supplied solution or reconstituted according manufacturer's instructions.

Kinase assays. For Akt kinase assays, active recombinant Akt1 (1µg), immunoprecipitated HA-Akt ΔPH (~1µg), or active recombinant MEK (1µg) was added to the immunoprecipitated ASK1 in a MOPS buffer (20 mM MOPS, 25 mM ß-glycerophosphate, 5 mM EGTA, 1mM sodium orthovanadate, 1 mM DTT, 10 mM $MgCl_2$, 10 mM $MnCl_2$) with 100 mM ATP. For IKK kinase assays, active recombinant IKKß (0.5 µg) was added to immunoprecipitated ASK1 in a kinase buffer (8 mM MOPS, 200 mM EDTA, 15 mM $MgCl_2$) with 100 mM ATP. For direct *in vitro* IKK kinase assay, a purified ASK1 C-terminal fragment (5 µg) was incubated with various concentrations of active recombinant IKKß in a kinase buffer with 100 mM ATP. Kinase reactions were performed at 37˚C for 10-30 min. All kinase reactions were terminated by boiling in SDS sample buffer. Ser967 phosphorylation was determined by immunoblotting.

Endogenous ASK1 immunoprecipitation. COS7 or MEF cells were grown on 10cm plates to confluency 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 5mL of an ASK1 specific antibody (F-9, Santa Cruz) and 25 µl of a 50% slurry of protein G sepharose at 4˚C for 4-6 hr, rotating slowly. Samples were washed 500 µL of lysis buffer, and beads were isolated by quick centrifugation. The supernatant was discarded and the process was repeated three times. Protein levels were assessed by immunoblotting.

Purification of ASK1 C-terminus from *E. Coli*. One colony of bacteria harboring an expression plasmid for GST-ASK1 C-terminal fragment (ASK1 K939-T1374) were grown in 500mL of ZYP-5052 autoinduction media (1% N-Z-amine AS, 0.5% yeast extract, 50 mM Na_2HPO_4, 50 mM KH_2PO_4, 25 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.2X metals, 0.5% glycerol, 0.05% glucose, 0.2% a-lactose)overnight at 37˚C, shaking. The sample was spun down at 14,000 rpm for 15 min and the pellet was resuspended in 10mL of 1x PBS (1M DTT, 100mM PMSF, 5M NaCl) and sonicated 6 times, 30 min at 4˚C on ice. The sample was centrifuged for 10 min at14,000 rpm, 6˚C. The supernatant was mixed with 2 mL of a 50% slurry of glutathione sepharose (GE Healthcare) in 1X PBS and rotated slowly at 4C for 2 hr. Samples were eluted with elution buffer (20mM reduced glutathione, 0.25 mM PMSF, 5mM DTT, 0.1% Triton X). Samples were concentrated by centrifugation and dialyzed into storage buffer (10mM HEPES, 150mM NaCl, 10% glycerol).

Apoptosis assays. Caspase activity was evaluated using 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. Caspase 3 specific activity was determined by normalizing each sample to protein concentration. Annexin V assay was evaluated according to manufacturer's protocol (BD Pharmingen). Percentage of Annexin V- and 7-AAD-positive cells were determined with the Guava Flow Cytometer using Guava Nexin software.

Time-resolved fluorescence resonance energy transfer. 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% NP40). GST-Terbium (HTRF) conjugated antibody was used couple GST-IKKß as a FRET donor. Venus-ASK1 served as a FRET acceptor. Samples were incubated at room temperature for 1 hr and FRET signal was measured on the Envision spectrophotometer (Excitation 337 nm and Emission 520 nm).

Statistical analysis. Data in bar graphs are presented as mean with standard deviation represented by error bars. n values are given in figure legends.

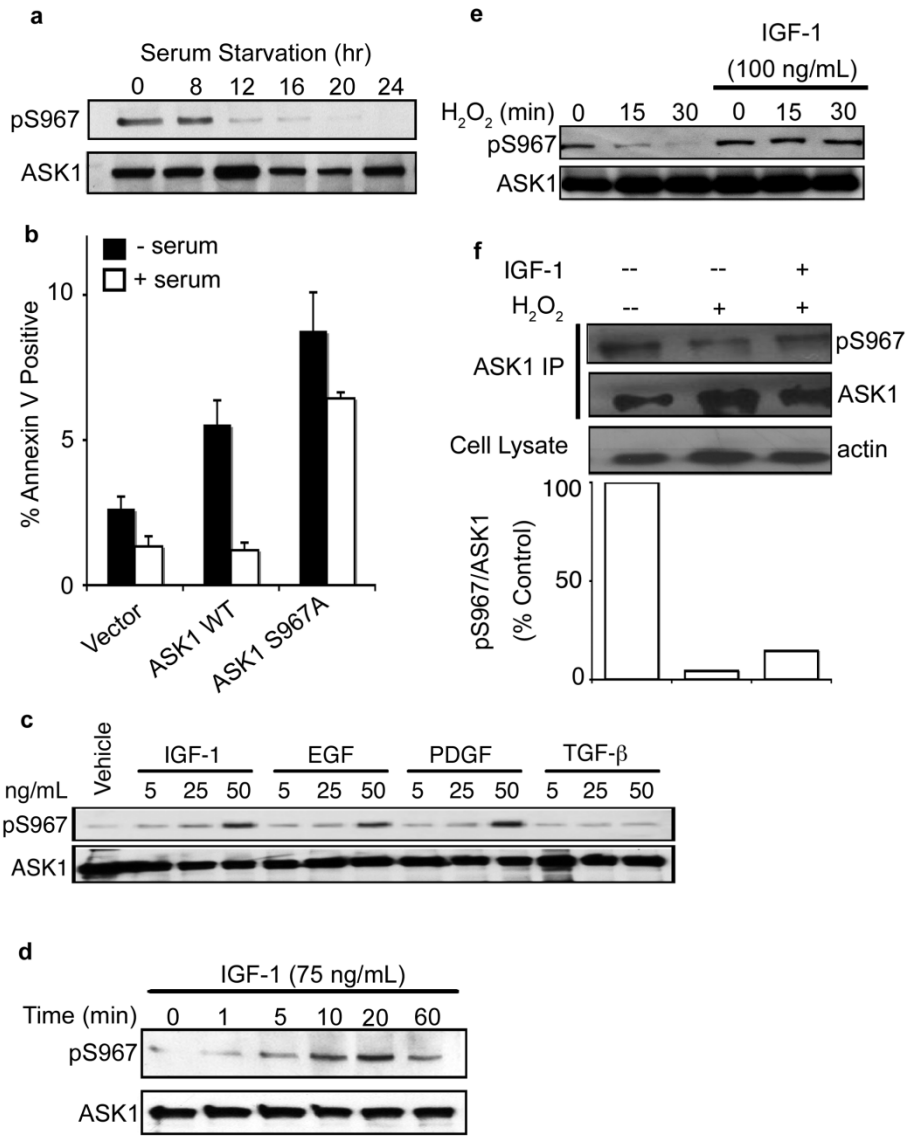
Results

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 (59; 197). Stress signals often induce dephosphorylation of Ser967 and promote cell death, and, indeed, Ser967 was dephosphorylated upon serum withdrawal (Fig. 3.1-1A) and by exposure to ROS, stimuli known to increase ASK1 kinase activity (42; 49; 59). Consistent with this notion, serum withdrawal enhanced ASK1-mediated apoptosis (Fig. 3.1-1B).

To reveal pathways that control this molecular sensing system, we examined factors that might enhance phosphorylation of ASK1 Ser967. Reintroduction of serum

Figure 3.1-1. Phosphorylation of ASK1 at Ser967 is dynamically regulated. (A-D) COS7 cells were transfected with HA-ASK1 or HA-ASK1 S967A. (A) Ser967 phosphorylation was monitored after serum starvation for indicated times. (B) Serum starvation induced apoptosis was measured by staining with Annexin V and 7-AAD after 24 hr. Data represent mean +/- standard deviation. n=5. (C) COS7 cells were serum starved for 24 hr and treated indicated doses of IGF-1, EGF, PDGF, TGF-ß or vehicle (PBS with 0.1%BSA) for 20 min. (D) Following 24 hr of serum starvation, cells were treated with IGF-1 or vehicle as indicate. (E-F) COS7 cells were treated with H_2O_2 (1 mM) in the presence of IGF-1 (100ng/mL) or vehicle (PBS + 0.1%BSA). pSer967 of overexpressed (E) or endogenous immunoprecipitated ASK1 (F) was measured. pSer967 and total ASK1 protein were detected by Western blot (A,C-F) and quantified with ImageJ (F).


a
Serum Starvation (hr)
0 8 12 16 20 24
pS967
ASK1
b
- serum
+ serum
% Annexin V Positive
10
5
0
Vector
ASK1 WT
ASK1 S967A
c
Vehicle
IGF-1
EGF
PDGF
TGF-β
ng/mL
5 25 50 5 25 50 5 25 50 5 25 50
pS967
ASK1
d
IGF-1 (75 ng/mL)
Time (min) 0 1 5 10 20 60
pS967
ASK1
e
IGF-1
(100 ng/mL)
H2O2 (min) 0 15 30 0 15 30
pS967
ASK1
f
IGF-1 -- -- +
H2O2 -- + +
ASK1 IP
pS967
ASK1
Cell Lysate
actin
pS967/ASK1
(% Control)
100
50
0

after serum starvation restored ASK1 Ser967 phosphorylation, leading to the hypothesis that there may be a pro-survival 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. 3.1-1B), indicating that serum's pro-survival 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: epidermal growth factor (EGF), insulin-like growth factor 1 (IGF-1), platelet-derived growth factor (PDGF), and transforming growth factor ß (TGF-ß). Treatment of cells with EGF, IGF-1, and PDGF, but not TGF-ß, resulted in marked phosphorylation of Ser967 (Fig. 3.1-1C), 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 dose and time dependent manner (Fig. 3.1-1D). Notably, IGF-1 was also able to block ROS induced dephosphorylation of ASK1 (Fig. 3.1-1E). Furthermore, IGF-1 could indeed impair stress induced dephosphorylation of endogenous ASK1 (Fig. 3.1-1F) 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 pro-survival role in many settings and down-regulates ASK1 activity through phosphorylation of Ser83 (49). Similar to Ser83, the region surrounding Ser967 of ASK1 fits nicely within the defined phosphorylation motif of Akt (RxRxxSxx), suggesting the

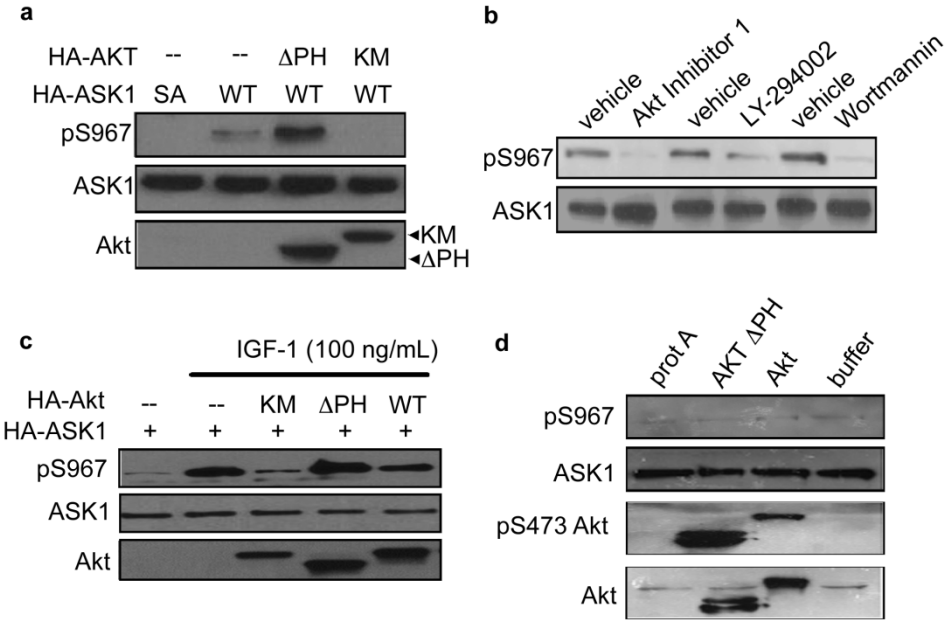
site could potentially be modified by Akt. In order to investigate whether Akt mediates IGF-1 induced Ser967 phosphorylation, constitutively active Akt (Akt ΔPH) or a catalytically inactive Akt mutant (Akt KM: K179M) was utilized. Expression of Akt ΔPH resulted in dramatic increase in Ser967 phosphorylation, while Akt KM expression led to marked decrease in Ser967 phosphorylation (Fig. 3.1-2A).

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. 3.1-2B), 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 ΔPH or Akt KM) were treated with IGF-1. While active Akt enhanced phosphorylation of ASK1 Ser967, overexpression of the dominant negative Akt blunted IGF-1's ability to increase ASK1 phosphorylation (Fig. 3.1-2C). 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 (198-200), it seemed likely that Akt also phosphorylates ASK1 directly. We tested this notion with an *in vitro* kinase assay. Active, recombinant Akt or immunoprecipitated HA-Akt ΔPH was utilized to phosphorylate ASK1 as a substrate. Surprisingly, no transfer of phosphate from ATP to Ser967 ASK1 was detected (Fig. 3.1-2D). After confirming the activity of recombinant

Figure 3.1-2. Akt/PKB promotes phosphorylation of ASK1 at Ser967. (A-C) COS7 cells were transfected with HA-ASK1, wild type Akt (Akt WT), constitutively active Akt (Akt ΔPH), and/or catalytically inactive Akt (Akt KM). (A) 48 hr after transfection, cells were serum starved for 24 hr. (B) COS7 cells transfected with HA-ASK1 (WT) or HA-ASK1 S967A (SA) grown in the presence of serum were treated with wortmannin (200 nm), LY294002 (30 μM), Akt inhibitor 124005 (40 μM), or DMSO for 30 min. (C) 48 hr after transfection, cells were serum starved for 24 hr and treated with IGF-1 (100 ng/mL) for 20 min. (D) An *in vitro* kinase reaction for ASK1 phosphorylation was performed with immunoprecipitated HA-Akt ΔPH or active recombinant Akt. pS967 ASK1 and total protein levels in (A)-(D) were analyzed by western blot.


a
HA-AKT -- -- ΔPH KM
HA-ASK1 SA WT WT WT
pS967
ASK1
Akt
◂KM
◂ΔPH
b
vehicle
Akt Inhibitor 1
vehicle
LY-294002
vehicle
Wortmannin
pS967
ASK1
c
IGF-1 (100 ng/mL)
HA-Akt -- -- KM ΔPH WT
HA-ASK1 + + + + +
pS967
ASK1
Akt
d
prot A
AKT ΔPH
Akt
buffer
pS967
ASK1
pS473 Akt
Akt

Akt with an antibody directed against phosphorylated Ser473, a phosphorylation event indicative of Akt activity, we came to the conclusion that Akt does not likely phosphorylate ASK1 directly at Ser967.

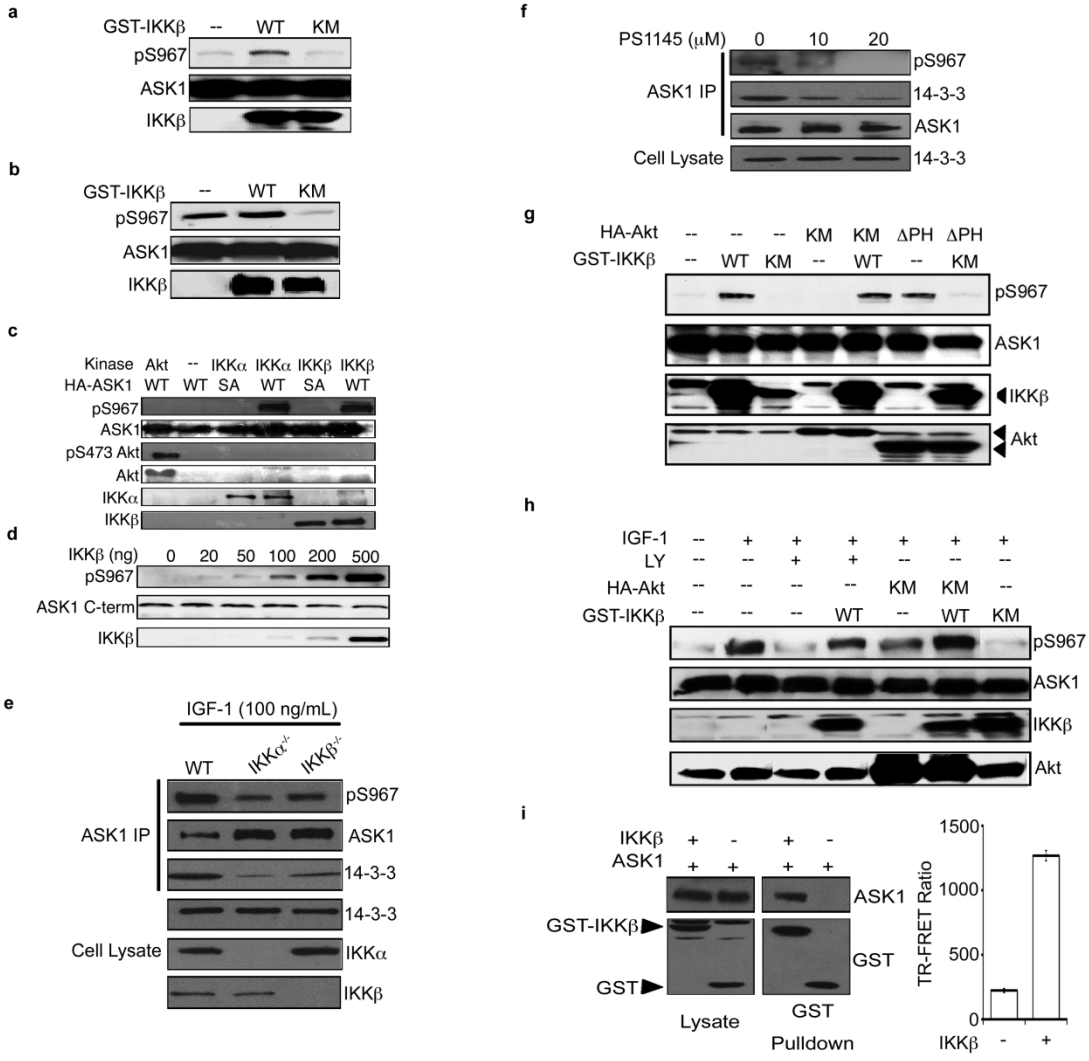
IKK mediates growth factor and Akt-induced phosphorylation of ASK1

After discovering Akt is likely to be upstream of the kinase responsible for phosphorylation of ASK1 at Ser967, kinase substrates of Akt were investigated. Notably, Akt activates the prominent pro-survival kinase, IKK. While IKK plays a vital role in NF-κB activation, the importance of IKK in the regulation of other signaling events is becoming increasingly evident (116). Thus, we tested the premise that IKK phosphorylates ASK1. Co-transfection of COS7 cells with wild type IKK (IKK WT) prevented serum starvation induced dephosphorylation of ASK1 (Fig. 3.1-3A). Similarly, when cells were co-transfected with a catalytically inactive form of IKK (IKK K44M), a decrease of phosphorylated Ser967 was observed (Fig. 3.1-3B), suggesting the kinase activity of IKK is critical in maintaining ASK1 Ser967 phosphorylation.

To determine whether IKK can directly catalyze the phosphorylation of ASK1 at Ser967, an *in vitro* kinase assay was performed. Interestingly, IKKß, as well as IKKα phosphorylated immunoprecipitated ASK1 *in vitro* (Fig. 3.1-3C) a phenomenon not observed with purified MEK or Akt (Fig. 3.1-2D). Furthermore, purified IKKß phosphorylated recombinant ASK1 in a dose dependent manner in a reconstituted biochemical system (Fig. 3.1-3D), confirming ASK1 as a direct substrate of IKKß.

To validate a role of IKK as a Ser967 kinase, a genetic and a pharmacological approach were employed. Knockout of IKKα or IKKß decreased IGF-1 induced ASK1

Figure 3.1-3. IKK phosphorylates ASK1 at Ser967. (A-B) COS7 cells were transfected with HA-ASK1 and GST-IKKβ (IKK WT) or catalytically inactive GST-IKKβ (IKK KM). 24 hr after transfection, media was replaced with DMEM (A) or DMEM containing 10% FBS (B). (C-D) An in vitro kinase assay was performed by adding increasing doses of active recombinant IKKβ to immunoprecipitated ASK1 (C) or purified recombinant ASK1 C-terminal fragment (K939-T1374) (D). (E) MEF cells (WT, IKKα$^{-/-}$, and IKKß$^{-/-}$) were serum starved (24 hr) and treated with IGF-1 (100 ng/mL) for 20 min. Endogenous ASK1 was immunoprecipitated to monitor pS967 status and 14-3-3 association. (F) Immunoprecipitation of endogenous ASK1 from COS7 cells serum starved and treated with PS1145 for 24 hr prior to treatment with IGF-1 (100 ng/mL) for 20 min. (G-H) pS967 ASK1 was monitored in COS7 cells transfected with HA-ASK1, GST-IKKβ (IKK WT), catalytically inactive GST-IKKβ (IKK KM), constitutively active HA-Akt (Akt ΔPH), and/or catalytically inactive HA-Akt (Akt KM) and serum starved for 24 hr (G) or treated with IGF-1 (75 ng/mL) or vehicle (PBS + 0.1%BSA) for 20 min following pre-treatment with LY294002 (30 μM) or vehicle (DMSO) for 2 hr (H). (I) The presence of ASK1 was assessed in GST-IKKß complexes isolated from COS7 cells transfected with HA-ASK1 and GST-IKKß 48 hr after transfection (left). Interaction was also assessed by time resolved fluorescence resonance energy transfer (TR-FRET). Lysates of COS7 cells transfected with either Venus-ASK1 or GST-IKKß were mixed as indicated, and fluorescence was measured with the Envision spectrophotometer (right). (A-I) pS967 ASK1 and total protein levels were assessed by Western blot.


a
GST-IKKβ -- WT KM
pS967
ASK1
IKKβ
b
GST-IKKβ -- WT KM
pS967
ASK1
IKKβ
c
Kinase Akt -- IKKα IKKα IKKβ IKKβ
HA-ASK1 WT WT SA WT SA WT
pS967
ASK1
pS473 Akt
Akt
IKKα
IKKβ
d
IKKβ (ng) 0 20 50 100 200 500
pS967
ASK1 C-term
IKKβ
e
IGF-1 (100 ng/mL)
WT
IKKα-/-
IKKβ-/-
ASK1 IP
pS967
ASK1
14-3-3
14-3-3
Cell Lysate
IKKα
IKKβ
f
PS1145 (μM) 0 10 20
ASK1 IP
pS967
14-3-3
ASK1
Cell Lysate
14-3-3
g
HA-Akt -- -- -- KM KM ΔPH ΔPH
GST-IKKβ -- WT KM -- WT -- KM
pS967
ASK1
IKKβ
Akt
h
IGF-1 -- + + + + + +
LY -- -- + + -- -- --
HA-Akt -- -- -- -- KM KM --
GST-IKKβ -- -- -- WT -- WT KM
pS967
ASK1
IKKβ
Akt
i
IKKβ + - + -
ASK1 + + + +
ASK1
GST-IKKβ
GST
GST-IKKβ
GST
Lysate
GST
Pulldown
TR-FRET Ratio
1500
1000
500
0
IKKβ - +

Ser967 phosphorylation and 14-3-3 interaction (Fig. 3.1-3E). Additionally, incubation of cells with a small molecule IKK inhibitor, PS1145, abolished IGF-1 induced Ser967 phosphorylation (Fig. 3.1-3F). Thus, it is likely that IKK acts downstream of Akt to phosphorylate ASK1 at Ser967.

To confirm the order of IKK with respect to Akt within the IGF-1 to Ser967 signaling axis, we carried out a series of combination experiments with various constitutively active and dominant negative mutants. While dominant negative IKK KM inhibited Ser967 phosphorylation induced by the constitutively active Akt ΔPH, dominant negative Akt KM failed to block active IKK-catalyzed Ser967 phosphorylation (Fig. 3.1-3G), placing Akt upstream of IKK. As shown in Figure 3.1-1, 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. 3.1-3H). However, active IKK overcame the effect of PI3K and Akt inhibitors, restoring phosphorylation of ASK1 at Ser967 (Fig. 3.1-3H). Together, these data establish a novel signaling axis by which IKK acts downstream of Akt to phosphorylate ASK1 at Ser967.

IKKβ 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 pull-down assay, ASK1 is found in the IKKβ protein complex (Fig. 3.1-3I). Then, we utilized time-resolved fluorescence resonance energy transfer (TR-FRET) technology, which can detect protein interactions within 100Å. Indeed, incubation of IKKβ with

ASK1 led to an increase in FRET signal (Fig. 3.1-3I). These results indicate that ASK1 and IKKβ likely interact directly.

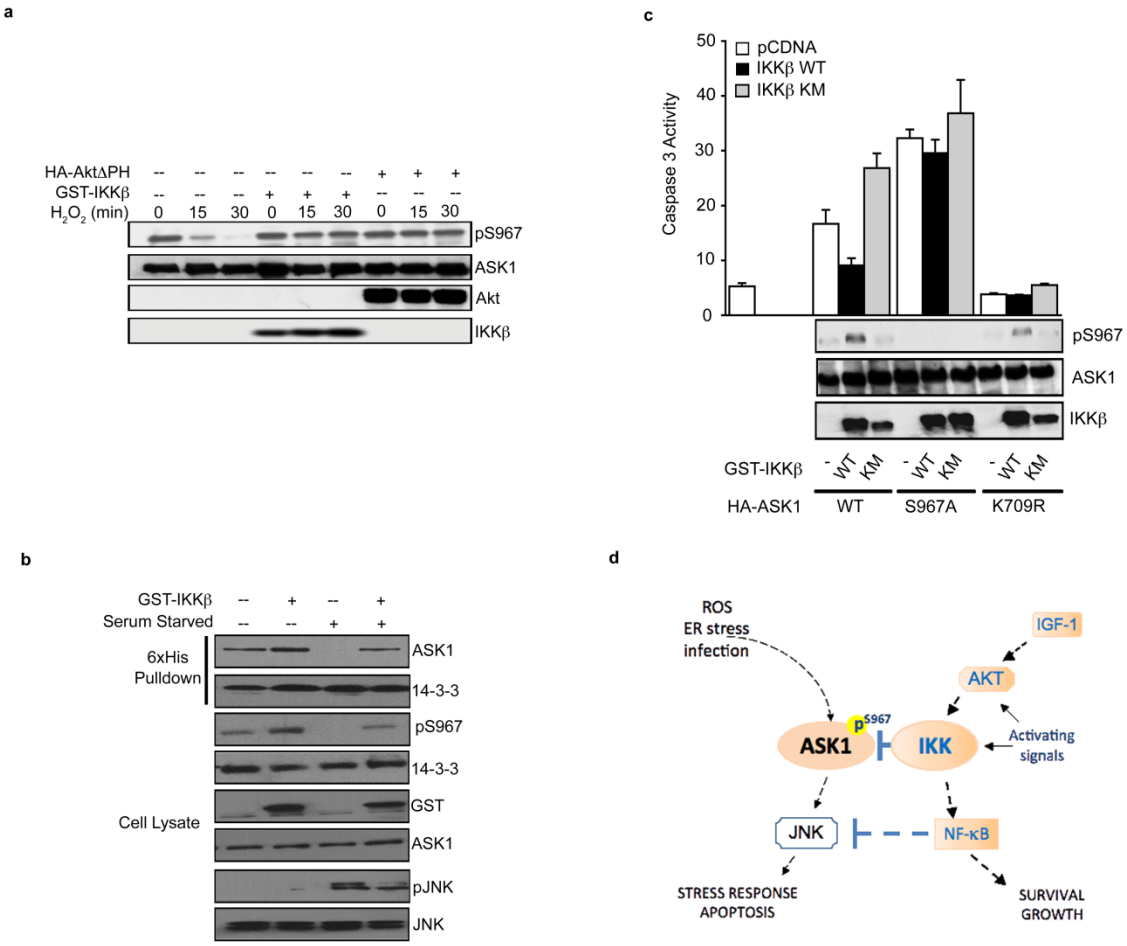
The IGF-1/Akt/IKK signaling antagonizes H_2O_2 –induced ASK1 activation

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 Figure 3.1-1, H_2O_2 triggered dephosphorylation of ASK1 at Ser967, which can be reversed by IGF-1. Like IGF-1, expression of Akt ΔPH, as well as IKK, effectively blocked dephosphorylation of Ser967 by H_2O_2, supporting a role of Akt and IKK in mediating the IGF-1 effect (Fig. 3.1-4A, Fig. 3.1-3H). Similarly, serum starvation reduced pSer967 of ASK1 and the subsequent 14-3-3 association (Fig. 3.1-4B). Significantly, overexpression of IKK reversed Ser967 phosphorylation as well as 14-3-3 association with ASK1 (Fig. 3.1-4B), keeping ASK1 in an inhibited state. 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 pro-death and stress signals such as ROS and serum starvation.

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

Figure 3.1-4. The IGF-1/Akt/IKK pathway prevents stress-induced ASK1 function. (A) COS7 cells transfected with GST-IKKß or HA-Akt ΔPH and treated with H_2O_2 (1 mM) as indicated and pSer967 ASK1 was monitored by Western blot. (B) COS7 cells transfected with HA-ASK1, 6xHis-14-3-3γ and/or GST-IKKß. Cells were serum starved (16 hr) and 6xHis-14-3-3 complexes were isolated Ni+ beads. Dissociation of HA-ASK1 and 6xHis-14-3-3 was monitored by Western blot. (C), COS7 cells were transfected with HA-ASK1 WT, HA-ASK1 S967A, or the catalytically inactive HA-ASK1 K709R and serum starved (24 hr). Caspase 3 activity was measured as (pmol p-Nitroaniline released/hour)/mg of protein. First bar indicates activity of the blank. Data represent mean ± SD. n=3. pS967 ASK1 and total protein levels were detected by western blot. (D) Model of ASK1 regulation by IKK.


a
HA-AktΔPH
GST-IKKβ
H2O2 (min)
0 15 30 0 15 30 0 15 30
pS967
ASK1
Akt
IKKβ
b
GST-IKKβ
Serum Starved
6xHis Pulldown
ASK1
14-3-3
pS967
14-3-3
Cell Lysate
GST
ASK1
pJNK
JNK
c
pCDNA
IKKβ WT
IKKβ KM
Caspase 3 Activity
50
40
30
20
10
0
pS967
ASK1
IKKβ
GST-IKKβ
WT
KM
HA-ASK1
WT
S967A
K709R
d
ROS
ER stress
infection
IGF-1
AKT
ASK1
pS967
IKK
Activating signals
JNK
NF-κB
STRESS RESPONSE
APOPTOSIS
SURVIVAL
GROWTH

IKK. Caspase 3 protease activity was monitored as readout for apoptotic cell death, and phospho-Ser967 levels in cell lysates was monitored by western blot in order to reveal the level of Ser967 phosphorylation during the treatment. Consistent with a pro-survival role of IKK, expression of IKK inhibited ASK1-induced apoptosis (Fig. 3.1-4C). However, it is possible that this inhibition is due to known pro-survival effects of IKK independent of ASK1. In order to address this possibility, we expressed the ASK1 mutant S967A to induce apoptosis. IKK had minimal effects on the apoptotic activity of this unphosphorylatable mutant (Fig. 3.1-4C), suggesting that IKK inhibits ASK1 apoptotic activity primarily through inducing phosphorylation at Ser967. Thus, phosphorylation of ASK1 at Ser967 may be a major mechanism through which IKK exerts its pro-survival and anti-stress regulatory role in the cell.

Discussion

The Ser/Thr protein kinases, IKKα and IKKß, were discovered as critical mediators of the NF-κB signaling pathway, which regulate immune responses, inflammation and programmed cell death (106; 116; 201). Diverse environmental signals can trigger the activation of IKK through various transmembrane receptors such as TNFα receptors, toll-like receptors, and IGF-1 receptor (116). While the paradoxical activation of both pro-survival and pro-death pathways by TNFα was puzzling at the beginning, the intricate crosstalk between the NF-κB and JNK pathways illustrates the demand for a well orchestrated signaling network for meaningful biological readouts. It has been established that IKK activation inhibits JNK signaling through NF-κB induced XIAP and GADD45 in a transcription dependent manner (172). Our report unravels a direct action

of activated IKK on ASK1, an upstream regulator of JNK, which establishes a crucial point of cross-talk between IKK-mediated immune response, inflammatory, and pro-survival pathways and ASK1-mediated stress responses. Our data support a dual regulatory mechanism of IKK on the ASK1/JNK stress signaling axis (Fig. 3.1-4D). Activation of IKK leads to phosphorylated IκB to induce a transcription-dependent inhibition of JNK through NF-κB and to phosphorylation and direct inhibition of ASK1, an activator of JNK, ensuring a precisely regulated signaling output.

ASK1 serves as a central signaling hub in mediating stress response and apoptosis induced by a number of stimuli, including cytokines, ROS, ER stressors, infectious agents, and cancer chemotherapeutics (17). 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 IGF1/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.

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-ß in Alzheimer's disease and angiotensin-II induced cardiac hypertrophy and injury (76; 202). 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 counter the action of ER stress-induced, ASK1-mediated neuronal cell death, as in polyQ diseases including Huntington's disease and spinocerebellar ataxias (203). Blocking ASK1 activity by IKK activation may alleviate the effect of polyQ induced ER stress. On the other hand, ASK1 also mediates apoptotic cell death triggered by a number of cancer therapeutics through ER stress, elevated ROS, or other mechanisms (204-208). Therefore, pathways that control ASK1 activity, such as the Akt/IKK signaling axis, are expected to 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 antagonist 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 (209).

While 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 (145-147). Our discovery of ASK1 as an IKK substrate 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.

Chapter 3.2: The NEMO binding domain of IKK mediates ASK1/IKK interaction without disruption of IKK/NEMO binding

Mary C. Puckett, Yuhong Du, Haian Fu

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Puckett, MC, Goldman, EH, Cockrell, LM, Huang, B, Kasinski, AL, Du, Y, Wang, C, Lin, A, Ichijo, H, Khuri, FR, and H. Fu. Integration of the Apoptosis Signal-Regulating Kinase 1-Mediated Stress Signaling with the Akt/PKB-IkB kinase cascase. 2013. MCB. *In press.*

Abstract

Dysregulation of cell survival and death pathways contributes to the pathogenesis of numerous diseases, from cancer to neurodegenerative diseases, and protein-protein interactions play a critical role in the proper regulation of these pathways. Here we describe one such interaction between the apoptosis signal regulating kinase 1 (ASK1) and the inhibitor of κB kinase β (IKKβ). ASK1 and IKKβ interact with one another independent of kinase activity, predominantly through the C-terminus of ASK1 and the NEMO binding domain (NBD) of IKKβ. While overexpression of IKKβ impairs ASK1 mediated apoptosis, disruption of binding by overexpression of an NBD peptide restores ASK1 function. Interestingly, IKKβ also interacts with ASK1 via its NBD in a manner distinct from NEMO. IKKβ appears to interact with ASK1 and NEMO simultaneously, and, while mutation of two key residues in NBD abolishes NEMO interaction with IKKβ, it has no effect on ASK1 interaction. Further, expression of NEMO has minimal effect on stress induced activation of ASK1. Understanding the interaction interface, its regulation, and the consequences of binding disruption could lead to the development of novel therapeutics.

Introduction

A finely tuned response to extracellular signals and the intracellular environment is critical in the maintenance of proper cell function. Cells constantly receive a mix of pro-growth and stress signals and must be able to accurately interpret these signals to activate proper intracellular signaling pathways. One critical sensor of extracellular and intracellular stress signals is the apoptosis signal-regulating kinase 1 (ASK1), which responds to stress signals such as cytokines, reactive oxygen species (ROS), and endoplasmic reticulum (ER) stress to initiate a mitogen activated protein kinase (MAPK) signaling cascade, leading to the activation of the Jun N-terminal Kinase (JNK) and/or p38 to elicit an appropriate cellular response (31).

Because ASK1 is a crucial mediator of stress signaling, its activity is tightly controlled. One important manner in which ASK1 is regulated is through protein-protein interactions, promoting or inhibiting ASK1 activity. Specifically, ASK1 can homodimerize or dimerize to with ASK2, leading to ASK1 activation (28; 40). TRAF family members can also bind ASK1, inducing an activating conformational change (43). Conversely, ASK1 function can be inhibited by a variety of protein interactions. Thioredoxin binds ASK1, keeping the kinase in an inactive state through oxidizable cysteine residues (43). And, when phosphorylated at Ser967, 14-3-3 family members can bind and inhibit the kinase (59; 187). Recently, we identified IKK as a novel protein partner for ASK1, which can bind and inhibition ASK1 function (Chapter 3.1).

IKKβ is most known for its role in canonical NF-κB signaling. In response to cytokines, the IKK complex, consisting of IKKα, IKKβ, and IKKγ (also known as the NF-κB essential modulator or NEMO) is formed (210). Formation of this complex allows

for activation of IKKβ and, in turn, the phosphorylation, ubiquitination and, degradation inhibitor of κB (IκB) proteins, which under inactive conditions keep NF-κB in an inactive state. The newly released NF-κB is then able to translocate to the nucleus and drive transcription of pro-survival and pro-inflammatory genes (116).

An essential component of the IKK complex is NEMO, without which canonical signaling cannot occur (135). Loss of NEMO has been linked to several devastating diseases (211). While the function of NEMO is not entirely understood, it is believed that the protein acts as a scaffold to promote complex formation and bridge interactions between IKK and activating or inhibitory binding partners (129). Recent studies have also shed light on NEMO's role in regulating interactions outside of the IKK complex (116). Here, we identify the interaction interface of ASK1 and IKKβ and provide evidence for a unique function of the NEMO binding domain of IKKβ in regulating this interaction.

Methods

Plasmids and reagents. HA-ASK1, Venus-ASK1, GST-IKKβ, GST-IKKα, and Flag-NEMO and all truncation mutants were used for mammalian protein expression. DNA was purified from E. coli with the Qiagen Maxi Kit. HA-ASK1 mutants and fragments were in pcDNA3 mammalian expression vector and have been previously described (187). PS1145 and tetracycline were obtained from Sigma.

Cell culture and transfections. COS7 and HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM, CellGro) with 10% fetal bovine serum (FBS, Atlanta

Biologicals) and 1% penicillin/streptomycin (Life Technologies) at 37ºC with 5% CO_2 in a humidified incubator. DNA transfection was performed with XtremeGENE HP (Roche) or FuGENE HD reagent (Promega) according to the manufacturer's instructions.

Cell lysis and immunoblotting. Cells were lysed 48 hr after transfection in 200 µL (GST pull-down and immunoprecipitations) or 60µL (TR-FRET) of 0.2% lysis buffer [0.2% Nonidet P-40 (NP-40); 10 mM HEPES, pH 7.4; 150 mM NaCl; 1:1000 dilution phosphatase inhibitor cocktail (Sigma), 1:1000 dilution protease inhibitor cocktail (Sigma)]. Proteins were resolved by SDS-PAGE and immuno-blotted with specific antibodies: GST (Z-9), ASK1 (F-9), and HA antibodies were obtained from Santa Cruz. Flag (M2) monoclonal antibody was obtained from Sigma. Secondary antibodies used were horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz) and horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz) for monoclonal and polyclonal primary antibodies respectively. Proteins were visualized using enhanced West Pico or West Dura (Thermo Scientific).

GST pull-down and immunoprecipitation assays. To perform GST pull-down assays, 160 µL of lysate was added to 25 µL of a 50% slurry of glutathione beads (GE Healthcare) in lysis buffer and samples were rotated slowly at 4ºC for 2-4 hr. For immunoprecipitations, 160 µL of lysate was added to 25 µL of a 50% slurry of Protein G beads (GE Healthcare) and samples were rotated slowly at 4ºC for 30 min to clear lysates. Supernatant was collected, added to 2 µL of anti-ASK1 antibody (F-9, Santa Cruz), and rotated slowly at 4ºC for 2 hr. 25 µL of 50% slurry of Protein G beads was

added, and samples were rotated an additional two hours. Beads were washed three times with 200 μL of lysis buffer to decrease nonspecific binding. Beads were then boiled in 20 μL of 2X SDS sample buffer for 5 min to recover bound proteins for analysis by SDS-PAGE and Western blot.

PC12 neurite outgrowth assay. To induce ASK1 expression, PC12 cells were cultured in DMEM with 1% horse serum. All cell treatments were carried out for three days, after which cells were fixed with 2% paraformaldehyde for 30 min and solubilized with 0.1% Triton X for 20 min. Fixed and solubilized cells were incubated with Alexa-Fluor488-phalloidin (Invitrogen) in 5% BSA at a final concentration of 1:1000 overnight. Nuclei were counterstained with 5μg/mL Hoechst dye (Invitrogen). Cells were washed three times with 1X PBS between each step. Fluorescence was measured with the ImageExpress spectrophotometer (Molecular Devices), and neurite outgrowth was quantified with MetaExpress software (Molecular Devices).

Time-resolved fluorescence resonance energy transfer. Cell lysates containing overexpressed proteins were transfected to 384-well black plates. Lysates were serially diluted in a reaction buffer (20 mM Tris-HCl pH 7.0, 50 mM NaCl, 0.01% NP40). GST-Terbium (HTRF) conjugated antibody was used couple GST-IKKβ as a FRET donor. Venus-ASK1 served as a FRET acceptor. Samples were incubated at room temperature for 1 hr and FRET signal was measured on the Envision spectrophotometer (terbium excitation: 337 nm, Venus emission: 520 nm).

Apoptosis assay. Annexin V/7-AAD staining was evaluated according to manufacturer's protocol (BD Pharmingen). Annexin V and 7-AAD positive cells were identified with the Guava Flow Cytometer and analyzed with the Guava Nexin software package.

Results

Interaction does not require Ser967 phosphorylation or kinase activation

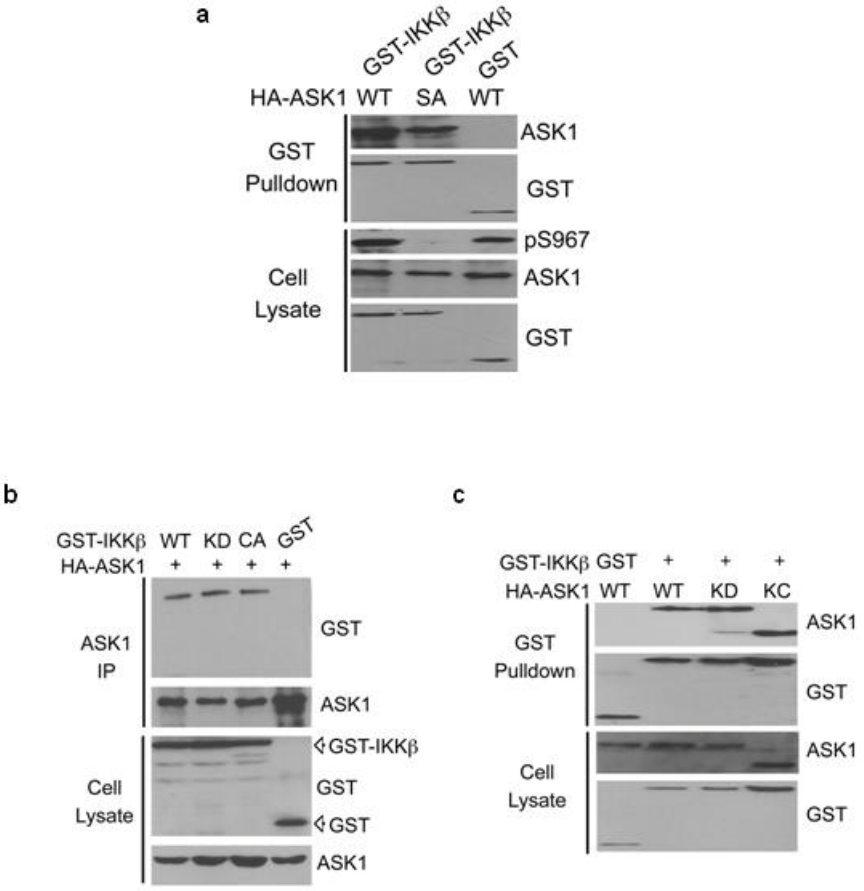
Since IKKβ can phosphorylate ASK1 at Ser967 and this phosphorylation event is sufficient to reduce ASK1 activity in response to upstream activating signals (Chapter 3.1), we first wanted to assess whether phosphorylation of this site was necessary for ASK1/IKKβ interaction. Interestingly, IKKβ showed comparable levels of binding with both wild type ASK1 and an unphosphorylatable mutant, ASK1 S967A, indicating that the ASK1/IKKβ interaction is not dependent on the phosphorylation state of this site (Figure 3.2-1A).

To look at this more generally, we probed whether the activation state of either kinase altered their interaction since ASK1 is known to exist in different complexes depending on its activation state (43). Neither ASK1 nor IKKβ showed a significant binding preference for a wild type (WT), kinase dead (KD), or constitutively active (CA) binding partner (Figure 3.2-1B-C).

IKKβ interacts with the C-terminus of ASK1

ASK1 and IKKβ can interact directly (Chapter 3.1), and this interaction is likely important for regulation of ASK1 by IKK. To further understand how these two kinases

Figure 3.2-1. Determinants of ASK1/IKK interaction. (A) Ser967 phosphorylation is not required for interaction. GST-IKKβ was overexpressed in COS7 cells with HA-ASK1 wild type (WT) or S967A mutant. GST-IKKβ complexes were isolated by GST pulldown and bound ASK1 was analyzed by Western blot. (B-C) Kinase activity is not required for interaction. GST-IKKß was overexpressed with HA-ASK1 wild type (WT), kinase dead (KD), or constitutively active (CA) and vice versa. (B) HA-ASK1 complexes were isolated by immunoprecipitation and bound IKKβ was analyzed by Western blot. (C) GST-IKKβ complexes were isolated by GST pulldown and bound ASK1 was analyzed by Western blot.


a
GST-IKKβ
GST-IKKβ
GST
HA-ASK1 WT SA WT
GST Pulldown
ASK1
GST
Cell Lysate
pS967
ASK1
GST
b
GST-IKKβ WT KD CA GST
HA-ASK1 + + + +
ASK1 IP
GST
ASK1
Cell Lysate
GST-IKKβ
GST
GST
ASK1
c
GST-IKKβ GST + + +
HA-ASK1 WT WT KD KC
GST Pulldown
ASK1
GST
Cell Lysate
ASK1
GST

interact, we set out to identify which region(s) of ASK1 interacted with IKKβ. ASK1 is comprised of a kinase domain flanked by N- and C-terminal coiled-coil containing domains, all of which have been implicated in ASK1 interactions with other proteins (Figure 3.2-2A). When IKKβ was overexpressed with ASK1 fragments, IKKβ only interacts with the C-terminus of ASK1 (Figure 3.2-3A). Because Ser967, the site at which IKKβ phosphorylates ASK1, is located within the C-terminus, this result was not surprising. Furthermore, the C-terminus of ASK1 contains multiple coiled coil domains (56), which IKKβ has been previously shown to use in protein-protein interactions (212).

ASK1 interacts with the kinase and NEMO binding domains of IKKβ

To determine the complementary region on IKKβ where ASK1 binds, we used a similar iterative deletion approach (Figure 3.2-2B). When full length ASK1 as overexpressed in combination with various IKKβ protein fragments, ASK1 showed interaction with two distinct regions of IKKβ. As anticipated for a kinase substrate, ASK1 interacted with the kinase domain of IKKβ. Interestingly, however, ASK1 also interacted strongly with the NEMO binding domain (NBD) of IKKβ (Figure 3.2-3B). Binding to this region of ASK1 was observed by both immunoprecipitation and TR-FRET (Figure 3.2-3C). In FRET studies, the NBD fragment showed even greater ASK1 binding that full length IKKß, indicating this may be the primary site for interaction with ASK1. Additionally, overexpressed ASK1 showed stronger interaction with IKKβ than IKKα (Figure 3.2-3D), which, though it shares similar structure to IKKβ, lacks most of the NBD region. Furthermore, overexpression of the NBD fragment was sufficient to

Figure 3.2-2. Structure of ASK1 and IKKβ. (A) Structure of ASK1. ASK1 Consists of a central kinase domain flanked by N- and C-terminal regulatory domains. (B) Structure of IKKβ. IKKβ consists of an N-terminal kinase domain and C-terminal NBD domain with a central domain containing HLH and LZ motifs.

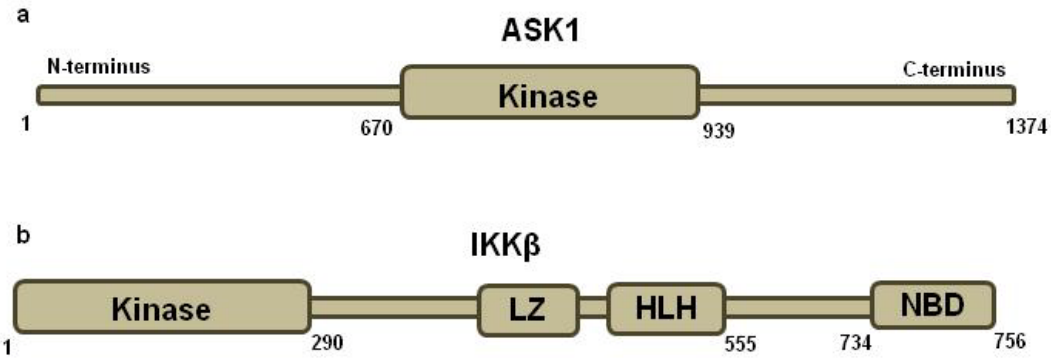
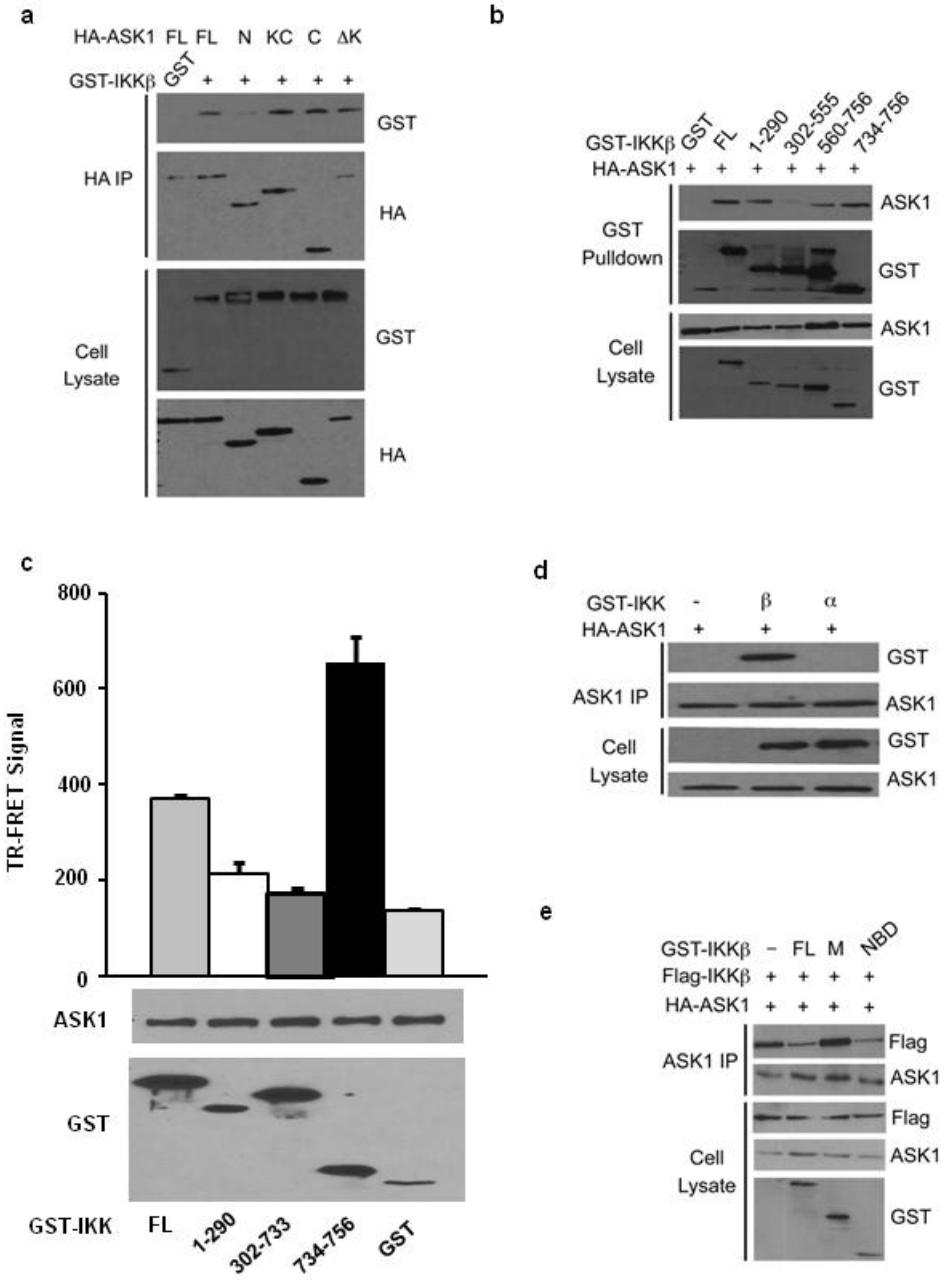

a
ASK1
N-terminus
C-terminus
Kinase
1
670
939
1374
b
IKKβ
Kinase
LZ
HLH
NBD
1
290
555
734
756

Figure 3.2-3. Interaction interface of ASK1 and IKK. (A) IKKβ interacts with the C-terminus of ASK1. Full length GST-IKKβ was co-expressed with indicated HA-ASK1 fragments. GST-IKKβ complexes were isolated by GST pulldown, and bound ASK1 fragments were assessed by Western blot. (B) ASK1 interactions with the kinase domain and NBD of IKKβ. Full length HA-ASK1 was co-expressed with indicated constructs of GST-IKKβ. HA-ASK1 complexes were isolated by immunoprecipitation and bound IKKβ fragments were assessed by Western blot. (C) ASK1 interacts most strongly with the NBD of IKKβ. HEK293T cell were transfected as indicated. Cell lysates were mixed with GST-Terbium conjugated antibody, and fluorescence was measured with the Envision spectrophotometer. Protein levels were assessed by Western blot. (D) ASK1 preferentially interacts with IKKβ over IKKα. HA-ASK1 and GST-IKKβ or GST-IKKα were co-expressed. GST-IKK complexes were isolated by GST pulldown and levels of bound ASK1 were assessed by Western blot. (E) ASK1/IKKβ interaction can be inhibited by an NBD peptide. HA-ASK1 and Flag-IKKβ were co-expressed with or without GST-IKKβ, GST-IKKβ NBD (734-756) or GST-IKKβ M (302-555). ASK1 complexes were isolated by immunoprecipitation, and bound IKKβ was assessed by Western blot.


a
HA-ASK1 FL FL N KC C ΔK
GST-IKKβ GST + + + + +
HA IP
GST
HA
Cell Lysate
GST
HA
b
GST-IKKβ GST FL 1-290 302-555 560-756 734-756
HA-ASK1 + + + + + +
GST Pulldown
ASK1
GST
Cell Lysate
ASK1
GST
c
TR-FRET Signal
800
600
400
200
0
ASK1
GST
GST-IKK FL 1-290 302-733 734-756 GST
d
GST-IKK - β α
HA-ASK1 + + +
ASK1 IP
GST
ASK1
Cell Lysate
GST
ASK1
e
GST-IKKβ – FL M NBD
Flag-IKKβ + + + +
HA-ASK1 + + + +
ASK1 IP
Flag
ASK1
Cell Lysate
Flag
ASK1
GST

compete away binding to full length IKKβ (Figure 3.2-3E), supporting the role for this region in ASK1/IKKβ interaction.

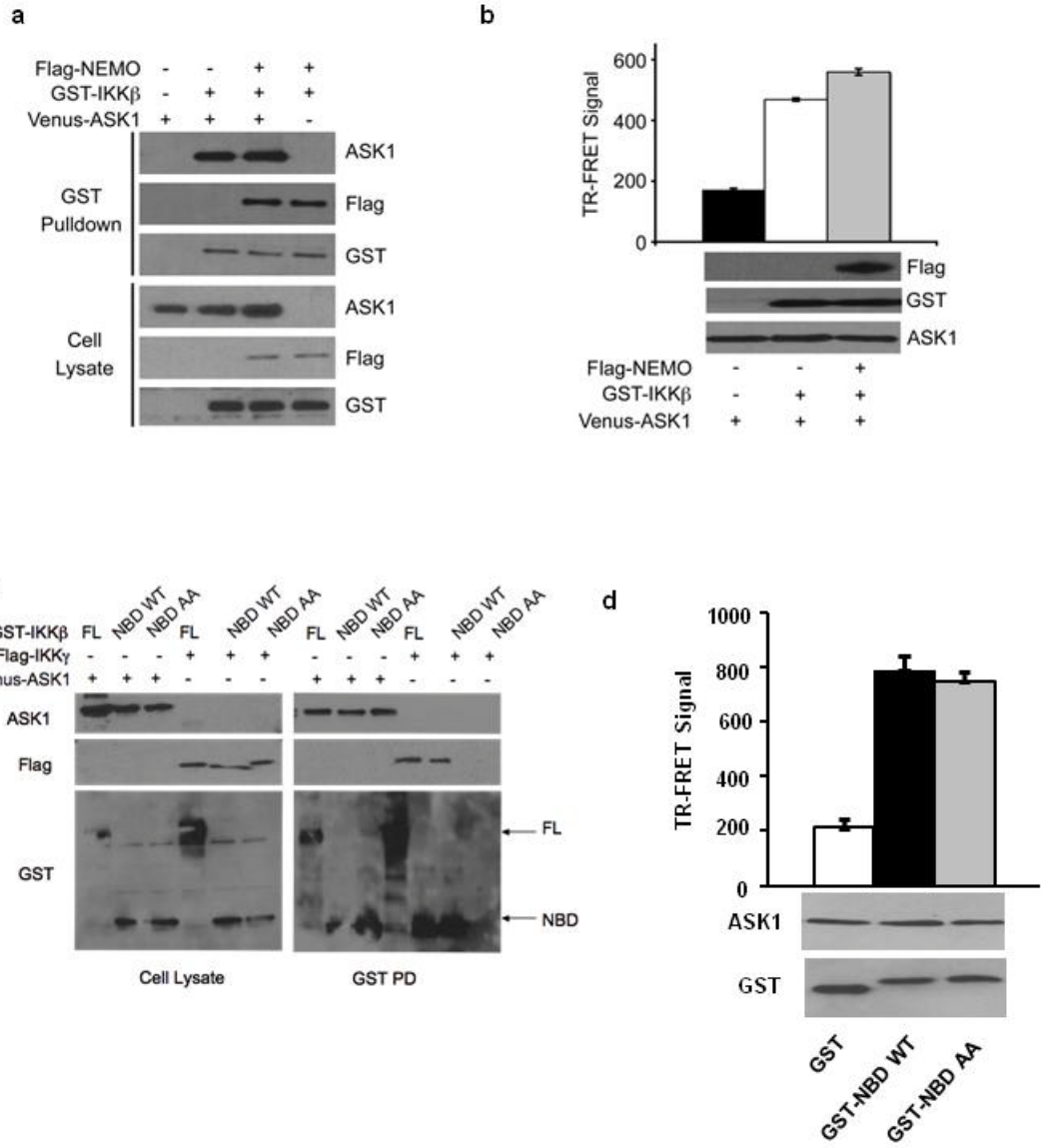
NEMO and ASK1 form a complex with IKK

The NEMO binding domain (NBD) region of IKK, as its name implies, was first identified as the interaction region of IKKγ (129; 130), also known as the NF-κB Essential Modulator (NEMO). Because ASK1 also most strongly interacts with this region of IKKβ, we hypothesized that ASK1 and NEMO would compete for IKKβ binding. To test this, we co-expressed all three of these proteins and assessed binding by both GST pull-down and TR-FRET. Surprisingly, IKKβ showed strong interaction with both ASK1 and NEMO simultaneously, and ASK1 and IKK showed stronger interaction via TR-FRET in the presence of NEMO, indicating that NEMO may facilitate this interaction (Figure 3.2-4A-B). NEMO expression is known to be important for IKKβ interaction with other proteins (137; 138), and it may be playing a similar role for ASK1.

Disruption of NEMO binding site does not alter ASK1 interaction

Previous studies have shown that two particular tryptophan residues are important for NEMO/IKKβ interaction and that mutation of these residues in IKKβ is sufficient to abolish NEMO interaction (213). To address whether ASK1 utilizes these same residues for IKKβ binding, we created IKKβ fragments with these tryptophan to alanine mutations. While NBD fragments containing these mutations no longer showed binding to NEMO as expected, they retained ASK1 interaction (Figure 3.2-4C). ASK1 retained

Figure 3.2-4. IKK forms a complex with ASK1 and NEMO. (A-B) NEMO does not disrupt ASK1/IKK interaction. Venus-ASK1, GST-IKKβ, and Flag-NEMO were co-expressed in HEK293T cells. (A) GST-IKK complexes were isolated by GST pull-down and levels of bound ASK1 were assessed by Western blot. (B) Cell lysates were mixed with GST-Terbium conjugated antibody, and fluorescence was measured with the Envision spectrophotometer. (C-D) Mutation of the NBD does not alter ASK1 binding. Venus-ASK1 or Flag-NEMO was overexpressed with GST-IKKβ, GST-IKKβ NBD WT, or GST-IKKβ NBD AA. (C) GST-IKK complexes were isolated by GST pull-down and levels of bound ASK1 were assessed by Western blot. (D) Cell lysates were mixed with GST-Terbium conjugated antibody, and fluorescence was measured with the Envision spectrophotometer.


a
Flag-NEMO - - + +
GST-IKKβ - + + +
Venus-ASK1 + + + -
GST Pulldown
ASK1
Flag
GST
Cell Lysate
ASK1
Flag
GST
b
TR-FRET Signal
600
400
200
0
Flag
GST
ASK1
Flag-NEMO - - +
GST-IKKβ - + +
Venus-ASK1 + + +
c
GST-IKKβ FL NBD WT NBD AA FL NBD WT NBD AA FL NBD WT NBD AA FL NBD WT NBD AA
Flag-IKKγ - - - + + + - - - + + +
Venus-ASK1 + + + - - - + + + - - -
ASK1
Flag
GST
FL
NBD
Cell Lysate
GST PD
d
TR-FRET Signal
1000
800
600
400
200
0
ASK1
GST
GST
GST-NBD WT
GST-NBD AA

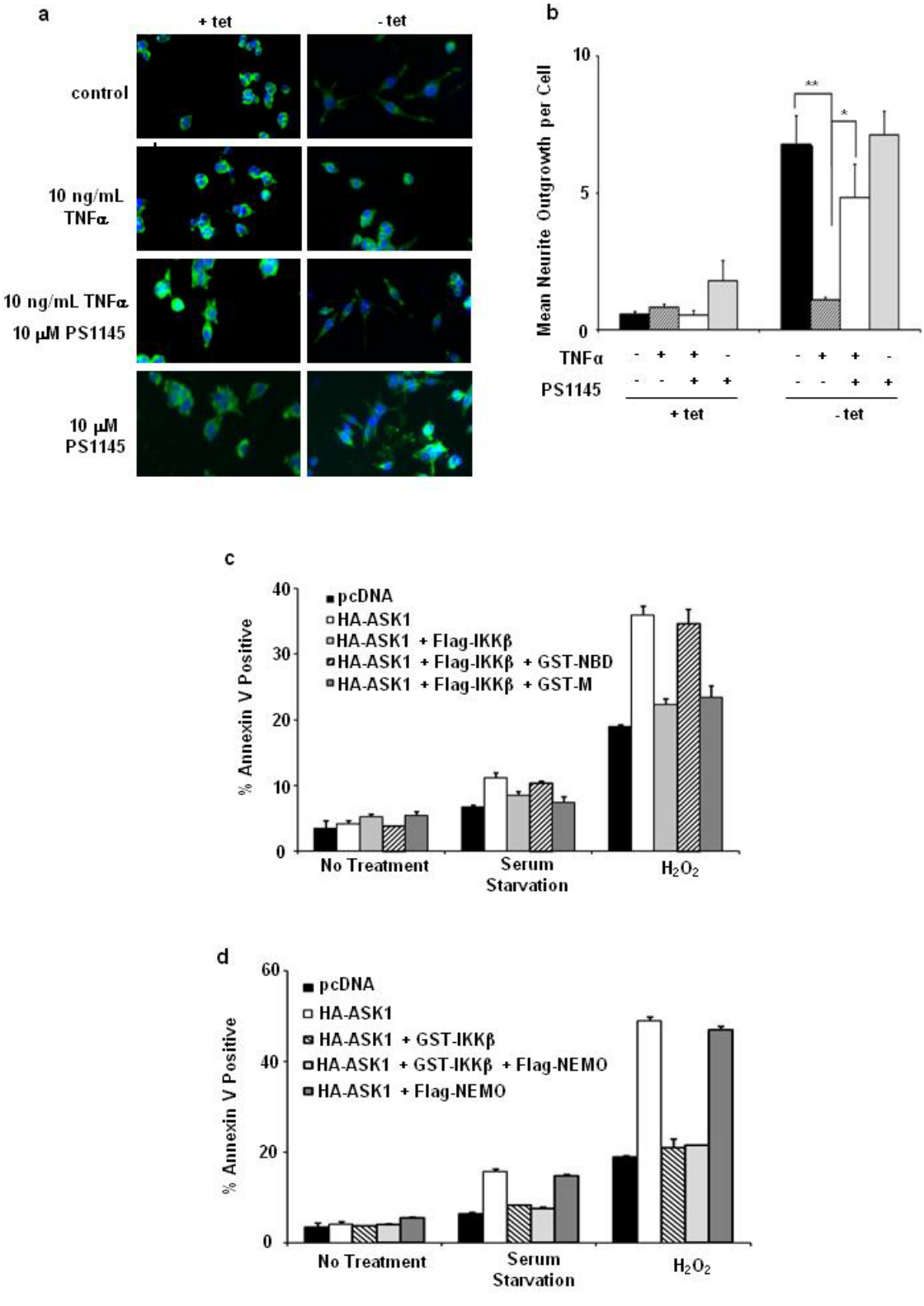
interaction to these fragments even by TR-FRET (Figure 3.2-4D), indicating that ASK1 may interact with distinct residues within the NBD.

IKK suppresses ASK1-mediated neurite outgrowth

To confirm that IKK could impair the biological function of ASK1, we utilized PC12 cells with inducible expression of ASK1ΔN, a constitutively active ASK1 mutant. Expression of ASK1ΔN promotes neurite outgrowth (214). ASK1-induced neurite outgrowth serves as a biological readout for ASK1 function. PC12 cells were treated with TNFα to activate IKK, and IKK activity was monitored by NF-κB translocation. IKK activation significantly decreased ASK1 induced neurite outgrowth (Figure 3.2-5A-B). Furthermore, treatment of cells with the IKK inhibitor PS1145 restored ASK1 induced neurite outgrowth to control levels.

While ASK1 and IKK can be activated by TNFα, ASK1 is primarily activated through TNFR1 signaling (215), whereas IKK can become activated in response to TNFR1 and TNFR2 signaling (216). While TNFR1 is ubiquitously expressed, TNFR2 expression is more limited (217). Previous research has shown, however, that TNFα can promote cell survival in PC12 cells through TNFR2 (218). In our model, TNFα may be preferentially activating TNFR2, leading to activation of IKK and subsequent ASK1 inhibition. Previous studies have also indicated that TNFα 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.

Figure 3.2-5. Disruption of ASK1/IKK interaction increases ASK1 mediated apoptosis. (A) IKK activation inhibits ASK1-mediated neurite outgrowth. PC12 cells were cultured in the presence or absence of tetracycline were treated with TNFα (10ng/mL) and/or PS1145 (10μM) as indicated. Following treatment, cells were fixed and stained with phalloidin. (B) Quantification of neurite outgrowth in (A). Data represent mean ± standard deviation. n=5. *$p<0.05$, **$p<0.02$. (C) NBD peptide restores ASK1-mediated apoptosis. COS7 cells were transfected with HA-ASK1 and Flag-IKKβ with or without GST-IKKβ NBD or GST-IKKβ M. 48 hr after transfection, cells were treated with 100μM H_2O_2 for 2 hr. Following treatment, apoptosis was measured by Annexin V/7-AAD staining. (D) NEMO does not alter ASK1-mediated apoptosis. COS7 cells were transfected with HA-ASK1 and GST-IKKβ with or without Flag-NEMO. 48 hr after transfection, cells were treated with 100μM H_2O_2 for 2 hr. Following treatment, apoptosis was measured by Annexin V/7-AAD staining.


a
+ tet
- tet
control
10 ng/mL TNFα
10 ng/mL TNFα
10 μM PS1145
10 μM PS1145
b
Mean Neurite Outgrowth per Cell
10
5
0
**
*
TNFα
PS1145
+ tet
- tet
c
% Annexin V Positive
40
30
20
10
0
pcDNA
HA-ASK1
HA-ASK1 + Flag-IKKβ
HA-ASK1 + Flag-IKKβ + GST-NBD
HA-ASK1 + Flag-IKKβ + GST-M
No Treatment
Serum Starvation
H2O2
d
% Annexin V Positive
60
40
20
0
pcDNA
HA-ASK1
HA-ASK1 + GST-IKKβ
HA-ASK1 + GST-IKKβ + Flag-NEMO
HA-ASK1 + Flag-NEMO
No Treatment
Serum Starvation
H2O2

Inhibition of interaction promotes apoptosis

Because phosphorylation of ASK1 at Ser967 has a direct effect on ASK1-mediated apoptosis (59) (Chapter 3.1) and overexpression of IKKβ is sufficient to reduce apoptosis mediated by wild type ASK1 but not an S967A mutant (Figure 3.2-5C), we wanted to assess whether disruption of ASK1/IKKβ binding could have a similar affect. In fact, overexpression of the NBD fragment of IKKβ was capable of reversing the inhibition of apoptosis caused by IKKβ while a fragment of IKKβ unable to bind ASK1 had no effect (Figure 3.2-5C). Furthermore, while NEMO expression alone had no effect on ASK1 mediated apoptosis, supporting the hypothesis that IKKβ interacts with ASK1 and NEMO through distinct mechanisms (Figure 3.2-5D).

Discussion

We have previously reported that IKK can inhibit ASK1 function through phosphorylation at Ser967 (Chapter 3.1), and here we describe the details of the interaction between these two kinases. Under physiological conditions, this inhibitory interaction between IKK and ASK1 may allow for an innately fine-tuned response to TNFα signaling by IKK, allowing for manipulation of both ASK1 and NF-κB activity. Knockout of IKKβ specifically has been shown to increase TNFα induced apoptosis (122; 219), possibly by relieving ASK1 of inhibitory phosphorylation at Ser967 and driving ASK1/JNK mediating apoptosis. Additionally, diseases that show upregulation of IKK activity and insensitivity to pro-apoptotic signals may promote cell survival not only through increased transcription of NF-κB target genes, but through inhibition of ASK1 and its downstream effector kinases.

Furthermore, the NBD of IKK is important for ASK1/IKK interaction and NEMO itself seems to facilitate ASK1/IKK interaction, promoting IKK mediated regulation of ASK1 and suggesting a novel role for both the well-characterized NEMO binding domain of IKK and NEMO. While NEMO was originally identified as a critical mediator of canonical NF-κB signaling and is required for IKK complex formation, both NF-κB promoting and inhibiting functions of the protein have been identified (137; 138). It is a logical extension that this protein may facilitate protein-protein interactions outside of the NF-κB realm with respect to IKK function. Additionally, NEMO may allow for additional regulation of ASK1 by IKK due to its ability to scaffold other protein-protein interactions. For example, NEMO has been shown to bring PP2A in contact with IKK (220), a phosphatase known to dephosphorylate ASK1 (61), which could serve as an additional layer of ASK1 regulation.

Further, mutation of the NBD abolished its interaction with NEMO as previously reported (213), but it had no effect on ASK1 interaction. This indicates that within the 22 amino acid region that comprises the NBD at least two distinct elements mediate IKKβ interactions with other proteins. Because overexpressed IKKα showed significantly reduced interaction with ASK1 as compared to IKKβ, it is likely that the region of the NBD not present in IKKα is important for ASK1 binding. Whether this is the case, however, remains to be addressed. Nevertheless, the fact that the IKKβ interacts with ASK1 and NEMO through distinct regions suggests the possibility of specifically disrupting ASK1/IKK interaction without impairing NEMO interaction and vice versa.

While protein-protein interactions are not traditionally considered to be ideal drug targets due to their large contact surfaces and lack of distinctive grooves or pockets (4), a

peptide consisting of the NBD of IKKβ has been used as a therapeutic in a number of studies (221). Addition of this peptide to cells has been shown to block IKKβ induced activation of NF-κB in cells (131), and it has even been used in rodent models of rheumatoid arthritis, where it decreased inflammation (163). In contrast to small molecular inhibitors, disruption of interactions by the NBD peptide can specifically impair canonical IKK/NF-κB signaling, leaving non-canonical signaling by IKKα unaffected, which may skew the effects of the peptide toward preventing the pro-survival and pro-inflammatory functions of this pathway, both of which are suspected to be important in cancer progression (222). Similarly, there significant crosstalk potential between NF-κB and ASK1 signaling, and some of the functions of the NBD peptide may be due at least in part to disruption of ASK1/IKK interaction. Notably, use of the NBD peptide has been shown to resensitize cancer cells to TNFα induced apoptosis (223), a phenomenon mediated by ASK1/JNK signaling.

Use of the NBD peptide may be so powerful because it plays a dual role, blocking pro-survival signaling through NF-κB and promoting apoptotic signaling through ASK1. Disruption of ASK1/IKK interaction may allow ASK1 to regain responsiveness to upstream stress signaling in patho-physiological states were normal apoptotic signaling has been circumvented, allowing for induction of apoptosis by mechanisms that were previously unavailable. As our knowledge of the unique interplay between ASK1 and IKK signaling grows, so does the potential for the development of therapies specifically targeting this interaction.

Chapter 4: Discussion

4.1 General properties of ASK1 interaction with ASK2 and IKK

Though ASK1 interacts with a variety of proteins which regulate its function, it predominantly does so through its N- and C-terminal coiled coil domains, and these protein-protein interactions have both positive and negative effects on ASK1 function. Interaction of thioredoxin with ASK1's N-terminus prevents activation of the kinase, whereas interaction of TRAF proteins with its C-terminus facilitates an active conformation (43). The protein-protein interactions presented in this dissertation are no exception; ASK1 interacts with both ASK2 and IKK through its C-terminus (Figures 2.2-2, 3.2-3), likely through its coiled coil domains. Due to the sequence homology of ASK1 and ASK2, it is also not surprising that the C-terminus of ASK2 mediates its interaction with ASK1 (Figure 2.2-1). Similarly, the NBD region of IKK, which mediates ASK1 interaction (Figure 3.2-3), is known to interact with the coiled coil domains of other proteins, like NEMO (129). Taken together, these data support the hypothesis that the C-terminus of ASK1 is a critical point of interaction with other proteins that regulate ASK1 function.

4.2 IKK regulation of ASK1 could explain JNK/NF-κB crosstalk

IKK's interaction with ASK1 is of particular interest because it points to crosstalk between two major signaling pathways, the MAPK and NF-κB signaling pathways. Though previous studies have indicated NF-κB can inhibit JNK activation by transcriptional means (170), our data suggest an additional mechanism of JNK regulation upstream of NF-κB. IKK, an upstream member of this signaling pathway, can inhibit ASK1, an upstream activator of JNK, by direct interaction and phosphorylation (Figure

3.1-3). Additionally, disruption of ASK1/IKK interaction prevents IKK from inhibiting ASK1-mediatied apoptosis (Figure 3.2-5), which involves activation of JNK, indicating that the interaction of these two proteins is essential for IKK-mediated regulation of JNK signaling.

Furthermore, activation of IKK is known to be a powerful inducer of pro-growth and pro-survival signaling (116). This may be true in part because IKK not only promotes pro-survival and growth signaling through the activation of NF-κB but also blocks pro-apoptotic signaling through the inhibition of ASK1. Both knockout of IKK or expression of a kinase dead IKK mutant diminished Ser967 phosphorylation of ASK1 (Figure 3.1-3), which is linked to 14-3-3 binding and ASK1 inhibition, indicating that IKK is important in the regulation of ASK1 by this phosphorylation event. Similarly, a mutant of ASK1, S967A, which cannot be phosphorylated by IKK, showed increased ASK1-mediated apoptosis in response to serum starvation (Figure 3.1-1), indicating that IKK may play a role in keeping ASK1 inhibited under basal conditions and that the interaction between ASK1 and IKK may play an important role in maintaining the balance of survival and death within cells. Overall, this dual mechanism of ASK1/JNK inhibition by both NF-κB mediated transcriptional activation and direct action of IKK on ASK1 ensures a finely tuned response to upstream signals.

4.3 Therapeutic significance of ASK1 protein-protein interactions

The pathology of many diseases rests on an imbalance of cell survival and cell death, and both ASK1 and IKK have been implicated in a variety of conditions. Identifying whether ASK1 phosphorylation at Ser967 or IKK interaction is altered in

disease could shed light on the biological mechanisms underlying disease formation and progression. Additionally, understanding which diseases have altered ASK1/IKK regulation could reveal potential targets for therapeutic development. ASK1 is thought to contribute to neuronal death in a number of neurodegenerative diseases including Huntington's, Parkinson's, and Alzheimer's diseases (77). Similarly, ASK1 has been implicated in cell death associated in ischemia-reperfusion injury, which occurs during a myocardial infarction or stroke (77). If a mechanism for promoting ASK1/IKK interaction could be identified, one might be able to reduce ASK1- mediated apoptosis in response to the ER stress and ROS production seen in these diseases. Such a mechanism could serve as target for novel drug development in neurodegenerative and cardiovascular diseases.

Conversely, IKK is known to promote tumorigenesis by a number of mechanisms (157). The majority of research into IKK's role in cancer has focused on its ability to activate NF-κB, which can in turn drive transcription of pro-survival and pro-growth genes. A number of additional substrates of IKK, however, have been identified that may contribute to its capacity for pro-tumorigenic signaling (209). One key additional mechanism by which IKK mediates survival and drives growth in cancer cells may be the inhibition of ASK1. In the absence of IKK, TNFα promotes cell death in cancer cells by activating JNK, a downstream member of the ASK1 signaling pathway (223). This could very well be due to increased ASK1 signaling due to a lack of inhibition by IKK. Impairing ASK1/IKK interaction or IKK phosphorylation of ASK1 could be a method to re-establish apoptotic signaling in response to stress signals in cells that have lost sensitivity to these pathways. Understanding whether ASK1 is in fact phosphorylated at

Ser967 by IKK at higher levels in cancer cells could shed light on whether IKK is acting to promote survival by this mechanism. Further, if overactive IKK does contribute to impaired ASK1-mediated apoptosis in cancer cells, this interaction could be targeted with the currently existing NBD peptide or with a novel small molecule inhibitor of the interaction to reinstate proper cell death signaling.

4.4 Some NBD peptide functions may be due to disruption of ASK1/IKK interaction

Previous studies have shown the NBD peptide is capable of disrupting IKK/NEMO interaction, and, in turn, IKK-mediated signaling (221). Use of this peptide in a rat model of RA reduced both inflammation and hyperplasia (163). While the peptide did decrease markers of NF-κB activation, the decrease in RA symptoms could also be due to reactivation of ASK1, which could counteract hyperplasia induction with increased apoptosis in response to pro-inflammatory cytokines.

Additionally, the NBD peptide has been used to modulate the survival of cancer cells. While cancer cells often develop resistance to chemotherapy-induced apoptosis, use of the NBD peptide has been shown to reverse this insensitivity in breast cancer cells (223). Though this effect has been attributed to reduced NF-κB activation leading to decreased pro-survival gene transcription, the peptide may also be blocking IKK's interaction with ASK1, and, subsequently restoring ASK1's ability to induce apoptosis in response to stress signals.

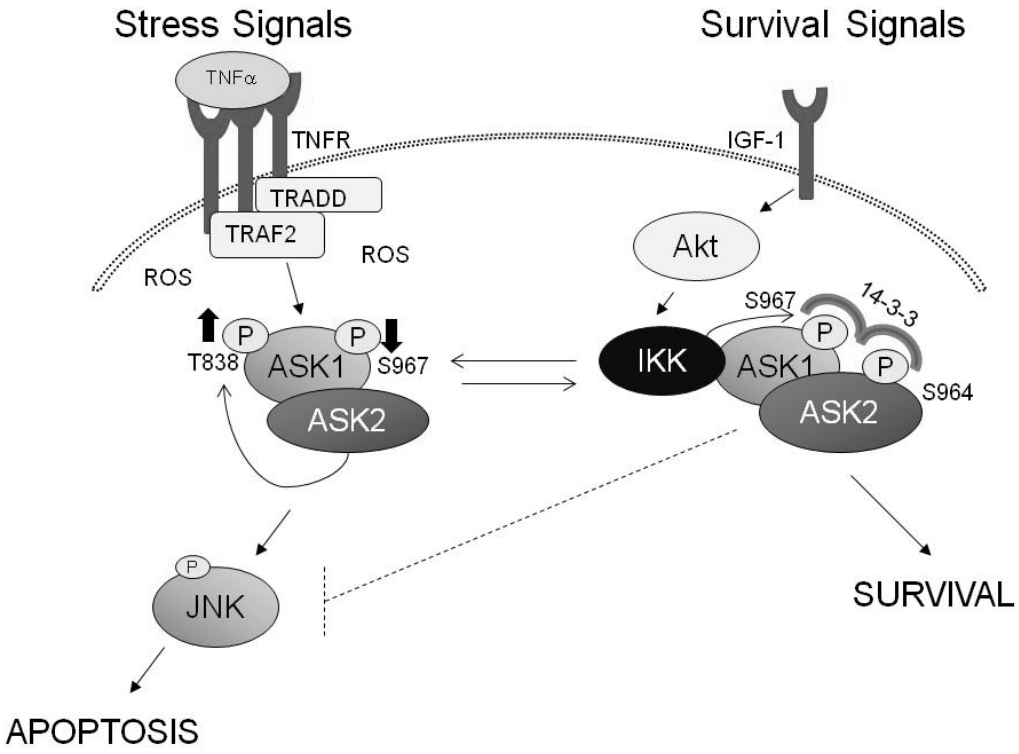
4.5 Conclusions and future directions

ASK1 is a critical mediator of stress signaling. In chapter 2, we showed that ASK2 can both promote and inhibit ASK1 function through distinct protein-protein interactions. Previous studies have shown that ASK2 can activate ASK1 by phosphorylation (40), and, indeed, our data suggest that ASK1/ASK2 interaction is important for ASK1 response to ROS (Figure 2.2-3 and 4-1). Interestingly, however, our data also reveal that once phosphorylated at Ser964, ASK2 can inhibit ASK1 by promoting interaction with 14-3-3, with knockdown of ASK2 reducing ASK1/14-3-3 binding (Figure 2.1-4).

In addition to ASK2, a third member of the ASK family, ASK3, has been identified (58). Very little is known, however, about the relationship of this new family member to ASK1 or ASK2. Similarly, more ASK family members may exist that simply have not yet been identified. Understanding the interactions between all of these family members is critical to understanding how the ASK family regulates stress induced apoptosis and other functions, and more studies are needed to determine how these kinases affect one another and how these interactions are regulated.

In chapter 3, we showed that IKKβ is a key regulator of ASK1 function by increasing ASK1 phosphorylation at Ser967 and promoting 14-3-3 interaction (Figures 3.1-3, 4 and 4-1) and that overexpression or activation of IKKβ can impair ASK1-mediated apoptosis and neurite outgrowth (Figure 3.2-4). What remains to be determined, however, is whether ASK1 reciprocally regulates IKK function. Several recent studies have shown that ASK1 can reciprocally phosphorylate proteins that impair its kinase activity, including PDK1, STRAP, and Pim1 (36; 37; 50). Because ASK1 and IKK are

Figure 4.1 Model of ASK1 regulation by ASK2 and IKK. In response to stress signals, ASK1 can interact with ASK2, which can increase ASK1 activity, promoting ASK1 phosphorylation at T838 and reducing phosphorylation at S967. In response to pro-growth and survival signaling, ASK1 phosphorylation at S967 and 14-3-3 binding is increased through multiple mechanisms. IKK can interact with and directly phosphorylate ASK1 at S967, promoting 14-3-3 interaction and ASK1 inhibition. Once phosphorylated at S964, ASK2 can also interact with 14-3-3, and this interaction promotes ASK1/14-3-3 binding and, subsequently, ASK1 inhibition.


Stress Signals
Survival Signals
TNFα
TNFR
TRADD
TRAF2
ROS
ROS
IGF-1
Akt
S967
14-3-3
P
T838
ASK1
S967
IKK
ASK1
P
S964
ASK2
ASK2
P
JNK
SURVIVAL
APOPTOSIS

both activated in response to TNFα and other stimuli (168), the two kinases may function to regulate one another in order to elicit an appropriate cellular response. Further studies need to be performed to identify potential ASK1 phosphorylation sites on IKK, and, if any are found, the effect they have on IKK function.

Additionally, our data suggest that IKKβ interactions with ASK1 via a region distinct from NEMO since ASK1 is capable of binding a NEMO-binding deficient mutant of the NBD. Whether NEMO truly plays a role in IKK regulation of ASK1, however, remains to be determined. Overexpression of NEMO alone had no effect on ASK1-mediated apoptosis. This is not proof, however, that loss of NEMO would be equally as benign with respect to ASK1 function. Further studies, such as knockout or knockdown of NEMO, need to be undertaken in order to sufficiently characterize NEMO's role in ASK1/IKK interaction. Additionally, understanding the precise region of the NBD that mediates ASK1 interaction could be useful in the development of a peptide that specifically disrupts ASK1/IKK interaction without altering NEMO interaction. Such a peptide could serve as a tool to better understand the relationship between these two proteins and how their interaction affects overall cell biology.

Through the data presented in this dissertation, we have shown that protein-protein interactions are critical to the proper regulation of ASK1 function and that, at least in the case of ASK2 and IKK, alteration of these interactions has functional consequences (Figures 2.1-4, 2.2-3, 3.2-5). ASK1 function has been implicated in a wide variety of diseases (77), and targeting ASK1 protein-protein interactions could be a method to regulate ASK1 function therapeutically. While peptides are not ideal drug candidates—though the NBD peptide has been used therapeutically in animal models

(221)—they could function as tools to screen for small molecule inhibitors of ASK1 protein-protein interactions. Modulating ASK1 activity through the disruption of specific interactions without globally altering kinase activity could allow for development of therapeutics specific to particular diseases. Furthermore, such therapies could allow for ASK1 activity to be targeted under patho-physiological conditions with altered protein-protein interactions, all while minimizing the effect on normal cell function. Because ASK1 plays important roles in both normal cell physiology and pathological conditions, distinct classes of ASK1 protein-protein interaction inhibitors could be useful in a wide variety of diseases.

Chapter 5: References

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