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Lisa Montoya Cockrell Date

Mechanisms Controlling the
Apoptosis Signal-Regulating Kinases (ASK) 1 and 2 Signalosomes

By

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Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
Program in Molecular and Systems Pharmacology

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Apoptosis Signal-Regulating Kinases (ASK) 1 and 2 Signalosomes

By

Lisa Montoya Cockrell
B.S., University of North Carolina at Wilmington, 2001

Adviser: Haian Fu, Ph.D.

An abstract of
a dissertation submitted to the Faculty of the Graduate School of Emory University
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Abstract

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Mitogen-activated protein kinase (MAPK) cascades transmit biological signals to elicit cellular responses. Apoptosis Signal-Regulating Kinase (ASK) 1 is a tightly regulated MAP3K whose activation results in stress responses, including cell death. The ASK1 signalosome represents the collection of proteins which both regulate and direct ASK1 activation and suppression. ASK2 is a newly defined member of the ASK1 signalosome. In this dissertation, it was discovered that (i) ASK2 directs the suppression of ASK1 through engagement of the cell regulatory protein 14-3-3; and (ii) ASK2 interaction with ASK1 is required for 14-3-3-mediated suppression. It was shown that ASK2 directly binds to 14-3-3, through a phosphorylation-regulated interaction mapped to serine 964 of ASK2. ASK1, ASK2, and 14-3-3 exist in a specific ternary complex dynamically controlled by phosphorylation. A 14-3-3 binding defective mutation in ASK2 greatly diminished the amount of ASK1 in the 14-3-3 complex. Through dissociation of 14-3-3, ASK1 becomes activated, evidenced both by a decrease in phosphorylation of serine 967 and an increase in downstream activation of the c-Jun N-terminal kinase. Inhibition of the ASK1/ASK2 interaction dramatically decreased the amount of ASK1 in the 14-3-3 complex, confirming the importance of ASK2 in directing 14-3-3-mediated ASK1 suppression. This research has identified a novel mechanism controlling signal relay between the two MAP3Ks ASK1 and ASK2, and suggests a model whereby upstream signaling events couple ASK2 serine 964 phosphorylation to the ASK1 signalosome, through dual engagement of 14-3-3 proteins.

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LIST OF ABBREVIATIONS

3-NP	3-nitropropionic acid
6xHis	Hexahistidine
AANAT	Serotonin N-acetyltransferase
ADP	Adenosine diphosphate
AIP1	ASK1-interacting protein
Akt	Protein kinase B
ALG-2	Apoptosis-linked gene-2
ALS	Amyotrophic lateral sclerosis
Apaf-1	Apoptosis activating factor-1
AP-1	Activator protein-1
Arg	Arginine
Asn	Asparagine
ASK1	Apoptosis Signal-Regulating Kinase 1
ASK1 ΔCCC	ASK1 construct with deleted C-terminal coiled-coil domain
ASK1 ΔN	ASK1 construct with deleted N-terminal domain
ASK1-KD	ASK1 construct with kinase dead mutation
ASK2	Apoptosis Signal-Regulating Kinase 2
ASK2-KC	ASK2 construct with truncated N-terminal domain
Asp	Asparagine
ATF2	Activating transcription factor 2
ATG	Autophagy-related gene
ATP	Adenosine triphosphate
Bad	Bcl-2/Bcl-X_L associated death promoter
bFGF	Basic fibroblast growth factor
Bcl-2	B-cell lymphoma-2 protein
Bcr	Breakpoint cluster protein
Bim	Bcl-2-interacting mediator of cell death protein
BH3	Bcl-2 homolog domain 3
BSA	Bovine serum albumin
C. elegans	*Caenorhabditis elegans*
c-IAP	Cellular inhibitor of apoptosis
C-terminal	Carboxyl terminus
CaMKII	Calcium/calmodulin-dependent protein kinase II
CIIA	Caspase-activated DNase inhibitor that interacts with ASK1
Caspase	Cysteine-aspartic acid protease
Cdc	Cell division cycle phosphatase
CED	*C. elegans* death gene
CHIP	C-terminus of heat shock protein 70-interacting protein
CIP	Calf intestinal phosphatase

CK1α	Casein kinase 1α
Co-IP	Co-immunoprecipitation
Cos7	African green monkey kidney cell line
CREB	cAMP response element binding
D. melanogaster	*Drosophila melanogaster*
Daxx	Death-domain associated protein
DEAE	Diethylaminoethyl cellulose
DEVD	Caspase substrate with sequence Asp-Glu-Val-Asp
DISC	Death-inducing signaling complex
DMEM	Dulbecco's modified Eagle's medium
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
E. coli	*Escherichia coli*
E2	Estradiol
EDTA	Ethylene diamine tetra-acetic acid
EGL-1	*C. elegans* programmed cell death activator
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
ExoS	Exoenzyme S
Fas	Death receptor, member of the TNFα receptor superfamily
FasL	Fas ligand
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FIP200	Focal adhesion kinase family interacting protein of 200 kDa
FOXO	Forkhead transcription factor
Gα13	Alpha subunit of small G protein of 13 kDa
Gln	Glutamine
Glu	Glutamic acid
GPCR	G protein-coupled receptor
Grx	Glutaredoxin
GSPT1	G(1) to S phase transition protein 1
GST	Glutathione S-transferase
GSTM1-1	GST Mu1-1
H_2O_2	Hydrogen peroxide
HA	Hemagglutinin
hBD	Human beta-defensin
HeLa	Human cervical cancer cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic buffering agent
HPLC	High performance liquid chromatography
HIV-1	Human immunodeficiency virus-1

Hsp72	Heat shock protein of 72 kDa
Hsp90	Heat shock protein of 90 kDa
IAP	Inhibitor of apoptosis
IGF-1R	Insulin-like growth factor-1 receptor
IRE1	Inositol-requiring enzyme 1
IRS	Insulin receptor substrate
IP	Immunoprecipitation
JNK	c-Jun N-terminal kinase
kDa	Kilodaltons
LB	Luria-Bertani broth
LPS	Lipopolysaccharide
Lys	Lysine
MAPK	Mitogen-activated protein kinase
MAP2K	MAPK kinase
MAP3K	MAPK kinase kinase
MAPKAPK	MAPK-activated protein kinase
MBP	Myelin basic protein
MEF	Mouse embryonic fibroblast
MEK	MAPK-ERK kinase
MIA	Microtubule-interfering agent
MNK	MAPK-interacting kinase
MPTP	1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine
MSK	Mitogen and stress activated kinase
mTOR	Mammalian target of rapamycin
N-terminal	Amino terminus
Nef	HIV negative factor
NP-40	Nonidet P-40 or Igepal
p21	Cip1/WAF1
p38	MAPK of 38 kDa
PBS	Phosphate-buffered saline
PCD	Programmed cell death
PCR	Polymerase chain reaction
PFK-2	6-phosphofructo-2-kinase
PKA	Protein kinase A
PKD	Protein kinase D
PP2A	Protein phosphatase 2A
PP2B	Protein phosphatase 2B
PP2Cε	Protein phosphatase 2Cε
PP5	Protein phosphatase 5
PMSF	Phenylmethylsulfonyl fluoride

PolyQ	Polyglutamine repeat
PRAK	p38-regulated/activated kinase
Prx-1	Peroxiredoxin-1
QRS	Glutaminyl-tRNA synthetase
Ras-GAP	Ras GTPase-activating protein
Rb	Retinoblastoma protein
RIP1	Receptor-interacting protein kinase 1
ROS	Reactive oxygen species
SAHA	Suberoylanilide hydroxamic acid
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
Ser	Serine
SKRP1	Stress-activated protein kinase pathway-regulating phosphatase 1
SLK	Ste20-like kinase
SOCS1	Suppressor of cytokine signaling 1
SOD1	Superoxide dismutase 1
SUMO-1	Small ubiquitin-related modifier-1
TAZ	Transcriptional co-activator with PDZ-binding motif
TBS-T	Tris-buffered saline with Tween-20
Thr	Threonine
TLR	Toll-like receptor
TNFα	Tumor necrosis factor α
TNFR	TNFα receptor
TRAF	TNFα receptor-associated factor
Tris	tris(hydroxymethyl)aminomethane buffering agent
Trx	Thioredoxin
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
Tyr	Tyrosine
UPR	Unfolded protein response
Val	Valine
WT	Wild-type
YAP	Yes-associated protein

Chapter 1

Introduction and Background

I.) Cell Fate, a Critical Balance Between Survival and Death

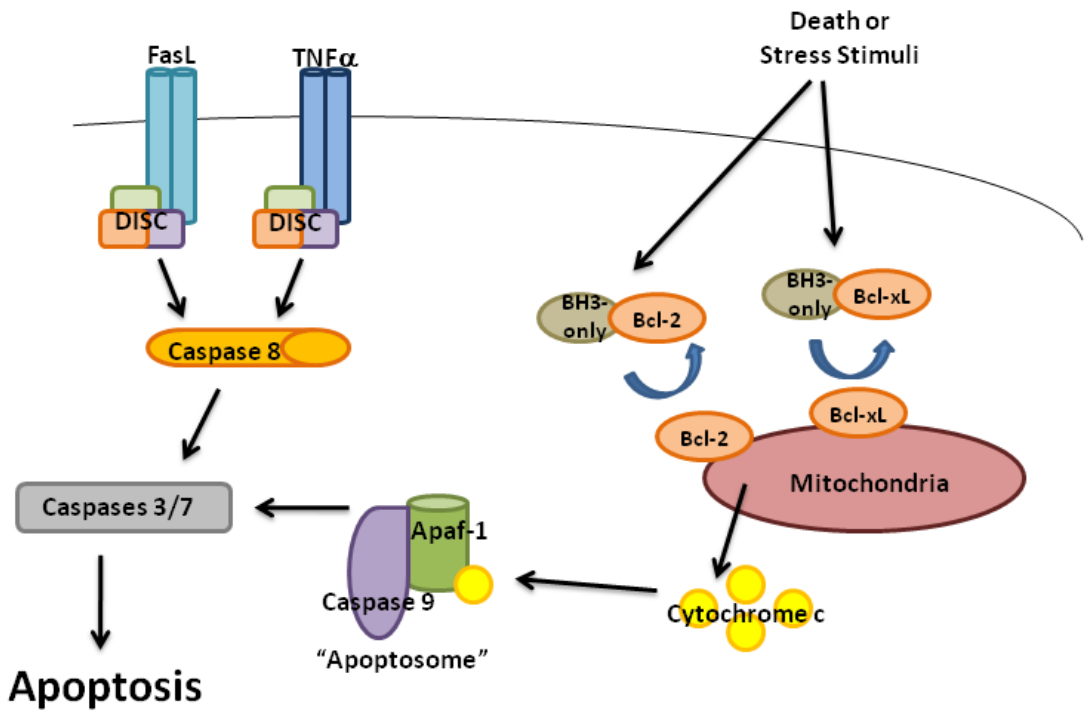
On a daily basis, cells are constantly bombarded by a multitude of pro-growth and pro-death signals, which they must continuously filter through in order to determine their own fate. Growth factors, hormones, and mitogens trigger extracellular induced signaling cascades which culminate in cellular survival, growth, and proliferative processes. Conversely, inflammatory and stress signals initiate pro-death processes. Historically, cell death has been categorized into relatively simple subgroups, depending on whether it occurs as a result of regulated or unregulated mechanisms. For example, necrosis is a chaotic and disorganized form of cell death long considered to occur in response to an overwhelming stress such as severe or acute injury (1). Emerging understanding of a growing body of evidence suggests that a number of cell death events occur as a regulated process, and are a direct result of programmed cell death (PCD).

i.) Apoptosis – Traditional Programmed Cell Death

Apoptosis, the most well-understood form of PCD, was first described in 1971 by Kerr as a type of "shrinkage necrosis" (2). This description referred to the morphological features which were later attributed to apoptosis, including nuclear fragmentation, chromatin condensation, membrane blebbing, and the formation of apoptotic bodies. The basic apoptotic protein signaling components were first described in the worm *Caenorhabditis elegans (C. elegans)*, through genetic studies (3). In *C. elegans*, apoptosis is initiated by the transcriptional upregulation of the pro-apoptotic protein EGL-1. EGL-1 binding to the anti-apoptotic protein CED-9 relieves the inhibitory effect

of CED-9 on the pro-apoptotic protein CED-4. Once activated, CED-4 then binds and activates the protease CED-3, which "executes" cell death by cleaving multiple downstream cellular targets. Exhaustive studies of apoptotic pathways in other eukaryotic cells have found a similar, but more complex, pathway conserved all the way to humans (4). In mammalian cells, two types of apoptosis cascades, the intrinsic and extrinsic pathways, have been described (Figure 1-1). Although they are triggered through distinct mechanisms, each of these pathways converges in their downstream signaling effectors. The intrinsic pathway is initiated through the introduction of stressful or death stimuli, which induce intracellular changes. In response to these stimuli, the pro-apoptotic BH3-only members of the Bcl-2 protein family antagonize the anti-apoptotic Bcl-2 family members, resulting in mitochondrial membrane damage. Mitochondrial permeabilization then leads to the release of cytochrome c, which participates with apoptosis activating factor-1 (Apaf-1) and cysteine aspartic acid protease (caspase) 9 to form the apoptosome complex. Within this complex, caspase 9 becomes conformationally activated, allowing it to continue on to cleave and activate downstream caspase executioners, including the caspases 3 and 7. Mammalian cells also possess components of an extrinsic apoptotic pathway, which are triggered through the extracellular stimulation of death receptors. Two of the most well understood death receptors, CD95/Fas and tumor necrosis factor α receptor 1 (TNFR1), are triggered by the ligands Fas and tumor necrosis factor α (TNFα), respectively (5). Although these receptors are distinct, ligand binding induces similar effects, including receptor dimerization, followed by the recruitment of intracellular adaptor proteins to the receptor. The resulting intracellular signaling complexes formed, referred to as death-induced

Figure 1-1. Major signaling events in apoptosis. Two forms of apoptotic cell death have been characterized in mammalian cells. The intrinsic pathway is initiated by the production of either extracellular or intracellular stress or death stimuli. In response, the pro-apoptotic BH3-only proteins interact with the anti-apoptotic Bcl-2 proteins, sequestering them away from the mitochondria, where they normally exert a protective effect. Subsequent mitochondrial damage results in mitochondrial membrane permeability, leading to the release of cytochrome c, which complexes with Apaf-1 and caspase 9 to form the apoptosome. Within the apoptosome, caspase 9 becomes conformationally activated, whereby it goes on to cleave and activate the downstream apoptotic executioners caspases 3 and 7. The extrinsic pathway is initiated by the binding of cytokines or pro-inflammatory ligands to death receptors, inducing receptor dimerization. Subsequently, adaptor proteins are recruited to death domains within the intracellular portion of the receptor, forming protein complexes referred to as DISCs. DISC formation leads to the cleavage and activation of caspase 8, which in turn activates the executioner caspases 3 and 7.


Death or
Stress Stimuli
FasL
TNFα
DISC
DISC
Caspase 8
BH3-
only
Bcl-2
BH3-
only
Bcl-xL
Bcl-xL
Bcl-2
Mitochondria
Caspases 3/7
Apaf-1
Caspase 9
"Apoptosome"
Cytochrome c
Apoptosis

signaling complexes (DISCs), trigger activation of caspase 8, which in turn cleaves the executioner caspases, including caspase 3 and caspase 7, thus linking the extrinsic and intrinsic apoptotic machinery (6).

ii.) Novel Mechanisms of Programmed Cell Death

Although the first and most fundamentally understood example of PCD was apoptosis, recent work has also begun to define novel examples of regulated forms of cell death. One of these, autophagy, is characterized by an accumulation of double-membrane enclosed vesicles, referred to as autophagosomes (7). These autophagosomes result from an intracellular catabolic mechanism designed to rid cells of degraded cytosolic components, dangerous protein aggregates, and unusable expired organelles. Autophagosomes fuse with lysosomes, whose acidic environment helps to degrade their contents. Autophagy has been established as having an important role in maintaining normal cell homeostasis, in which it removes the damaged organelles or misfolded protein aggregates dangerous to cell survival (8). However, cells have also been found to undergo autophagic cell death under stress conditions, when apoptosis is not an option (9). Although the mechanisms of autophagic cell death still remain to be elucidated, several genes have been identified as necessary. Importantly, these autophagy-related (*ATG*) genes are widely conserved from yeast through mammals, indicating that this process may be a fundamentally critical form of PCD.

Necroptosis is a newly described form of PCD. The concept of necroptosis arose when it was observed that cells underwent a non-apoptotic form of cell death when presented with known apoptotic stimuli (10-12). However, this only occurred in the

setting where apoptotic pathways were inhibited. The morphological features of necroptosis are similar to those attributed to necrosis, including organelle swelling, mitochondrial dysfunction, cell membrane permeabilization, and lack of DNA fragmentation. Although the signaling pathways resulting in necroptosis are unknown, the adaptor receptor-interacting protein kinase 1 (RIP1) has been shown to be an important mediator of necroptosis initiated by TNFα (13). Upon translocation to the mitochondria, RIP1 may be important in the rapid mitochondrial dysfunction which characterizes necroptosis. Future studies aimed at elucidating the mechanisms of necroptosis will be aided by the recent discovery of necrostatins, specific small molecule inhibitors of necroptosis (14). In fact, using necrostatin-1, Degterev and colleagues established necroptosis as a distinct mechanism of delayed response to ischemic brain injury in mice.

II.) ASK1, an Important Mediator of Stress-Induced Apoptosis

i.) Discovery

The discovery of apoptosis signal-regulating kinase 1 (ASK1) was first described in two separate reports, both of which used a degenerate polymerase chain reaction (PCR)-based strategy to identify new kinases. In one report by Ichijo and colleagues, degenerate PCR primers oriented to conserved catalytic subdomains within the serine/threonine kinase family were used to identify a full-length cDNA clone with an apparent amino acid sequence of 1,374 amino acids and a calculated molecular mass of 155 kilodaltons (kDa) (15). Wang and colleagues identified similar characteristics of a

full-length cDNA using degenerate PCR primers against regions conserved in the catalytic domains of the mammalian kinase MAP3K1 and the yeast kinase STE11 (16).

A predicted catalytic kinase domain was identified in the central region of this novel cDNA sequence. Phylogenetic comparison suggested that the identified cDNA clone was most closely related to the mitogen-activated protein kinase kinase kinase (MAP3K) family, sharing close amino acid similarities within the catalytic domain. In fact, when it was expressed in yeast lacking the SSK2 and SSK22 kinases, it was sufficient to complement yeast growth, suggesting it could function as a MAP3K in yeast cells (15). It was then also found to function as a MAP3K in mammalian COS7 cells, when it was shown to selectively activate the mitogen-activated protein kinase (MAPK) pathways leading to c-Jun N-terminal kinase (JNK) and p38 activation. Because it displayed pro-apoptotic characteristics, as described below, this newly identified MAP3K was designated ASK1 (15).

ii.) Evolutionary Conservation

Comparison of the overall amino acid sequence similarity between human and mouse ASK1 revealed they were highly conserved, with 91.9% similarity (17). The two sequences were nearly identical, 98.9%, within their respective kinase domains. A database search revealed that ASK1 also shared homology with sequences from the nematode *C. elegans* and the fly *Drosophila melanogaster (D. melanogaster)*. Compared with mouse ASK1, the overall and kinase domain amino acid similarity for *D. melanogaster* was 40.2% and 72.6%, respectively, and for *C. elegans* was 29.1% and 62.2%, respectively. Later, mouse ASK1 was also found to share 81% overall amino

acid identity with the chick ASK1 sequence (18). Together, this suggests that ASK1 is a highly conserved MAP3K, and therefore its function may be a general and critical feature shared among organisms.

iii.) Structure

ASK1 was found to contain a single central catalytic domain, flanked on either side by long amino (N-) and carboxyl (C-) terminal sequences. The N- and C-terminals each contain a coiled-coil domain, which are hypothesized to mediate multiple functions, including protein interactions and protein stabilization (19). For example, the coiled-coil domain in the C-terminus of ASK1, located from amino acids 1236 to 1293, is noted to allow formation of an ASK1 homodimer under basal cellular conditions (20). This C-terminal coiled-coil domain was also shown to be essential for ASK1 enzymatic activity (20), and an ASK1 construct lacking the C-terminal coiled coil domain (ASK1 ΔCCC) was unable to induce activation of either p38 or JNK (21). The ASK1 ΔCCC construct also displayed a reduced activation status when a putative activating phosphorylated residue was monitored (21). One explanation for this lack of activity may be due to the reduced ability of ASK1 ΔCCC to participate in protein-protein interactions. In fact, compared with an approximately 1,500 kDa high molecular weight complex formed by the ASK1 full-length sequence, ASK1 ΔCCC forms a much smaller protein complex of only 500 kDa (21).

The crystal structure of the human ASK1 catalytic domain, in complex with the generic protein kinase inhibitor staurosporine, was recently solved, allowing further understanding of the structure-function relationships within this protein (22). Compared

with other protein kinases, ASK1 shares a prototypical domain structure within the catalytic domain (23, 24). The catalytic domain is comprised of a small lobe, made up of five β-sheets and a α-helix, and a large lobe made up almost entirely of α-helices. The two lobes are connected by a hinge region containing the ATP-binding site, which in this case was occupied by the competitive ATP inhibitor staurosporine (25). Two hydrogen bonds were responsible for anchoring the lactam moiety of staurosporine into the hinge region, which was hypothesized to mimic the pattern of hydrogen bonding to the adenine base portion of ATP. The ASK1 catalytic domain was found to dimerize in a head-to-tail fashion, whereby the N-terminal portion of one kinase domain interacted with the C-terminal portion of the second kinase domain, and vice versa. Multiple hydrogen and hydrophobic bonds were shown to participate in the formation of this catalytic domain dimer. Interestingly, this dimerization occurred independently of the dimerization noted within the ASK1 C-terminal coiled-coil domain, as the recombinant protein used in the crystal structure analysis lacked the C-terminal in its entirety.

The recombinant ASK1 used in the crystal structure analysis was found to be catalytically active in vitro, and thus the presence of putative autophosphorylation sites could be determined. Mass spectrum analysis revealed three apparent autophosphorylation sites within the catalytic domain. One site was identified as Thr 813, located within the dimer interface. Although considered inaccessible under basal conditions, it is possible that this site could become more easily accessible upon structural changes occurring after ASK1 activation. A second site, Thr 838, was located within the activation segment of the catalytic domain. Importantly, phosphorylation at this site was previously shown to be essential in the oxidative stress-induced activation of

ASK1 (20). A more detailed description of ASK1 regulation by phosphorylation at Thr 838 is described in section II-vi-b. Phosphorylation of Thr 842, the third identified site, was speculated to induce formation of hydrogen bonds to Lys 805 in the catalytic loop. Site-directed mutagenesis of each of these threonine residues to alanine significantly affected ASK1 function in a cell-based reporter gene assay. The ASK1 T838A mutation had the most dramatically reduced effect, in agreement with previous results (20). However, ASK1 T813A and ASK1 T842A also significantly reduced ASK1 function in this reporter gene assay, suggesting that all three autophosphorylation sites were important for full activation of ASK1. Interestingly, these mutations did not directly affect the catalytic activity of ASK1, as each mutant maintained *in vitro* kinase activity similar to the wild-type protein. Instead, it is possible that phosphorylation at each site differentially regulates the recruitment of binding partners to ASK1, and in this way each mutation was able to uniquely affect ASK1 activity.

iv.) Targets and Downstream Signaling Pathways

Two major MAPK cascades are activated upon ASK1 activation (26, 27). The first of these occurs through phosphorylation of the mitogen-activated protein kinase kinase (MAP2K) 4 or MAP2K7, which culminates in activation of the MAPK JNK, of which at least three isoforms exist (JNK1-3). MAP2K4/7 activates JNK through concomitant phosphorylation at Thr 183 and Tyr 185, located in a consensus TxY motif within the catalytic domain. The second major MAPK cascade activated upon ASK1 activation leads to phosphorylation and activation of MAP2K3 or MAP2K6, which then sequentially result in activation of the MAPK p38. Four isoforms of p38 have been

identified (p38α, β, δ, γ). Unlike the MAP2Ks leading to JNK activation, MAP2K3/6 display more substantial substrate selectivity, with MAP2K3 preferentially activating the p38α and β isoforms, while MAP2K6 can strongly activate all four p38 isoforms. The p38 MAPKs are activated upon phosphorylation of the Thr and Tyr phosphoacceptor amino acid sequence TGY.

Transcription factors are some of the most noted downstream effectors of JNK and p38 activation. The JNK and p38 MAPKs are the dominant kinases responsible for recruitment and regulation of the transcription factor activator protein-1 (AP-1). Both JNK and p38 can also phosphorylate activating transcription factor 2 (ATF2), a member of the cAMP response element binding (CREB) subfamily of transcription factors. Furthermore, activated p38 MAPKs lead to phosphorylation and activation of a number of downstream protein signaling kinases, including MAPK-activated protein kinase 2/3 (MAPKAPK2/3), p38 regulated/activated kinase (PRAK), mitogen- and stress-activated kinase 1/2 (MSK1/2), and MAPK-interacting kinase 1/2 (MNK1/2).

v.) Substrate Consensus Phosphorylation Sequence

The sequence specificity of the ASK1 substrate phosphorylation site was determined by screening a peptide library with a recombinant ASK1 protein (22). This combinatorial peptide library contained sequences constructed surrounding a fixed central phosphoacceptor site, to assess the relative contribution of every amino acid residue at each of nine (-5 to +4 relative to the phosphoacceptor site) positions (28). ASK1 clearly displayed a preference for threonine as a phosphoacceptor, although it was also capable of phosphorylating serine-containing peptides. No phosphorylation of

tyrosine was observed. The +1 site seemed to be most important in determining ASK1 selectivity, as the kinase preferred either non-aromatic hydrophobic or aromatic residues at this location. Additionally, ASK1 displayed a less stringent selectivity for glutamine at the -2 position, and either serine, arginine, or tyrosine at the +2 position. Interestingly, ASK1 also strongly phosphorylated peptides which contained threonine residues at either the +2 or -2 position. However, Bunkoczi and colleagues acknowledged that this result also could be interpreted as an artifact of the presence of multiple phosphorylatable residues within the peptide.

Using these data together, a putative preferred ASK1 substrate sequence motif was deduced, TG**pT**YT, where the tyrosine in the +1 position could be replaced with phenylalanine, valine, or tryptophan. However, it is important to note that while the three identified autophosphorylation sites within ASK1 (Thr 813, Thr 838, and Thr 842) all contain this ASK1 motif in relative concordance, the four known direct ASK1 substrates (MAP2K4, MAP2K7, MAP2K3, and MAP2K6) do not contain this motif surrounding the identified ASK1-phosphorylated residues. ASK1 could overcome this discrepancy when phosphorylating its downstream substrates through multiple mechanisms. For example, the formation of a tight complex could induce close proximity of ASK1 with its MAP2K substrate, allowing ASK1 to recognize the critical residues in the MAP2K. Alternatively, structural changes in the MAP2K activation segment may occur upon interaction of the MAP2K docking site with ASK1, which could compensate for the correct residues. Evidence for this latter mechanism recently came with the discovery that the activation segment of the MAPK extracellular signal-regulated kinase (ERK) undergoes significant rearrangement upon occupation of its docking site (29).

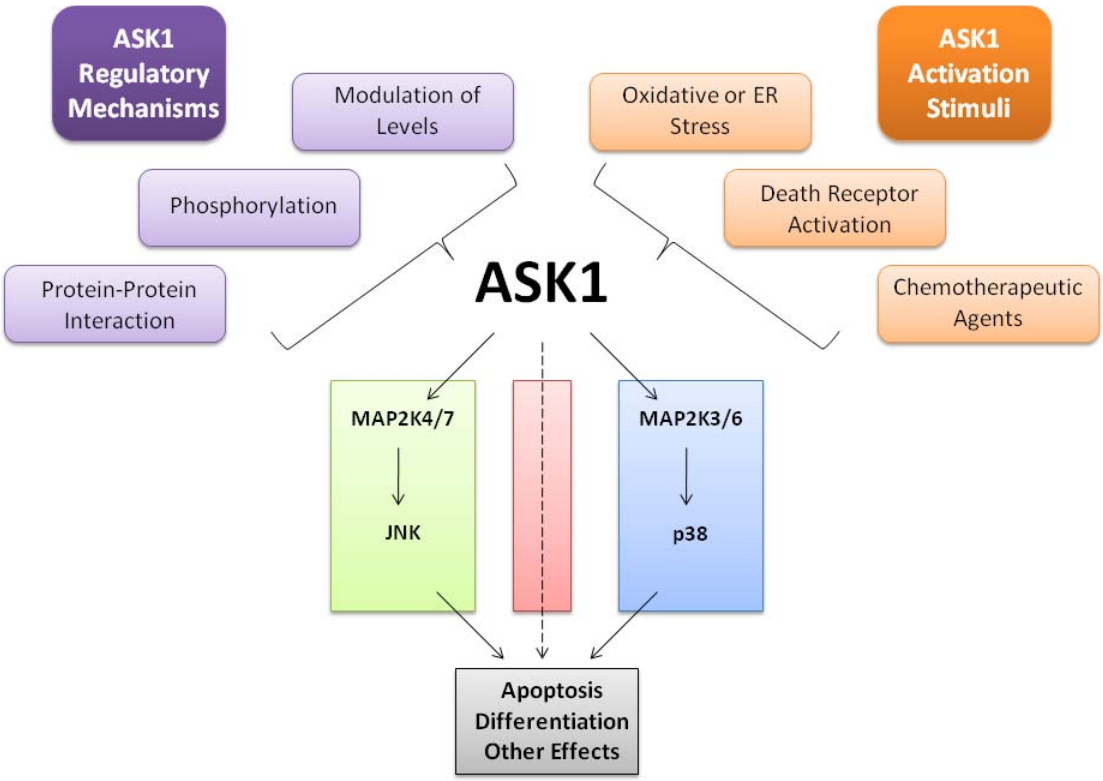
vi.) Mechanisms of ASK1 Regulation

Because ASK1 is such an important mediator of cell death, its activation is tightly controlled. Several mechanisms of ASK1 regulation have been identified, including modulation of ASK1 levels, phosphorylation of the ASK1 protein, and ASK1 binding partners (Figure 1-2).

a.) Modulation of ASK1 Levels

Several lines of evidence have suggested that ASK1 expression is dynamically regulated, both temporally and spatially. The first indication that ASK1 was differentially regulated during development came when Tobiume and colleagues reported a spatiotemporal-specific expression of ASK1 in the developing tissues of the mouse embryo (17). Interestingly, strong ASK1 expression was noted in the suprabasal layer of the epidermis and the hypertrophic region of the vertebrate body, both of which require apoptotic cell death for renewal of skin (30) and bone (31), respectively. Conversely, ASK1 expression was later shown not to be strictly localized to areas of increased apoptosis during development, with the exception of the inter-digital region, in mice (18). This could mean that during development, the ASK1 protein has functions separate from apoptotic regulation. ASK1 expression was also found to be responsive to fibroblast growth factor (FGF) treatment in the developing chick facial mesenchyme; in fact, FGF was shown to be critical for ASK1 expression in that tissue (18). However, this may be a species-specific phenomenon, as the same requirement for FGF was not apparent in the developing mouse embryo.

Figure 1-2. ASK1 signaling. The MAP3K ASK1 is responsive to numerous cell stress stimuli, including oxidative stress induced by ROS, ER stress, activation of death receptors, and chemotherapeutic agents. Upon activation, ASK1 can trigger several downstream pathways, which culminate in the phosphorylation and activation of the MAPKs JNK and p38. Although apoptosis is the major effect attributed to ASK1 activation, other effects have been identified, including cell differentiation. Because of its critical importance in determining cell fate, ASK1 itself is tightly regulated through multiple mechanisms, including modulation of gene and protein expression, protein phosphorylation, and protein-protein interactions.


ASK1 Regulatory Mechanisms
Modulation of Levels
Phosphorylation
Protein-Protein Interaction
ASK1 Activation Stimuli
Oxidative or ER Stress
Death Receptor Activation
Chemotherapeutic Agents
ASK1
MAP2K4/7
JNK
MAP2K3/6
p38
Apoptosis
Differentiation
Other Effects

Using an immunohistochemical approach, changes in ASK1 protein expression during wound healing and disease were monitored using several animal models. First, Funato and colleagues used an injured rat palate model (32). In the uninjured rat palate, only weak cytoplasmic staining of ASK1 in the keratinocytes was observed. However, 2 days after mucoperiosteal injury, intense ASK1 protein expression was detected at the edge of the migrating epithelium. After this increased expression, by day 5, active epithelial apoptosis was apparent using a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method. Importantly, p38 activation correlated with sites of increased ASK1 expression, suggesting that induced ASK1 expression and activation of its downstream pathways is an important mechanism during tissue regeneration after acute epithelial injury. Over the course of the following days as re-epithelialization occurred, ASK1 expression gradually decreased, through day 14, when wound healing was completed. In a rat spinal cord injury model, Nakahara and colleagues then discovered that ASK1 expression was dependent on the tissue examined (33). In the gray matter, ASK1 expression peaked as early as 24 hours post-injury, while it was maximal 3 days after injury in the white matter. Activated forms of JNK and p38 were correlated to the same cells which displayed increased ASK1 expression; these cells also were found to undergo apoptosis. Finally, Minn and colleagues recently showed a possible involvement of induced ASK1 expression in a Huntington's disease mouse model (34). In this model, 3-nitropropionic acid (3-NP), an irreversible mitochondrial complex II inhibitor, was used to induce oxidative stress and subsequent striatal damage, mimicking the clinical features of Huntington's disease. 3-NP was found to induce ASK1 expression in the injured striatum, and cells with high ASK1 expression colocalized with

cells displaying JNK activation and TUNEL positivity.

Changes in ASK1 expression may occur through multiple mechanisms. Perhaps the best characterized mechanism of increased ASK1 expression is through induction of *ASK1* gene transcription. The *ASK1* gene has been shown to be a target of the E2F family of transcription factors. Oligonucleotide arrays first identified *ASK1* as a direct target of the E2F family, and *ASK1* was shown to be highly induced by E2F1 (35.6-fold), E2F2 (5.6-fold), and E2F3 (18.1-fold), specifically (35). A subsequent study showed that overexpression of E2F1 resulted in increased ASK1 mRNA expression, which correlated with increased ASK1 protein expression (36). Conversely, RNA interference of E2F1 reduced ASK1 expression. A non-canonical E2F recognition site was isolated 12 bp upstream of the putative *ASK1* gene start site, and E2F family members, including E2F1, associated *in vivo* with this region during the cell cycle. Mutation of this site rendered *ASK1* nonresponsive to E2F regulation, suggesting *ASK1* was a specific target of E2F. A second study later found two putative E2F1 binding sites located within the *ASK1* gene promoter (37). E2F1 activation by the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) also resulted in substantial upregulation of *ASK1* gene expression in a colon cancer cell line, suggesting *ASK1* was a physiological target of E2F1 (38). Interestingly, increased ASK1 expression was shown to be involved in SAHA-induced apoptosis, but was not associated with increased activation of the prototypical ASK1 downstream effectors JNK and p38. Instead, *ASK1* induction by E2F1 was linked to a positive feedback mechanism, whereby it increased E2F1-induced expression of the pro-apoptotic target Bim. This could be a cell-specific effect, as a study by Hershko and colleagues further characterized the interaction between E2F1 and

ASK1, showing that not only *ASK1* was a target of E2F1, but the p38 phosphatase *Wip1* gene was also (39). While E2F1-induced ASK1 expression resulted in increased p38 activation and apoptosis, induction of *Wip1* served as a negative feedback regulator to this pathway, inhibiting E2F1-ASK1-p38-induced apoptosis.

Ubiquitination and proteasome-mediated degradation seems to be another important mechanism to maintain tight regulation of ASK1 protein levels. The first evidence that ASK1 underwent ubiquitination came when it was shown that the thioredoxin (Trx) protein induced ASK1 ubiquitination and subsequent degradation in endothelial cells (40). Overexpression of Trx significantly increased the amount of ubiquitinated ASK1 in these cells. Furthermore, association of Trx with ASK1 was found to be necessary for this phenomenon, as an N-terminal truncated version of ASK1 (ASK1 ΔN) incapable of binding Trx did not undergo ubiquitination. Trx-induced ASK1 ubiquitination was inhibited both by concomitant overexpression of TNFα receptor associated factor (TRAF) 2, an ASK1-activating protein which participates in dissociation of Trx from ASK1, and TNFα treatment, which induces association of endogenous TRAF2 with ASK1. Together, these data show that Trx-mediated ASK1 ubiquitination and degradation is dependent on association of Trx with ASK1, and suggests this targeted degradation is an important mechanism by which Trx mediates inhibition of ASK1 activation. Further insight into the mechanism of ASK1 protein degradation came when He and colleagues demonstrated that the suppressor of cytokine signaling 1 (SOCS1) interacted with ASK1 in endothelial cells (41). The SOCS protein family has been shown to be involved in numerous examples of regulated protein degradation (42). Indeed, association of SOCS1 with ASK1 correlated with decreased

levels of ASK1 protein expression (41). Moreover, phosphorylation of ASK1 Tyr 718 was found to be critical for SOCS1 interaction, although the kinase responsible for this phosphorylation is as of yet unidentified. SOCS1-induced ASK1 degradation was noted only under unstimulated conditions. Conversely, TNFα treatment induced dephosphorylation of Tyr 718 of ASK1, leading to a reduction of ASK1 interaction with SOCS1 and stabilization of the ASK1 protein. Further support for the importance of SOCS1 in regulating ASK1 levels was shown in endothelial cells derived from SOCS1-deficient mice, which had increased ASK1 expression and activation.

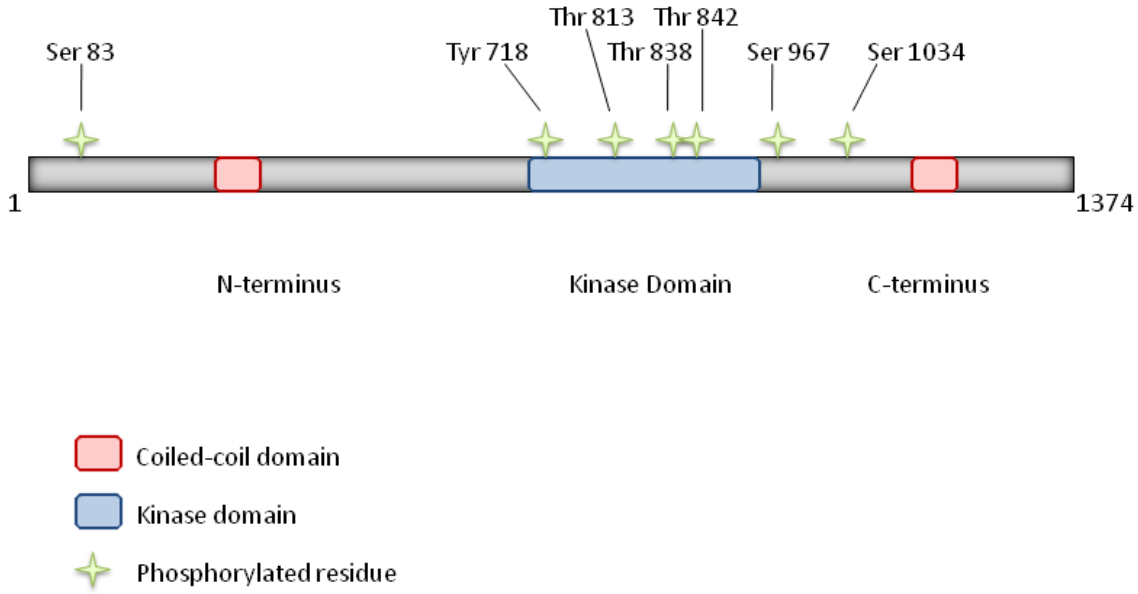
Recently, other mechanisms of regulating ASK1 expression have also been described. For example, expression of a constitutively activated form of the G protein, Gα13, was found to result in increased ASK1 expression (43). Constitutively activated Gα13 reduced the rate of ASK1 ubiquitination and degradation induced by prolonged thrombin exposure in human umbilical vein endothelial cells. This was shown to occur through dissociation of ASK1 from the C-terminus of Hsp70-interacting protein (CHIP), a cochaperone and E3 ubiquitin ligase involved in ASK1 ubiquitination (44). Finally, a report by Stordal and Davey argued that proteasome-mediated ASK1 degradation is a phenomenon restricted to ectopically overexpressed ASK1 (45). Instead, endogenous ASK1 was shown to exist in a cleaved form under basal, nonapoptotic cell conditions. These cleaved forms were not dependent on proteasome-mediated degradation, as the proteasome inhibitor lactacystin did not inhibit cleavage. Although this observation has not been confirmed in separate reports, it is an important point to consider when interpreting ASK1 studies which utilize exogenous ASK1 expression.

b.) Phosphorylation of ASK1

Metabolic labeling experiments have revealed ASK1 to be highly phosphorylated in cells under basal cell survival conditions (46), suggesting that phosphorylation may be a critical step in maintaining ASK1 regulation. To date, five phosphorylation sites have been described on ASK1 (Figure 1-3), in addition to two putative autophosphorylation sites which have not been characterized (detailed in section II-iii). All but one, Tyr 718, is a serine or threonine residue. The kinases and phosphatases responsible for regulating Tyr 718 phosphorylation remain to be identified. However its importance in determining ASK1 protein stability, as described in section II-vi-a, makes determining the regulation of this site an important step in understanding ASK1 activation.

Three of the identified serine phosphorylated residues are considered to be inactivating phosphorylation events. One of these, phosphorylation of Ser 83, has been well characterized to have an inhibitory effect on ASK1 activation. First reported by Kim and colleagues, ASK1 was found to contain a consensus Akt phosphorylation site (RGRGS**pS**V) surrounding Ser 83 (47). An *in vitro* kinase assay confirmed that ASK1 was an Akt substrate, when it was shown that recombinant Akt could phosphorylate a purified ASK1 fragment containing Ser 83, but was unable to phosphorylate the same fragment with mutated S83A. Further, endogenous levels of Akt and ASK1 were found to interact in epithelial tumor cell lines. This interaction was not affected by Akt activation status, and the ASK1 S83A mutation did not diminish interaction with Akt. Importantly, Ser 83 phosphorylation by Akt was then shown to have a functional effect. When co-expressed with wild-type ASK1, Akt was able to diminish hydrogen peroxide (H_2O_2)-mediated ASK1 activation. Conversely, ASK1 S83A remained unaffected, and

Figure 1-3. Phosphorylation sites identified within the ASK1 protein. To date, seven phosphorylation sites have been identified in ASK1. One of these, Tyr 718, mediates ASK1 protein degradation by inducing SOCS1 binding. Three of the residues (Thr 813, Thr 838, Thr 842) have been identified as sites of autophosphorylation. Of these, only Thr 838 has been well characterized. Phosphorylation at Thr 838 is considered to be a positive regulation, resulting in increased ASK1 catalytic activity. Thr 838 was first described to be a site of trans-autophosphorylation, which occurs subsequent to homodimerization of the ASK1 catalytic domains. More recently, ASK2 in a heteromeric complex with ASK1, has also been suggested to directly phosphorylate ASK1 at this site. Phosphorylation of Ser 83 by the pro-survival kinase Akt results in reduced ASK1 activation. Similarly, Ser 967 phosphorylation by an unidentified kinase leads to recruitment of 14-3-3 to the ASK1 C-terminus, resulting in diminished ASK1 kinase activity and ASK1-induced apoptosis. Both PP2A and PP2B have been suggested as Ser 967-directed phosphatases. Ser 1034 phosphorylation also occurs via unknown mechanisms, but phosphorylation at this site is linked with reduced ASK1 catalytic activity.


Ser 83
Tyr 718
Thr 813
Thr 842
Thr 838
Ser 967
Ser 1034
1
1374
N-terminus
Kinase Domain
C-terminus
Coiled-coil domain
Kinase domain
Phosphorylated residue

was still able to undergo full activation by H_2O_2 treatment. Akt overexpression was also shown to protect cells from ASK1-induced JNK activation, an effect that was negated with the expression of the ASK1 S83A construct. The physiological importance of Akt in mediating reduced ASK1 activity has been confirmed in numerous other reports, as well. For example, in an ovarian cancer cell line, overexpression of constitutively activated Akt was shown to decrease the sensitivity of these cells to the DNA damaging antineoplastic agent cisplatin (48). The mechanism of this desensitization occurred through an ASK1-mediated pathway, and it was surmised that overexpression and overactivation of Akt in cancer cells could lead to suppression of ASK1 activity, thereby leading to increased resistance to chemotherapy-induced apoptosis in tumor cells. Similar results were also noted in an ovarian cancer cell line treated with the chemotherapeutic agent paclitaxel, when it was shown that estradiol (E2) treatment significantly attenuated paclitaxel-induced apoptosis through a mechanism involving Akt activation and subsequent ASK1 Ser 83 phosphorylation (49). This mechanism was found to be specific for an Akt-ASK1 pathway, as E2 was unable to diminish paclitaxel-induced apoptosis in the setting of overexpressed ASK2 S83A. Akt-mediated inhibition of ASK1 was also noted to be an important mechanism of survival in human islet cells (50), and involved in the antioxidant selenite-induced neuroprotection against ischemic brain injury (51). An important point in understanding the Akt regulation of ASK1 came when Zhang and colleagues reported that the chaperone protein heat shock protein (Hsp) 90 formed a ternary complex with Akt and ASK1 in unstimulated endothelial cells, suggesting that Hsp90 held Akt and ASK1 in close proximity (52). Furthermore, this ternary complex was also shown to stabilize the Akt and ASK interaction under

conditions of oxidative stress, in an attempt to protect cells from stress-induced apoptosis.

The first phosphorylation site described in ASK1 was Ser 967. Ser 967 is located within a putative 14-3-3 recognition motif, RSI**pS**LP, which is just C-terminal of the kinase domain within the ASK1 protein. Ser 967 was first discovered to be a phosphorylated residue in the ASK1 protein when Zhang and colleagues reported that mutation of this site to S967A reduced interaction with the phospho-serine/threonine binding protein 14-3-3, described in greater detail in section II-vi-c-1 (53). Ser 967 is phosphorylated under basal survival conditions in cells, as revealed by a phospho-specific antibody (54). Phosphorylation of Ser 967 was associated with a functional effect on the ASK1 protein, because the ASK1 S967A mutation displayed enhanced pro-apoptotic activity. A later report provided a mechanism for this enhanced pro-apoptotic activity by showing that the S967A mutation alone produced a 3-fold increase in ASK1 catalytic activity (54). Further, phosphorylation at Ser 967 was sensitive to dephosphorylation upon treatment with the reactive oxygen species (ROS) H_2O_2, which occurred concomitantly with an increase in ASK1 kinase activity, thus further linking Ser 967 dephosphorylation with ASK1 activity. Phosphorylation of ASK1 Ser 967 may be a critical event in the physiological regulation of ASK1 activity, as multiple reports have linked phosphorylation at this site with an ASK1-induced cellular effect. For example, treatment of breast cancer cell lines with the antioxidants EUK8 and N-acetyl cysteine was found to inhibit dephosphorylation of Ser 967 (55), which agreed with the study from Goldman and colleagues showing Ser 967 phosphorylation was sensitive to ROS (54). In another study, ASK1 Ser 967 dephosphorylation was considered to be a mechanism of diabetic cardiomyopathy (56). Phosphorylation of ASK1 Ser 967 has also

been proposed to be a key component of laminar flow-mediated inhibition of cytokine signaling in endothelial cells (57). While the kinase responsible for ASK1 Ser 967 remains to be identified, two candidate phosphatases have been described. Both in a purified *in vitro* system as well as mammalian cell lysates, the protein phosphatase 2B (PP2B), or calcineurin, was capable of dephosphorylating ASK1 at this site, resulting in enhanced cardiomyocyte apoptosis (58). However, this was in direct conflict with data from Goldman and colleagues, which showed that the calcineurin-specific phosphatase inhibitor cyclosporine A was incapable of preventing H_2O_2-induced Ser 967 dephosphorylation (54). Another report subsequently identified protein phosphatase 2A (PP2A) as the Ser 967 phosphatase (59), which correlates with the ability of PP2A inhibitors to prevent H_2O_2-induced Ser 967 dephosphorylation (54). Thus, ASK1 Ser 967 dephosphorylation may be a cell type-specific event, dependent on the signaling events and phosphatases present in the relevant cellular context.

One of the most highly phosphorylated sites in ASK1, identified through trypsin digestion followed by microcapillary reverse-phase high performance liquid chromatography (HPLC) coupled to nanoelectrospray tandem mass spectrometry, was Ser 1034 (46). Intriguingly, mutation of this site to alanine induced a dramatic change in the pro-apoptotic potential of ASK1. When expressed in cells, the ASK1 S1034A mutant displayed increased apoptotic activity, similar to that attributed to ASK1 S967A. This enhanced apoptotic activity was explained by an increase in both ASK1 autophosphorylation as well as trans-phosphorylation of the MAP2K3-p38 kinase cascade. Interestingly, mutation of ASK1 to S1034E, in which case the glutamate residue was surmised to serve as a phosphomimetic, dramatically reduced both the kinase activity

and pro-apoptotic activity, down to levels of the wild-type protein. This confirmed the fundamental importance of phosphorylation at this site for regulating ASK1 catalytic activity. Although the kinases and phosphatases responsible for mediating Ser 1034 phosphorylation are as of yet unidentified, it was determined that interaction with both Akt and 14-3-3 were unaffected by the S1034A mutation. This suggests that the regulation of Ser 1034 phosphorylation occurs through an independent and unique mechanism. One possibility is deduced from the residues surrounding Ser 1034, PP**pS**P, a motif recognizable by proline-directed kinases (60).

Thr 838 is the single confirmed phosphorylation site within ASK1 associated with a positive regulatory role. This site was first identified when it was shown that ASK1 was a direct substrate for protein phosphatase 5 (PP5), both *in vitro* and *in vivo* (61). This threonine residue lies within the activation loop of the ASK1 kinase domain. Using a phospho-specific antibody against Thr 838, phosphorylation at this site was correlated with increased ASK1 activity, induced by ROS (20). PP5 was determined to be an essential phosphatase responsible for dephosphorylating ASK1 at this residue, and PP5 association with ASK1 reduced H_2O_2-induced ASK1 activation (61). Additionally, co-expression of PP5 suppressed ASK1-induced apoptosis resulting from H_2O_2 treatment. More recently, the protein phosphatase 2C epsilon (PP2Cε) was also found to be capable of direct dephosphorylation at Thr 838 *in vitro* (62, 63). The differential association of these phosphatases with ASK1 was dependent upon the cellular conditions, suggesting that these two phosphatases have unique roles in regulation of ASK1. Several reports have shown a physiological role of PP5 in the dephosphorylation and suppression of ASK1 activity (64, 65). Additionally, one report detailed a link between rat cerebral

ischemia and increased phosphorylation of Thr 838, confirming the functional role of this phosphorylation in tissues (66). This site was originally proposed to be a substrate for trans-autophosphorylation, occurring after H_2O_2-induced conformational changes created a new interface, allowing for trans-autophosphorylation (20). However, Thr 838 was also shown to not exclusively be a substrate for trans-autophosphorylation, but also was able to undergo transphosphorylation. Recently, a candidate kinase for this site was identified as apoptosis signal-regulating kinase 2 (ASK2) (67), in a model described in section III-iv-a.

c.) ASK1 Protein-Protein Interactions

Numerous protein interacting partners have been described for ASK1 (Table 1-1). This section contains a description of selected ASK1 binding partners, chosen for their well characterized and unique effects upon ASK1. Several are discussed in other sections of this introduction, and therefore will not be discussed in detail here, including the redox-sensitive proteins Trx and glutaredoxin (Grx). ASK2, one of the newest identified ASK1 interacting partners, is a major component of this thesis, and therefore is fully described in section III.

1.) 14-3-3

One of the most well characterized ASK1-binding proteins with an inhibitory effect on ASK1 is 14-3-3. 14-3-3, described in full detail in section IV, is a cell regulatory protein that recognizes its numerous target proteins through a conserved phosphorylated serine or threonine motif (68). In the case of ASK1, binding to 14-3-3

Table 1-1. Summary table of identified ASK1 interacting partners. A comprehensive list of identified ASK1 binding proteins. Listed here are summaries of the mechanism of regulation or effect on ASK1 induced by the binding partner.

Interacting Protein	Full or Alternate Protein Name	Mechanism or Effect of Binding to ASK1	References
Positive Effectors of ASK1 Activation			
AIP1	ASK1-interacting protein	Binds to ASK1 at dephosphorylated Ser 967, inducing release of 14-3-3 from ASK1 by recruitment of PP2A	(59, 69, 70)
ASK2	Apoptosis Signal-Regulating Kinase 2/MAP3K6	Forms heteromeric complex with ASK1, in which it is stabilized by ASK1 in a kinase-independent manner; ASK2 also phosphorylates ASK1 at Thr 838 (also see negative effectors)	(67, 71, 72)
β-arrestin 2		Acts as a scaffold to link ASK1 with MAP2K4 and JNK3, bringing this MAPK module under the control of the angiotensin II type 1A GPCR	(73)
CaMKII	Calcium/Calmodulin Kinase II	Phosphorylates ASK1 at unknown residue, mediating Ca^{2+}-induced ASK1 activation	(74-76)
Daxx	Death-domain associated protein	Fas-induced interaction with ASK1 disrupts an inhibitory intramolecular interaction between the N- and C-termini of ASK1, allowing ASK1 oligomerization	(77, 78)
FIP200	Focal Adhesion Kinase Family Interacting Protein of 200 kDa	Acts as a scaffold to facilitate TRAF2 interaction with ASK1 in response to TNFα	(79)
Gα13	G α protein 13	Binds to the N- and C-termini of ASK1, preventing ASK1 degradation	(43)

Interacting Protein	Full or Alternate Protein Name	Mechanism or Effect of Binding to ASK1	References
Positive Effectors of ASK1 Activation (continued)			
GSPT1	G(1) to S Phase Transition Protein 1	Enhances ASK1 autophosphorylation and diminishes 14-3-3 association	(80)
PKD	Protein Kinase D	Interacts with ASK1 C-terminus in response to H_2O_2, leading to ASK1 dephosphorylation at Ser 967 and 14-3-3 dissociation	(81)
PP2A	Protein Phosphatase 2A	Dephosphorylates ASK1 at Ser 967, promoting dissociation of 14-3-3	(59)
PP2B	Protein Phosphatase 2B/Calcineurin	Dephosphorylates ASK1 at Ser 967, promoting dissociation of 14-3-3	(58)
Rb	Retinoblastoma protein	Interacts with ASK1 through an LXCXE motif, which is required for ASK1-induced apoptosis	(82)
SKRP1	Stress-activated protein kinase pathway-regulating phosphatase 1	Functions as a scaffolding partner to enhance association between ASK1 and MAP2K7	(83)
SLK	Ste20-like Kinase	Enhances ASK1 phosphorylation at unknown sites, leading to increased ASK1-p38 activation	(84)
TRAF2/6	TNFα Receptor-Associated Factor 2/6	Enhances N-terminal homophilic interaction between ASK1 oligomers	(85-87)

Interacting Protein	Full or Alternate Protein Name	Mechanism or Effect of Binding to ASK1	References
	Negative Effectors of ASK1 Activation		
14-3-3		Binds to phosphorylated Ser 967, protects this site from ROS-mediated dephosphorylation	(53, 54, 88)
Akt	Protein Kinase B	Phosphorylates ASK1 at Ser 83, protecting from chemotherapy-induced ASK1 activation	(47-50, 52)
ASK2	Apoptosis Signal-Regulating Kinase 2/MAP3K6	Directs formation of a ternary complex between 14-3-3 and ASK1, leading to ASK1 inhibition (also see positive effectors)	(89)
Cdc25A	Cell division cycle phosphatase 25A	Diminishes ASK1 homo-oligomerization, independent of its phosphatase activity	(90)
CHIP	C-terminus of heat shock protein 70-interacting protein	Induces ASK1 ubiquitination and degradation, and translocation of Daxx into the nucleus	(44)
c-IAP	Cellular Inhibitor of Apoptosis	Ubiquitin protein ligase which is required for TNFR2-induced ASK1 degradation	(91)
CIIA	Caspase-activated DNase (CAD) Inhibitor that Interacts with ASK1	Inhibits ASK1 oligomerization	(92)
GSTM1-1	Glutathione S-Transferase Mu1-1	Protects ASK1 from heat shock-mediated activation	(93)
Grx	Glutaredoxin	Binds to C-terminus of ASK1; protects cells from glucose deprivation-induced metabolic oxidative stress by suppressing redox sensitive activation of ASK1	(94, 95)

Interacting Protein	Full or Alternate Protein Name	Mechanism or Effect of Binding to ASK1	References
Negative Effectors of ASK1 Activation (continued)			
Nef		HIV-1 protein that prevents Trx release from ASK1 and therefore interferes with Fas and TNFα-induced pro-apoptotic signaling in virally infected host cells	(96)
H-Ras		Inhibits ASK1 in a MEK/ERK and PI3K/Akt –independent manner	(97)
Hsp72	Heat Shock Protein 72	Blocks ASK1 homo-oligomerization in response to mild heat shock	(98)
Hsp90	Heat Shock Protein 90	Stabilizes Akt and ASK1 in close proximity	(52)
IGF-1R	Insulin-like Growth Factor Receptor Type I	Association with ASK1 leads to phosphorylation of tyrosine residues in the N-terminus of ASK1	(99)
p21	Cip1/WAF1	Cytoplasmic p21 binds to and is phosphorylated by ASK1; inhibits ASK1 in a cell-cycle independent manner	(100, 101)
PP5	Protein phosphatase 5	Dephosphorylates ASK1 at Thr 838, reducing ASK1 activity	(61)
QRS	Glutaminyl-tRNA synthetase	Associates with ASK1 through the kinase domain in a glutamine-dependent manner	(102)

Interacting Protein	Full or Alternate Protein Name	Mechanism or Effect of Binding to ASK1	References
Negative Effectors of ASK1 Activation (continued)			
Prx-1	Peroxiredoxin-1	Binds to Trx binding domain in ASK1 and inhibits ASK1 in a redox-sensitive manner	(103)
Raf-1		Binds to the N-terminus of ASK1 and inhibits ASK1 in a manner independent of both Raf-1 catalytic activity and MEK-ERK pathway activation	(104)
SOCS1	Suppressor of cytokine signaling 1	Induces ASK1 tyrosine phosphorylation, leading to subsequent ASK1 protein degradation in response to TNFα signaling	(41)
SUMO-1	Small ubiquitin-related modifier-1	Inhibits homo-oligomerization in a sumoylation-independent manner	(105)
Trx	Thioredoxin	Maintains ASK1 in an inactive conformation through binding to the ASK1 N-terminus in a reduced form and preventing full oligomerization of the protein in an ROS-sensitive manner	(87, 106, 107)

has been found to occur through a primary site involving Ser 967 (RSI**pS**LP) (53). 14-3-3 binding to ASK1 results in a negative effect on the pro-apoptotic potential of the ASK1 protein. In fact, mutation of this site to a nonphosphorylatable alanine (ASK1 S967A) results in a protein with dramatically enhanced apoptotic ability. ASK1 binding to 14-3-3 is a common feature to all seven mammalian 14-3-3 isoforms, and co-expression of all 14-3-3 isoforms suppresses ASK1-induced apoptosis (88). The mechanism for 14-3-3-mediated inhibition was found to include suppression of the kinase activity of ASK1, as the ASK1 S967A mutation, incapable of binding to 14-3-3, displays increased basal kinase activity by 3-fold compared to wild-type (54). Interestingly, Ser 967 phosphorylation was found to be sensitive to ROS (54), and therefore stimuli-driven dephosphorylation at Ser 967 may reveal an important mechanism for ASK1 activation. This was confirmed when it was shown that TNFα also activates ASK1, at least in part, through dissociation of 14-3-3 from ASK1 (57). Recently, the interaction of 14-3-3 with ASK1 was found to be further regulated by the ASK1-interacting protein 1 (AIP1), described in more detail in section II-vi-c-2. The G(1) to S phase transition protein 1 (GSPT1) also may positively regulate ASK1 activation by enhancing dissociation of 14-3-3 from ASK1 (80). The role of 14-3-3-mediated inhibition of ASK1 activity has been shown to be important under diverse physiological conditions. Mice expressing a dominant negative 14-3-3 construct in postnatal cardiac tissue were found to experience increased ASK1 activation, and were shown to undergo p38-dependent apoptosis (108, 109). Additionally, the induction of diabetes in these mice was shown to also induce dephosphorylation of ASK1 at Ser 967, providing a possible mechanism for diabetes-induced cardiomyopathy (56). Steady laminar flow was hypothesized to help protect

endothelial cells from apoptosis induced by pro-inflammatory cytokines, such as TNFα, a mechanism traced to increased association of ASK1 with 14-3-3 (57). Finally, the association of 14-3-3 with ASK1 was also found to be sensitive to ischemic spinal cord injury in mice (110).

2.) AIP1

ASK1-interacting protein 1 (AIP1) was discovered by Zhang and colleagues as an ASK1 binding partner in the Ras GTPase-activating protein (Ras-GAP) family (70). AIP1 normally exists in a tightly closed conformation, in which intramolecular interactions occur between the N- and C-terminus of the protein. Upon TNFα stimulation, AIP1 begins to unfold, allowing association of the AIP1 N-terminus with the ASK1 C-terminus. Under these conditions, AIP1 enhances the dissociation of 14-3-3 from the C-terminal portion of ASK1, where it interacts through ASK1 Ser 967, as described in section II-vi-c-1. Zhang and colleagues followed this report with another study showing that under basal conditions, AIP1 is normally found in complex with the TNFR1 in resting endothelial cells (111). Only after TNFα stimulation is AIP1 released from TNFR1, resulting in cytoplasmic translocation and formation of a protein complex with other proteins, including TRAF2 and RIP1. RIP1, a serine/threonine protein kinase, was actually shown to phosphorylate AIP1 at Ser 604, creating a 14-3-3 recognition site (112). Phosphorylation and subsequent 14-3-3 association was suggested to facilitate the open conformation of AIP1, allowing it to more readily interact with the TRAF2-based cytoplasmic protein complex and ASK1. Most recently, AIP1 was also found to exist in complex with PP2A (59). TNFα-induced association of AIP1 with ASK1 recruited

PP2A to the ASK1 C-terminus, where it functioned to dephosphorylate ASK1 at Ser 967 and induce dissociation of 14-3-3 from ASK1. Cells from AIP1 knockout mice have revealed that AIP1 activation of ASK1 is a critical component of endoplasmic reticulum (ER) stress-induced ASK1 activation (113).

3.) $p21^{Cip1/WAF1}$

p21, a cell cycle checkpoint protein, was identified to be an ASK1-interacting partner in monocytes by Asada and colleagues (101). Interestingly, while nuclear p21 exhibits cell cycle characteristics, only cytosolically located p21 interacts with ASK1. A truncated p21 protein, lacking the nuclear localization sequence, has no cell cycle regulatory function but remains capable of interacting with ASK1. This interaction is considered to have a negative effect on ASK1 activity, and this truncated p21 protein, through enhanced binding with ASK1, produces an apoptotic-resistant cell phenotype. Schepers and colleagues followed this study with a separate report, showing that a high percentage of acute monocytic leukemia blasts displayed constitutive cytoplasmic p21 expression (114). Additionally, these blasts had decreased sensitivity to VP16-induced apoptosis, suggesting that cytoplasmic p21-induced ASK1 inhibition could be a mechanism of apoptotic evasion in tumor cells. Zhou and colleagues established that one mechanism by which cells induced cytoplasmic localization of p21 was through Akt-mediated phosphorylation of p21 at Thr 145 (115). This suggests that Akt may have a dual role in inhibition of ASK1 – first through direct phosphorylation of ASK1 at Ser 83 (described in section II-vi-b), and second through an indirect mechanism, via phosphorylation and cytoplasmic localization of the ASK1 inhibitory protein p21.

Intriguingly, a separate study by Zhan and colleagues found that ASK1 could phosphorylate p21 both *in vitro* and *in vivo* at Ser 98 (100). Phosphorylation at this site was determined to be critical for association with, and inhibition of, ASK1. Another study reported that p21-induced ASK1 suppression served as a mechanism of resistance to rapamycin treatment (116).

4.) Daxx

The death domain associated protein Daxx mediates a Fas ligand (FasL)-induced apoptotic cascade which culminates in JNK activation and cell death (117, 118). Daxx was found to specifically potentiate ASK1 activation, and a kinase dead version of ASK1 inhibited Daxx-mediated apoptosis (77). The mechanism of this effect was attributed to interaction between ASK1 and Daxx, and in fact, this was shown to couple ASK1 to the Fas receptor apoptotic machinery. The ASK1 interaction with Daxx was found to be inhibited by the cell chaperone protein Hsp27, which blocked Daxx-mediated apoptosis (119, 120). One report suggested that Daxx-mediated apoptosis occurring through ASK1 was independent of the catalytic activity of ASK1, although this has not been confirmed (121). Instead, Ko and colleagues found another mechanism of ASK1-induced Daxx interaction, namely through cytosolic sequestration of Daxx (122). Song and colleagues followed this report by showing Daxx cytosolic localization also resulted from glucose deprivation (78). Recently, the ASK1-induced Daxx translocation was shown to be of particular physiological relevance in a 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP)-induced mouse model of Parkinson's disease (123). In this model, MPTP triggered ASK1 activation and subsequent Daxx translocation, resulting in apoptosis of

the dopaminergic neurons of the substantia nigra pars compacta.

5.) Raf-1

The proto-oncogene protein Raf-1 is a well characterized MAP3K that triggers activation of the downstream kinases MAP-ERK kinase (MEK) and ERK, in a distinct MAPK signaling module (124). Chen and colleagues first described an interaction between Raf-1 and ASK1, which was mapped to the N-terminal region of the ASK1 protein (104). Interestingly, this interaction occurred independently of the catalytic activity of Raf-1, suggesting a unique function of Raf-1 on ASK1. Furthermore, overexpression of Raf-1 was capable of attenuating ASK1-induced apoptosis, a process that was neither dependent on the catalytic activity of Raf-1 nor the Raf-1-induced activation of MEK and ERK. This was later suggested to be a physiologically relevant interaction, when it was shown that mice with a cardiac-specific Raf-1 knockout experienced a significant increase in apoptotic cardiomyocytes with a concomitant increase in ASK1 activity (125). More recently, a study by Alavi and colleagues showed that the treatment of endothelial cells with basic fibroblast growth factor (bFGF) induced ASK1 interaction with Raf-1, and this provided a mechanism for resistance of these cells to the genotoxic chemotherapeutic doxorubicin (126). While the mechanism of the Raf-1 interaction with ASK1 is still under investigation, the recent identification of apoptosis-linked gene-2 (ALG-2) as both a Raf-1 and an ASK1 substrate may provide one clue into this interaction (127). Raf-1 was shown to block the ASK1-specific phosphorylation of ALG-2, and this was suggested as a possible mechanism for how Raf-1 inhibits ASK1-dependent apoptosis.

d.) ASK1 Oligomerization

ASK1 was first shown to oligomerize by Tobiume and colleagues, who reported that ASK1 existed as a pre-formed homo-oligomer under basal conditions (20). The ASK1 C-terminal coiled-coil domain was found to be essential for the formation of this oligomer. Although existence of this oligomer was required for ASK1 activation, interaction through the C-terminal coiled-coil domain was not sufficient for full activation, and thus this pre-formed oligomer is considered inactive. Instead, H_2O_2 treatment induces a conformational change within the pre-formed ASK1 oligomer, creating a new dimerization interface through which ASK1 is able to undergo trans-autophosphorylation. This ROS-induced conformational change is thought to be due in part because of dissociation of Trx from the ASK1 N-terminus, whereby TRAF2 and TRAF6 enhance an N-terminal homophilic interaction between the ASK1 oligomer, occurring through the N-terminal coiled coil domains (87). A recent report also found that ASK2 and ASK1 heteroligomerize, in a model in which ASK2 may trans-autophosphorylate ASK1 and lead to ASK1 activation, described in section III-iv-b (67). However, this may not be a general mechanism of ASK1 activation, and may only occur in cell-type specific settings.

e.) The ASK1 Signalosome

To date, over thirty proteins have been identified as ASK1-interacting partners. The presence of two coiled-coil domains, one each in the N- and C-termini of ASK1, suggest that ASK1 is capable of interacting with multiple protein partners (19). To more

fully determine the extent of complex formation which ASK1 undergoes, gel filtration column chromatography was performed (21). Compared with the predicted molecular mass of monomeric ASK1, approximately 160 kDa, ASK1 overexpressed in cells formed a high molecular mass complex of > 1,500 kDa. This high molecular mass complex was designated the ASK1 signalosome. Importantly, the kinase dead version of ASK1 (ASK1-KD) formed a complex of similar mass, suggesting that ASK1 activation was not an integral determinant of complex formation. However, the ASK1 ΔCCC construct formed a complex of only 500 kDa, providing evidence that the C-terminal coiled-coil domain was necessary for determining a large proportion of the signalosome. A similar high molecular mass complex (1500-2000 kDa) was also observed with endogenous ASK1 in multiple cell lines under basal conditions, suggesting that formation of this signalosome was a general feature of ASK1. Interestingly, treatment of cells with H_2O_2 resulted in a dramatic increase in the molecular mass of the ASK1 signalosome, to > 3,000 kDa. This effect was attributed to the specific release of ROS by H_2O_2, as pre-treatment with antioxidants inhibited this shift. Treatment of cells with another known ASK1 activator, TNFα, also resulted in a similar shift to > 3,000 kDa. In this report, Noguchi and colleagues further showed that recruitment of both TRAF2 and TRAF6 to the larger ASK1 signalosome was at least partially responsible for the increase in the molecular mass of the complex.

vii.) Mechanisms of ASK1 Activation

ASK1 is responsive to numerous forms of stress stimuli (Figure 1-2), representing both intrinsic and extrinsic apoptotic signaling mechanisms.

a.) Oxidative Stress

A major source of cellular stress-induced apoptosis is triggered by oxidative stress, namely through the production and release of ROS, including H_2O_2 (128, 129). ROS have been shown to result in ASK1 activation through multiple mechanisms, and indeed, ASK1 was found to be critical for H_2O_2-mediated cell death, as ASK1 knockout mouse embryonic fibroblasts (MEFs) do not undergo H_2O_2-mediated cell death (27). One major mechanism by which ROS trigger ASK1 is through the alteration of redox-sensitive ASK1-interacting partners, namely Trx and Grx. Under basal reduced intracellular conditions, Trx is found to associate with the N-terminus of ASK1 (106). This association is thought to maintain ASK1 in a partially oligomerized, but not active conformation (20, 107), although Trx may also play a role in regulating ASK1 expression through proteasome degradation pathways, elaborated on in section II-vi-a (40). Upon oxidative stress, two cysteine residues within Trx are modified with sulfhydryl groups to form an oxidized version of Trx, which dissociates from ASK1. This dissociation is a prerequisite for the development of a fully oligomerized and active conformation of ASK1 (87). Similarly, under normal conditions, Grx interacts with the C-terminal portion of ASK1 (94). Under conditions of glucose deprivation, which triggers the formation of H_2O_2, Grx becomes oxidized and dissociates from ASK1, whereby ASK1 activity is increased (95, 130). Another major mechanism whereby ROS activates ASK1 is through dephosphorylation at Ser 967 and subsequent dissociation of the ASK1 inhibitory protein 14-3-3, as discussed in section II-vi-c-1 (54).

b.) Endoplasmic Reticulum Stress

A major source of intracellular-triggered stress comes from the unfolded protein response (UPR) elicited in the ER (131). The UPR arises under conditions in which the cell has an increased need to prevent misfolded proteins from successfully exiting the secretory pathway, which it responds to by expanding the capacity of the ER lumen. This triggers a state of increased ER stress, which when persistently activated leads to an increase in apoptosis. ASK1 was first linked to ER stress-induced apoptosis in neuronal cells, when it was shown that ASK1 knockout primary neurons successfully evaded ER stress-induced JNK activation and neuronal apoptosis (132). The mechanism for this critical role in ER stress-induced cell death was shown to occur through aggregation of polyglutamine (PolyQ) repeat fragments, and induction of proteasomal dysregulation, leading to formation of an active ASK1 complex comprised of inositol-requiring enzyme 1 (IRE1) and TRAF2. This was significant, as it had been previously shown that the transmembrane protein kinase IRE1 responded to ER stress by activating the ASK1 downstream target JNK, thus providing a link between IRE1 and ASK1 (133). More recently, Luo and colleagues added another layer to this story when they found that the ASK1 binding protein AIP1 formed a complex with IRE1, which led to association with ASK1 and subsequent ASK1 activation (113). Matsuzawa and colleagues reported that ASK1 knockout MEFs were resistant to pharmacologically induced ER stress by two agents, thapsigargin and tunicamycin (134). Significantly, ASK1-mediated ER stress-induced apoptosis was found to play a critical role in progression of familial amyotrophic lateral sclerosis (ALS). Here, induction of ASK1 activation was hypothesized to occur

through inducing an interaction between a mutated form of the superoxide dismutase (SOD1) and a component of the ER-associated degradation machinery Derlin-1 (135).

c.) Death Receptors

Aside from the intrinsically-triggered apoptotic pathways, extrinsic pathways are another major cause of the initiation of apoptosis signaling cascades. Extrinsically triggered apoptosis is primarily mediated through the activation of members of the death receptor family. Two family members in particular have been linked to activation of ASK1. The first of these is the pathway triggered by activation of the Fas (CD95) death receptor by the FasL (136). Once activated, the C-terminus of the Daxx protein specifically binds to the Fas intracellular death domain (137), which subsequently leads to interaction with and activation of ASK1, described in section II-vi-c-4 (77). Similarly the cytokine TNFα, the extracellular ligand for TNFR1, activates TNFR1 and recruits TRAF2 and TRAF6 to the TNFR1 (136). Both TRAF2 and TRAF6 have been shown to interact with and activate ASK1 in a TNFα-dependent manner (85, 86). This is believed to be mediated in part, at least, through recruitment of TRAF2 and TRAF6 to the N- and C-terminals of ASK1, facilitating complete homophilic interaction between ASK1 dimers and full ASK1 oligomerization. This allows subsequential activation of downstream ASK1 effectors (21, 87). Again, ASK1 was found to be essential for TNFα-induced cell death, as ASK1 knockout MEFs do not undergo TNFα-induced apoptosis (27).

d.) Chemotherapeutics

The first clue that ASK1 activity may be triggered by chemotherapeutic agents

came when Wang and colleagues reported various microtubule-interfering agents (MIAs) activate ASK1 (138). In this study, an induction of ASK1 kinase activity was noted with paclitaxel, docetaxel, vinblastine, and vincristine. Each of these agents have distinct mechanisms of tubulin inhibition and unique tubulin binding sites, suggesting a general mechanism of action by MIAs (139). Overexpression of ASK1-KD inhibited paclitaxel and vinblastine-induced JNK activation, suggesting that these drugs activated JNK through an ASK1-mediated pathway. This was confirmed in a second study by the same group, that went on to show that the apoptosis induced through the ASK1-JNK axis resulted from an early stage of MIA-induced cell damage, occurring within the first 16 hours of treatment of ovarian cancer cells (140). It was later found that ASK1 became activated after treatment of cells with paclitaxel, resulting in phosphorylation of the anti-apoptotic protein Bcl-2 (141). However, the link between ASK1 activation and Bcl-2 phosphorylation is still unclear. Chemotherapeutic activation of ASK1 is not a mechanism unique to MIAs. For example, the DNA damaging agent cisplatin was shown to induce ASK1 activation in an ovarian cancer cell line as early as 4 hours following drug treatment, which preceded caspase-dependent apoptosis (142). Rapamycin, a macrolide antibiotic both in clinical use and investigation for several diseases including cancer and transplant recovery, is a specific inhibitor of the mammalian target of rapamycin (mTOR) kinase (143). A major advancement in the understanding of rapamycin-induced apoptosis came when Huang and colleagues discovered that rapamycin induced rapid and sustained activation of ASK1 and its downstream target JNK (116). Interestingly, this phenomenon did not occur in cells with inducible expression of the ASK1-interacting protein p21, which bound to and inhibited ASK1

under these conditions. The mechanism for rapamycin-induced ASK1 activation was traced to inhibition of the negative ASK1 regulator PP5, thereby blocking ASK1 dephosphorylation (65). An antioxidant under clinical investigation as an anticancer chemotherapeutic, resveratrol, also was shown to induce ASK1 activation (144). Although the mechanism of this activation remains to be elucidated, it appears that FasL signaling may be at least partially responsible.

viii.) Consequences of ASK1 Activation

Once activated, ASK1 goes on to trigger cellular signaling cascades resulting in functional effects. Apoptosis was the first cellular effect attributed to ASK1 activation, and it is the function most often studied regarding ASK1. However, recent evidence suggests that the role of ASK1 may not be unique, and the presence of ASK1 in different cellular contexts can affect its function.

a.) Apoptosis

The first report of ASK1, by Ichijo and colleagues, identified it as a trigger of cellular apoptosis (15). Indeed, it is clear that exogenous expression of wild-type ASK1 alone is sufficient to induce cell death. This was found to be dependent on the kinase activity of ASK1, and was further shown to be enhanced by a constitutively activated N-terminally truncated ASK1 construct (ASK1 ΔN) (106). Upon activation, ASK1 triggers release of cytochrome c from the mitochondria, indicating a role in the relay of the intrinsic apoptotic signaling network (142, 145). This is followed by the downstream

activation of the executioner caspases 3 and 7, resulting in apoptosis. However, although the role of ASK1 in apoptosis has been clearly established, subsequent reports have detailed several non-apoptotic functions of ASK1 as well (Figure 1-2).

b.) Differentiation

The first indication of the role of ASK1 in cellular differentiation came with the discovery that constitutively active ASK1 ΔN could induce neurite outgrowth, resulting in differentiation of PC12 cells into mature neuronal cells (146). The mechanism of this was attributed to activation of the p38, but not JNK, downstream MAPK axis. ASK1 was confirmed to promote neuronal differentiation in a separate study by Faigle and colleagues, who again reported that that p38 was a critical effector of this process (147). Subsequently, ASK1 activation of p38 was found to play a similar role in keratinocyte differentiation, marked by increased expression of the differentiation markers transglutaminase-1, loricrin, and involucrin (148).

c.) Innate Immunity

ASK1 was shown to be an important component in innate immunity when it was reported that ASK1 activation was a critical step in the induction of macrophage apoptosis (149), an essential step in the diminishment of mycobacterial infection (150). In another example of its important role in innate immunity, ASK1-p38 activation was shown to play a critical role in the regulation of the innate immunity developed during keratinocyte differentiation, resulting in increased expression of the Toll-like receptor (TLR) 2 and stabilization of an immune complex formed by TLR2 and the immune

proteins human beta-defensin (hBD) and LL37 (151). In a mouse model of lipopolysaccharide (LPS)-induced septic shock, LPS was found to induce the formation of intracellular ROS, which resulted in the interaction of TRAF6 with ASK1, and subsequent activation of p38 (152). Interestingly, this was also shown to uniquely require activation of the TLR4, which led to the generation of ROS.

Separately, ASK1 has also been shown to have a key role in injury repair. The first indication that ASK1 was involved in wound repair came with the discovery that ASK1 expression was induced in regenerating rat palatal epithelium after acute injury (32). Subsequently, a unique role of ASK1 was discovered when it was reported that ASK1 knockout mice displayed significant delay in hair regrowth following wounding (153). This was attributed to a decreased infiltration and activation of macrophages.

ix.) ASK1 Involvement in Disease

Because of its varied and critical roles in cell fate, it is not surprising that several lines of evidence suggest that ASK1 is an important component of the pathophysiology of diseases in which dysregulation of cell fate pathways is triggering. The establishment of ASK1 as a critical mediator of multiple diseases suggests that ASK1 represents an attractive target in several disease settings. Listed here are choice examples in which ASK1 may have a causative role in the development of disease.

a.) Neurodegenerative Disorders

Evidence for a role of ASK1 in neurodegenerative diseases comes mainly from its

importance in dictating stress-induced neuronal cell death. For example, ASK1 is a necessary component for polyQ-triggered ER stress-induced cell death in PC12 cells, a neuronal cellular model (132). This is especially significant in light of the fact that polyQ repeats play a pathogenic role in the development of several disorders, including Huntington's disease and spinocerebellar ataxia. However, ASK1-induced cell death is not restricted to this cell type, as it was also a critical mediator of apoptosis in retinal neurons as well (154). This was followed by a report detailing the importance of ASK1 in motor neuron apoptosis, suggesting ASK1 was also involved in the loss of motor neurons that is a hallmark of ALS (155). Holasek and colleagues established that an ASK1-p38 pathway was responsible for this motor neuron-specific cell death, as opposed to an ASK1-JNK pathway (156). Significantly, an MPTP-induced mouse model of Parkinson's disease recently showed that ASK1 became activated as early as 12 hours following MPTP insult. This was followed by the initiation of downstream signaling cascades leading to JNK activation, which was abrogated by the co-administration of a thiol antioxidant (123). Together, these cell and animal model studies suggest that ASK1 has an important and causative role in neurodegenerative diseases; however, this will have to be confirmed with follow-up studies showing increased ASK1 activation in patients with neurological disorders.

b.) Cardiovascular Diseases

There is much evidence for a role of ASK1 in cardiovascular diseases mediated by cardiac hypertrophy. There are multiple examples which have laid the groundwork to show ASK1 mediates apoptosis in cardiac cells (157, 158). Several reports also provide a

mechanism for this ASK1-induced apoptosis, namely through dephosphorylation of Ser 967 and dissociation of the 14-3-3 inhibitor protein (56, 57, 108, 159). The G protein-couple receptor (GPCR) agonist angiotensin II has also been shown to induce cardiac hypertrophy in an ASK1-dependent manner. This was first illustrated in a cellular model (160), followed by an ASK1 knockout mouse model (161), which further showed an importance in coronary arterial remodeling. Because of its critical role in cardiomyocyte apoptosis, ASK1 is an attractive target for therapeutic development. Indeed, in a hamster model of hereditary dilated cardiomyopathy, transcoronary genetic transfer of a dominant negative ASK1 gene attenuated progression of cardiac ventricular remodeling, evidenced by suppression of fibrosis (162).

c.) Infectious Disease

The involvement of ASK1 in infectious disease is limited to only one example, but it may have a significant impact in the field of infectious disease. Geleziunas and colleagues described a complicated role of ASK1 in mediating human immunodeficiency virus-1 (HIV-1) viral infection (96). In this example, the HIV-1 protein Nef has a dual effect on ASK1. Nef induces the production of FasL expression on the surface of host T cells infected with HIV-1. It was shown that Nef directly interacts with ASK1 in these HIV-1-infected host T cells. This interaction inhibits the function of ASK1, ultimately resulting in diminished activation of the ASK1-JNK axis, and a decrease in ASK1-induced apoptosis. In this way, Nef protects the virally infected host cell from cell death triggered by a TNFα-induced ASK1-mediated response. Concomitantly, the induction of FasL by Nef also has consequential effects on the nearby uninfected bystander cells,

providing a mechanism for immune system evasion. Here, the FasL produced by the virally infected T cell may trigger activation of the Fas receptor on the bystander cells, leading to cell death in these uninfected cells through a presumably ASK1-dependent process.

d.) Diabetes

Activation of ASK1 by TNFα was shown to result in increased phosphorylation of the insulin receptor substrate (IRS) 1, a key mediator of the tight regulation involved in insulin signaling (163). Phosphorylation of IRS1 leads to its inhibition, resulting in an impairment of the insulin response, specifically in adipose cells (164). This phosphorylation was found to occur through ASK1 activation of JNK, a significant finding in light of previous results shown that JNK could promote insulin resistance through phosphorylation of IRS1 (165). Most recently, a role for ROS in the development of insulin resistance was also suggested, when it was reported that mitochondrial ROS triggered ASK1-JNK mediated phosphorylation and inhibition of IRS1 (166).

III.) ASK2, a Novel Component of the ASK1 Signalosome

i.) Discovery

ASK2 is the second identified member of the ASK1 family. It was first discovered as MAP3K6 in 1998 (72). In this report, Wang and colleagues used the yeast two-hybrid system to identify potential binding partners of ASK1. An ASK1 construct

containing only the kinase and C-terminal domains was fused with the DNA binding domain of the GAL4 gene, and used as bait with plasmid DNA from a HeLa cell cDNA library. Positive interactions were identified by assaying for β-galactosidase activity. Of several clones, one was further characterized, and found to be a novel cDNA sequence. This cDNA sequence was further shown to have high homology with several MAP3Ks, and therefore was termed MAP3K6. In fact, the kinase domain of MAP3K6 shares 37%, 42%, 43%, and 42% identity to the amino acids of the catalytic domains of MAP3K1, MAP3K2, MAP3K3, and MAP3K4. When the kinase domain is compared with MAP3K5, or ASK1, it is 82% identical. Additionally, MAP3K6 and MAP3K5 are 45% homologous overall. For these reasons, MAP3K6 has become referred to as ASK2. In addition to a central kinase domain, ASK2 also contains long N- and C-terminal flanking regions.

ii.) Gene Structure and Putative Isoforms

The ASK2 gene, located on chromosome 1 in the human genome (1p36.11), contains 28 exons and codes for a protein of 1288 amino acids. Although only one main isoform has been identified for human ASK2, studies of the mouse ASK2 sequence have identified two splice variants (167). Additionally, the 5' flanking region of the mouse ASK2 gene contains a strong suppressive element, suggesting that the expression of ASK2 is highly regulated. To date, these facts have not yet been confirmed in the human genomic ASK2 sequence.

iii.) Expression

Northern blot analysis of ASK2 mRNA has revealed limited information regarding expression of ASK2 (72). Among the examined human tissues, heart and skeletal muscle tissue contained the highest amount of ASK2 mRNA expression. Comparatively, lung, liver, kidney, testis, and spleen tissue produced weaker ASK2 mRNA signals. In this study, brain tissue contained no ASK2 mRNA. However, these results have not yet been confirmed, and future studies with more sensitive assays may reveal low levels of ASK2 mRNA expression in these cases of negative expression. Furthermore, no studies have been performed with antibodies specific for the ASK2 protein, to determine and compare actual protein levels within different tissues.

ASK2 protein cellular localization was determined using subcellular compartment extractions of HeLa cells (71). This showed ASK2 was expressed at relatively equal levels in the cytoplasm, nucleus, and membrane organelles including the mitochondria. Reduced expression was noted in the cytoskeletal extract.

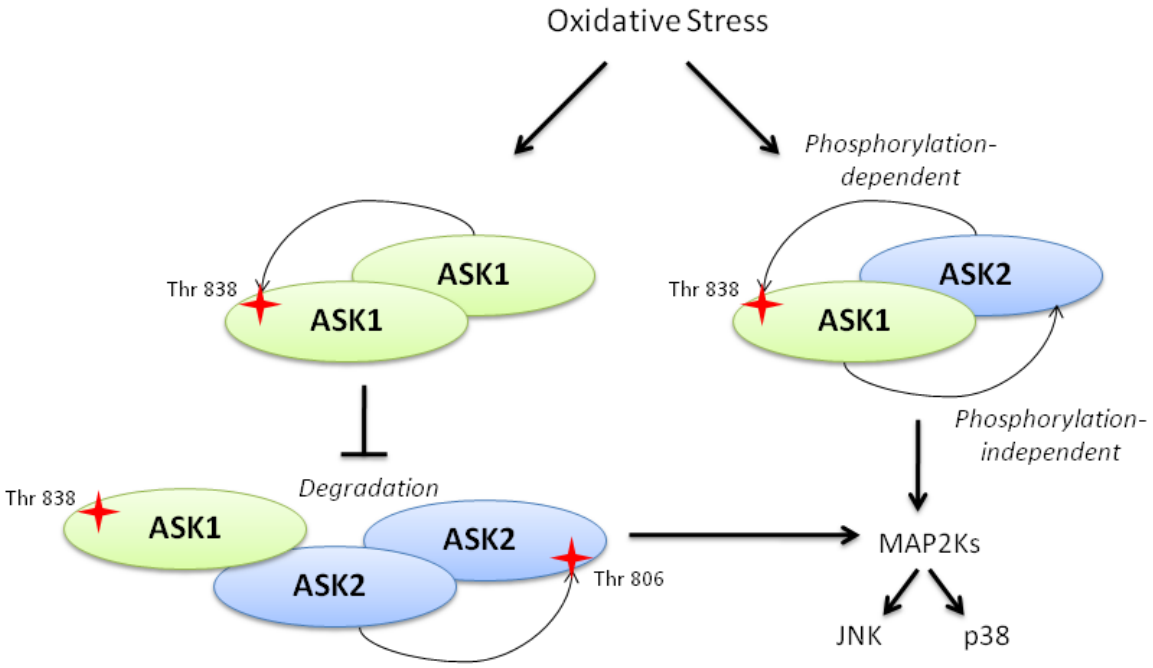
iv.) Functions of ASK2

Although it is a relatively new and unstudied protein, functional effects have been attributed to the ASK2 kinase in recent reports (Figure 1-4). Most of these have been gleaned from relatively few experiments, and it is likely that the functional role for ASK2 will expand in the near future.

a.) Enzymatic

Examination of the activation loop of ASK2 found that it varied by only one amino acid when compared to ASK1 (67). Additionally, the ASK2 sequence contains a

Figure 1-4. Current insights regarding ASK2 regulation and activation. Currently, two models exist for the regulation of ASK2 by ASK1. In the first model, ASK1 autophosphorylates and activates itself in response to oxidative stress. Activated ASK1 then binds to ASK2 homo-oligomers. This interaction stabilizes the ASK2 protein, preventing it from undergoing proteasomal-mediated degradation, and allowing it to undergo autophosphorylation at Thr 806. In the second model, ASK1 and ASK2 exist in a heteromeric complex. In this complex, ASK2 phosphorylates ASK1 at the Thr 838 residue, allowing full ASK1 catalytic activity to ensue. Simultaneously, ASK1 allosterically modulates ASK2 in a kinase-independent manner. This also results in ASK2 activation, possibly through ASK2 autophosphorylation as in the first model. These models are not necessarily mutually exclusive, and both trigger downstream MAPK cascades resulting in JNK and p38 activation.


Oxidative Stress
Phosphorylation-dependent
Thr 838
ASK1
ASK1
Thr 838
ASK2
ASK1
Phosphorylation-independent
Degradation
Thr 838
ASK1
ASK2
ASK2
Thr 806
MAP2Ks
JNK
p38

threonine residue (Thr 806) homologous to Thr 838 within ASK1, phosphorylation of which has been shown to be essential for full ASK1 activation (20, 61). The near identical nature of the activation loops between the two proteins also suggested that ASK2 enzymatic activity is regulated in a similar manner. Interestingly, ASK2 was found to undergo autophosphorylation, but this phenomenon only occurred in the presence of ASK1 (67). This suggests that while ASK2 has enzymatic activity, it may not be intrinsic and is instead only induced in the presence of ASK1. Importantly, ASK1 enzymatic activity was shown not to be necessary for this function, as the kinase-dead version of ASK1 (ASK1-KD) displayed the same effect. Phosphorylation of ASK2 at Thr 806 was then shown to be induced by oxidative stress, because H_2O_2 treatment of HEK293 cells overexpressing ASK2 led to a dramatic increase in Thr 806 phosphorylation, but only in the presence of co-transfected ASK1-KD (67). In another example of ASK2 kinase activity, ASK2 was also found to induce phosphorylation of ASK1 at Thr 838. This provided a mechanism for a previous observation, showing Thr 838 of ASK1 was a substrate for both trans-autophosphorylation as well as an unidentified kinase (20).

The MAP3K kinase activity of ASK2 was first discovered when it was shown to activate JNK in an *in vitro* kinase assay (72). However, unlike ASK1, it only resulted in weak activation of JNK. Additionally, ASK2 was unable to lead to activation of other MAPKs, including p38 and ERK. Of note, these *in vitro* kinase assays did not include a MAP2K, only the particular MAP3K and MAPK under investigation. Therefore, these assays may not have truly measured the MAPK-pathway activation potential of ASK2. To more fully determine if ASK2 functioned as a MAP3K, it was tested in an *in vitro*

kinase assay which contained both MAP2K6 and its substrate, p38 (67). When expressed alone, ASK2 was unable to activate p38 through sequential activation of MAP2K6. However, when co-expressed with ASK1-KD, ASK2 effectively led to activation of MAP2K6 and subsequent phosphorylation of p38, to approximately the same degree as ASK1 alone. Similar results were also obtained with the MAP2K4 and JNK kinases. These results were confirmed *in vivo* by monitoring phosphorylation of the endogenous p38 and JNK MAPKs in the presence of ASK2 and ASK1. siRNA knockdown of endogenous ASK2 in mouse B16 melanoma cells dramatically reduced the ability of H_2O_2 to induce JNK phosphorylation and activation, in spite of the continued presence of endogenous ASK1. This suggests that the MAP3K function of ASK2, and not just ASK1, was necessary for oxidative stress-induced activation of MAPK pathways leading to JNK activation.

b.) Heteromeric Complex with ASK1

Takeda and colleagues confirmed the interaction between ASK1 and ASK2, using both ectopically and endogenously expressed proteins (67). Intriguingly, these investigators also noted that endogenous ASK2 expression was dramatically reduced in MEF cells deficient in ASK1. This reduction in ASK2 expression was attributed to an increase in ASK2 degradation in the ASK1 knockout MEFs, as the proteasome inhibitors Lactacystin and MG132 both caused a reversion of this phenotype to levels similar to expression in ASK1 wild-type MEFs. Additionally, ectopic expression of ASK1 in ASK1 knockout MEFs rescued ASK2 degradation, confirming the requirement of the presence of ASK1 to maintain ASK2 stability. The kinase activity of ASK1 is not

necessary for this function, as ASK1-KD effectively stabilized ASK2 as well. The presence of the C-terminus of ASK1 was shown to be necessary in order for ASK1 to be found in complex with ASK2. In fact, an ASK1 construct containing only the C-terminus was sufficient to rescue ASK2 expression. This ASK1/ASK2 heteromeric complex was later confirmed in a separate report (71).

Taken together, ASK1 and ASK2 are proposed to have reciprocal functionality in complex together (Figure 1-4). When complexed with ASK2, ASK1 results in ASK2 protein stability, by a notably kinase-independent mechanism. Within the same complex, catalytically activate ASK2 results in phosphorylation of ASK1 at Thr 838, leading to full activation of the ASK1 protein. This latter model is not mutually exclusive of the previous model which proposed that ASK1 autophosphorylates itself on Thr 838 (20), and the use of either ASK1 or ASK2 as an ASK1 Thr 838 kinase may be dependent on cell and tissue-type specific expression of the ASK2 protein.

c.) Apoptotic Activity of ASK2

In addition to its activating functions of JNK and p38, two MAPKs well characterized for their ability to induce cell apoptosis, ASK2 was also assessed for its ability to induce caspase activity (67). Indeed, ASK2 expression led to increased cleavage of a caspase 3 DEVD substrate. This only occurred when co-expressed with full-length ASK1-KD, and not the C-terminus ASK1 construct, suggesting that aside from stabilization, an additional region within ASK1 is necessary to support the enzymatically active conformation of ASK2.

IV.) Biology of 14-3-3 Proteins

i.) Description

14-3-3 is a family of eukaryotic proteins that modulate diverse cellular signaling pathways by binding to a variety of client proteins. Through these interactions, 14-3-3 plays a critical role in a wide variety of cellular processes, including the regulation of cell survival and proliferation. The protein was christened with its numeric name because of its characteristic elution and migration patterns during two-dimensional DEAE-cellulose chromatography and starch gel electrophoresis, respectively (168). 14-3-3 family members are acidic proteins with an isoelectric point of 4.5 to 5, and an approximate molecular weight of 27-30 kDa (Table 1-2). Although first identified as a highly abundant brain protein, 14-3-3 is also expressed in a wide range of tissues and organs, indicating a general role in cellular function (68, 168-170). As further evidence of their fundamental importance, 14-3-3 proteins have been identified as critical regulators in virtually every eukaryotic organism studied, including yeast (171), *D. melanogaster* (172), *Arabidopsis* (173), and numerous mammals (174).

Using reversed-phase HPLC, seven mammalian isoforms of 14-3-3 have been identified (175). Each isoform is denoted by a Greek symbol (β, ε, η, γ, σ, τ, ζ) and encoded by a distinct gene (Table 1-2). Interestingly, no new 14-3-3 isoforms were revealed with the most recent sequencing completion of the human genome, suggesting that this list of seven isoforms is exhaustive in human (176). Sequence analysis has revealed a high degree of identity at the amino acid level (>50%) within and across known species. Alignment of 46 separate animal, plant, and fungal 14-3-3 sequences has

Table 1-2. Gene information for each human 14-3-3 isoform. The primary gene identifier is listed for each of the seven 14-3-3 isoforms, along with their associated accession numbers and chromosome location. The amino acid number corresponds to the number of amino acids present in each gene coding sequence, and the molecular weight corresponds to the predicted molecular weight of the protein.

Isoform	Primary Gene Symbol	Accession Number(s)	Chromosome Location	Molecular Weight (kDa)	Amino Acid Number
β	Ywhab	NM_003404 NM_139323	20q13.1	28.10	246
ε	Ywhae	NM_006761	17p13.3	29.18	255
η	Ywhah	NM_003405	22q12.3	28.21	246
γ	Ywhag	NM_012479	7q11.23	28.36	247
σ	Sfn	NM_006142	1p36.11	27.83	248
τ	Ywhaq	NM_006826	2p25.1	27.78	245
ζ	Ywhaz	NM_003406 NM_145690	8q23.1	27.76	245

revealed five stretches of amino acids highly conserved among isoforms within various species (177). The structural conservation between 14-3-3 isoforms reflects the presence of shared functions among these proteins, and also suggests that they may functionally replace each other (178). However, several isoforms seem to also have their own distinct role in cellular regulation. A major source of debate in the 14-3-3 community is the functional significance of each of the various isoforms, of which at least two are expressed in almost every known eukaryotic organism. Several theories have been proposed to explain the need for isoform multiplicity, including a requirement for a large amount of 14-3-3 protein, differences in isoform sub-cellular localization, and target protein binding specificity (174). Phylogenetic analysis of 14-3-3 isoform expression among species shows that one common theme shared is that multicellular organisms tend to express a greater number of isoforms compared with unicellular organisms. The most highly conserved isoform between species is 14-3-3ε, of which only one amino acid variant has been identified in mammals (176). 14-3-3σ is the most divergent isoform, a finding supported by its distinct cellular function. Each isoform except 14-3-3σ has also been found to undergo acetylation at the N-terminus, a characteristic which has been exploited to generate isoform-specific antibodies (179).

ii.) Crystal Structure Analysis

A major advance in our understanding of 14-3-3 came when the crystal structure of the 14-3-3ζ and 14-3-3τ isoforms were determined (180, 181). More recently, a comparative structural analysis of all seven human 14-3-3 crystal structures provided further insight into their similarities (182). Two major findings were produced from

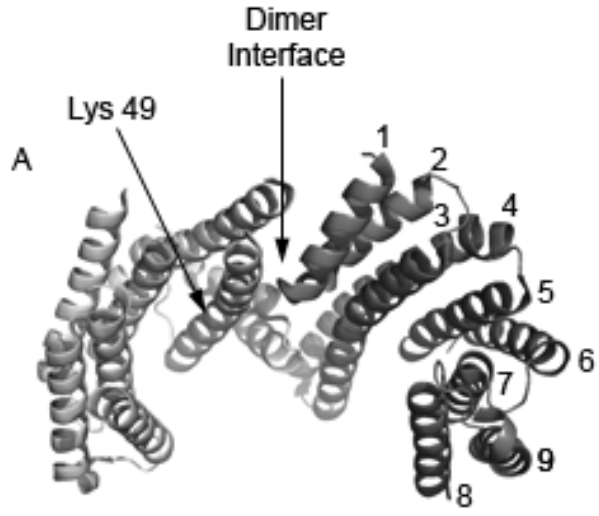
these structures – first, that 14-3-3 is a highly helical, dimeric protein, and second, that the helical subunits of the protein form a cup-like structure within each monomer (Figure 1-5). Additionally, a flexible C-terminal loop was identified that also mediates client protein binding.

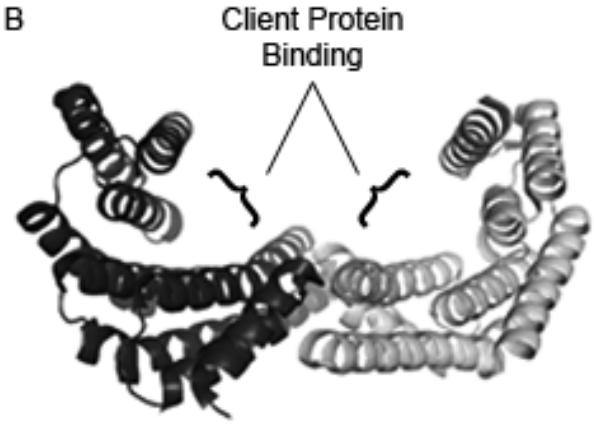
a.) Dimerization

One feature shared among isoforms is that 14-3-3 is a dimeric protein, and each monomeric subunit is comprised of nine antiparallel α-helices, separated by short loops. The N-terminus of each monomer is responsible for dimerization, whereby helix α1 of the first subunit forms an interaction interface with helices α3 and α4 of the second monomer. *In vitro* studies have further defined a 17 kDa fragment representing the most N-terminal amino acid residues that is sufficient for dimerization (183, 184). Although these N-terminal residues represent one of the most variable portions of the protein (183), several hydrophobic and hydrophilic residues are remarkably conserved among the mammalian isoforms. These residues (Leu 12, Ala 16, Val 62, Ile 65, and Tyr 82) have indeed been shown to be involved in maintaining the dimer interface (68). These characteristics may allow for specificity in determining homo- and heterodimerization among isoforms, which offers potential for a rich source of cellular regulation.

Determining the pattern by which isoforms undergo homo- and heterodimerization could be an important clue into the functional significance of the various mammalian 14-3-3 isoforms. Although not completely characterized, recent work has begun to define these patterns. While all 14-3-3 proteins exist as dimers, the stability of the dimer interaction differs among isoforms, with both 14-3-3ε and 14-3-3γ

Figure 1-5. Crystal structure of the human 14-3-3ζ isoform. (*a*) The ribbon structure shows each of the 9 α-helices labeled within one monomer (α1-α9), as well as the Lys 49 residue inside the amphipathic groove. *(b)* The bracket indicates the location of each of the ligand-binding amphipathic grooves. (Structure designed with MacPyMOL (DeLano Scientific, LLC, Palo Alto, CA), using published sequence data).


Dimer
Interface
Lys 49
A
1
2
3
4
5
6
7
8
9


B
Client Protein
Binding

displaying the highest stability, compared to 14-3-3β and 14-3-3η (182). In fact, 14-3-3ε seems to prefer heterodimerization, while 14-3-3σ predominantly forms homodimers. Differential salt bridge formation and unique residues at the dimer interface may account for the propensity of 14-3-3ε heterodimers and 14-3-3σ homodimers in cells. Co-immunoprecipitation studies have also added evidence for differential patterns of dimerization among isoforms. One study showed that ectopically expressed 14-3-3σ exclusively forms homodimers (185), confirming previous observations (186). Conversely, the 14-3-3ζ isoform was found to be capable of both homo- and heterodimerization with all isoforms except 14-3-3σ, arguing for the specificity of 14-3-3σ homodimerization. Interestingly, when five key residues (Ser 5, Glu 20, Phe 25, Glu 55, and Glu 80) within the 14-3-3σ N-terminus were mutated to their corresponding residues in the 14-3-3ζ isoform, the homodimerization selectivity of 14-3-3σ was lost, displaying a pattern more similar to 14-3-3ζ (185). Further, the homodimerization of 14-3-3σ was shown to be essential for its specific cell proliferation inhibitory function indicating that, at least in the case of this isoform, specific dimerization determines function. Future studies to evaluate the complete pattern of homodimerization and heterodimerization are needed in order to fully address the functional importance of isoform multiplicity.

b.) Amphipathic Groove

Another shared characteristic among isoforms is the presence of a cup-shaped structure within each 14-3-3 monomer, formed by a bundle of the α-helices 3 through 9. The inner face of the structure forms an amphipathic groove lined with conserved

hydrophilic amino acid patches on one side and hydrophobic residues on the other. Hydrophobic residues from helices α7 and α9, basic residues from α3, and both polar and charged residues from α5 all contribute to the formation of the amphipathic groove (180, 181).

The crystal structure solutions of 14-3-3 with the serotonin N-acetyltransferase (AANAT) protein (187), as well as with other peptides (182, 188-190), supports that the amphipathic groove is the primary site within 14-3-3 responsible for client protein binding. The amino acid residues lining the amphipathic groove are largely invariant among 14-3-3 isoforms, further indicating that binding to client proteins via the amphipathic groove is a common feature shared among 14-3-3 proteins. These residues include Lys 49, Arg 56, Arg 127, and Tyr 128, mutations of which diminish client protein binding. 14-3-3 crystal structures indicate the involvement of additional residues in the C-terminal region, such as Glu 208, in the interaction of 14-3-3 with full-length proteins, such as AANAT (187). The variant regions of the 14-3-3 structure may contribute to isoform-specific ligand binding. For example, the loop connecting helices α8 and α9 exhibits a divergent sequence in 14-3-3σ (191). Mutations of these divergent residues to those conserved in other isoforms appear to switch client protein binding preference. Structural analysis of the 14-3-3β isoform in both the apo (non-ligand bound) and ligand-bound forms has revealed that while the amphipathic groove could exhibit structural flexibility, allowing it to interact with a variety of client partners, alterations of the angle between the two monomeric subunits seems to confer client protein binding specificity (182).

c.) C-terminal Loop

Another insight into 14-3-3 proteins revealed by the crystal structure was that a flexible C-terminal loop interacts with basic residues within helix α3. Although this C-terminal loop is highly variant among isoforms, it is invariably comprised of acidic residues, suggesting a functional importance. One interesting model for the role of this C-terminal loop is that of a "gate-keeper" (180, 192). In this role, its interaction with helix α3 causes the loop to occupy the space at the top of groove. Ejection of this loop opens the "gate", allowing client proteins to interact within the amphipathic groove. Support for this theory came with the discovery that a truncated version of 14-3-3, in which this C-terminal tail was removed, binds interacting proteins and peptides to a greater degree than the wild-type version (193). Interestingly, this study suggested a hierarchy of 14-3-3 client protein interactions, in which the C-terminal loop effectively competes with low-affinity interacting partners for binding within the groove, but which itself is competed away by more high-affinity client proteins. The mechanisms which dictate this regulation by the C-terminal tail have not yet been discovered, but recent work has shown that at least in one organism, the protozoan *Giardia duodenalis*, 14-3-3 undergoes polyglycylation at the extreme C-terminus (194). Likewise, phosphorylation of Thr 232 has also been shown to negatively regulate 14-3-3 association with client partners (195). Interestingly, this site is located within the C-terminal loop, further implicating the role of this loop structure in regulating ligand binding. Currently, two putative kinases, the breakpoint cluster protein (Bcr) and casein kinase 1α (CK1α), are reported to phosphorylate 14-3-3 at this site (196, 197).

iii.) 14-3-3 Interactions with Client Proteins

The list of 14-3-3 client proteins has grown to beyond 200, thanks to several proteomics-based studies (198-201). The highly conserved nature of the residues lining the amphipathic groove suggests that 14-3-3 interacts with client proteins via a common mechanism. Early observations suggested that this mechanism occurred via phosphorylation of the client protein. It was speculated that a phosphorylated amino acid would be able to couple with basic residues within the groove, allowing a high affinity interaction to occur. Indeed, the first demonstration of this theory occurred when it was shown that 14-3-3 only bound to phosphorylated tryptophan hydroxylase, an enzyme involved in the synthesis of the neurotransmitter serotonin (202). A subsequent study found the same phenomenon was also true for binding to Raf-1 (203). Soon after, a pivotal report defined 14-3-3 as a specific and direct phosphoserine-binding protein, using synthesized peptides based on regions within Raf-1 (204). Indeed, 14-3-3 was discovered as the first class of protein modules that recognize phosphorylated serine or threonine motifs in a sequence specific manner (202, 204, 205). Phosphorylation in many client proteins in a sequence specific context increases the affinity of the client proteins to 14-3-3, leading to 14-3-3 association. This same study also defined a canonical consensus motif, RSx**pS**xP (where x symbolizes any amino acid), through which 14-3-3 can recognize and bind to Raf-1 and other client proteins (204).

Subsequent screening of peptide libraries defined two consensus 14-3-3 binding motifs, termed modes 1 and 2, both of which revolve around either a phosphorylated serine, or less frequently, a threonine residue (205). Importantly, mode 1 (RSx**pS/T**xP) closely approximated the previously identified RSx**pS**xP motif identified within Raf-1.

The mode 2 motif has the preferred sequences of RxY/F[+]**pS**[LEAM]P, where [+] indicates a basic residue. Many identified 14-3-3 client proteins contain these defined 14-3-3-recognition motifs, including Raf-1 (204), Bad (206), ASK1 (53), YAP (207), and TAZ (208). Although the mode 1 and 2 motifs appear to play a dominant role in mediating 14-3-3 binding to client proteins, other phosphorylated motifs have been identified. Most recently the SWTY, or mode 3 motif (**pS/T**$x_{(1-2)}$-COOH), was identified as a C-terminus consensus binding motif capable of interacting with 14-3-3 with a similar affinity to the canonical motif (209-211). Examples of proteins containing the mode 3 motif include the plant H(+)-ATPase and the Kir2.1 potassium channel protein. Other phosphorylated motifs have been identified, including variations of these motifs and a serine-rich motif (Rx_{1-2}**pS**x_{2-3}**pS**). A cautious note should be added that sometimes, the 14-3-3 recognition site does not conform to a defined, perfect motif. For example, 14-3-3 binding to the tumor suppressor p53 is mediated by a non-consensus motif (KGQST**pS**RH), which is further complicated because phosphorylation of the preceding serine actually decreases 14-3-3 recognition (212). Other examples include PFK-2 (RNY**pS**VGS) and the IGF-1 receptor (SVPLDPSASSS**pS**LP), whose binding is affected by the multitude of serines surrounding its recognition site (213, 214).

The nature of phosphorylation-induced 14-3-3 interactions with client proteins allows for the coupling of 14-3-3-regulated pathways to diverse signaling networks controlled by protein kinases and phosphatases. Multiple kinases have been identified which phosphorylate target proteins to induce 14-3-3 association, the majority of which fall into the basophilic AGC [including Akt and protein kinase A (PKA)] and the calcium/calmodulin-dependent kinase families (215). However, other kinases have been

implicated in mediating 14-3-3 client protein interaction as well. Conversely, the phosphatases PP1 and PP2A have been implicated as major mediators of 14-3-3 dissociation from client binding partners (216).

Although the importance of phosphorylation in mediating 14-3-3 interactions with client proteins is clearly established, several examples of non-phosphorylated binding have been discovered, including the bacterial Exoenzyme S (ExoS) ADP-ribosyltransferase and p190RhoGEF (217-220). Despite the lack of a phospho-group, these interactions have a high affinity. Competition experiments with phosphorylated client peptides suggest that they also interact via the amphipathic groove (217). One possibility to explain the lack of the typical phosphorylated motif is the presence of negatively charged residues, which may mimic the negatively-charged phosphate group (68). However, this theory was recently questioned when it was observed that mutations of the Asp residues within the 14-3-3 binding site on ExoS did not diminish its 14-3-3-dependent functions (221). Instead, a crystal structure solution of 14-3-3ζ with ExoS showed this interaction was largely dependent on hydrophobic interactions between the two proteins (188).

Some 14-3-3 client proteins, such as AANAT and the forkhead (FOXO) family of proteins, contain more than one 14-3-3 recognition motif. In this case, the 14-3-3 dimer can bind one client protein at multiple sites (182). It is noted that one high affinity motif often serves as a “gatekeeper” to induce 14-3-3 binding, allowing the interaction of 14-3-3 with a second low affinity motif (222). In another explanation, one 14-3-3 dimer may bind to two motifs in two separate proteins to induce a complex formation. Recent structural studies of the 14-3-3 dimer with the plant H+-ATPase shows that each

monomer simultaneously binds an H+-ATPase peptide and three 14-3-3 dimers were associated with a hexamer of the enzyme, raising the possibility that 14-3-3 proteins can coordinate oligomerization of its client proteins (223).

The importance of specific amphipathic groove residues in ligand interactions has been determined both through crystal structure solutions and mutational analysis (180-182, 205). The crystal structure solution of 14-3-3ζ complexed with a peptide containing a putative 14-3-3 phosphorylated motif from the polyoma middle-T antigen revealed several coordinating interactions between the phospho-group and 14-3-3 residues (205). Specifically, salt bridges were formed to residues in the hydrophilic portion of the groove (Lys 49, Arg 56, and Arg 127), while a hydrogen bond was apparent with the hydroxyl group of Tyr 128. The critical importance of the Lys 49 residue became increasingly apparent when it was shown that charge-reversal mutation of this positive residue to Glu dramatically disrupted interaction with Raf-1 and ExoS, and numerous other proteins (53, 89, 190, 224). Charge reversal mutations of other amphipathic groove residues, including the hydrophilic residues Arg 56, Arg 60, and the hydrophobic residues Val 176 and Leu 220, also affect binding to 14-3-3 client proteins, although to varying degrees and with varying specificity (88).

iv.) Regulation of 14-3-3 Proteins

Phosphorylation of the 14-3-3 protein may represent a common source of regulation, dictating both dimerization and client protein recognition. For example, 14-3-3ζ is phosphorylated on residue Ser 58 (225). Ser 58 lies within the dimerization interface of the N-terminus of 14-3-3, and phosphorylation at this site disrupts the

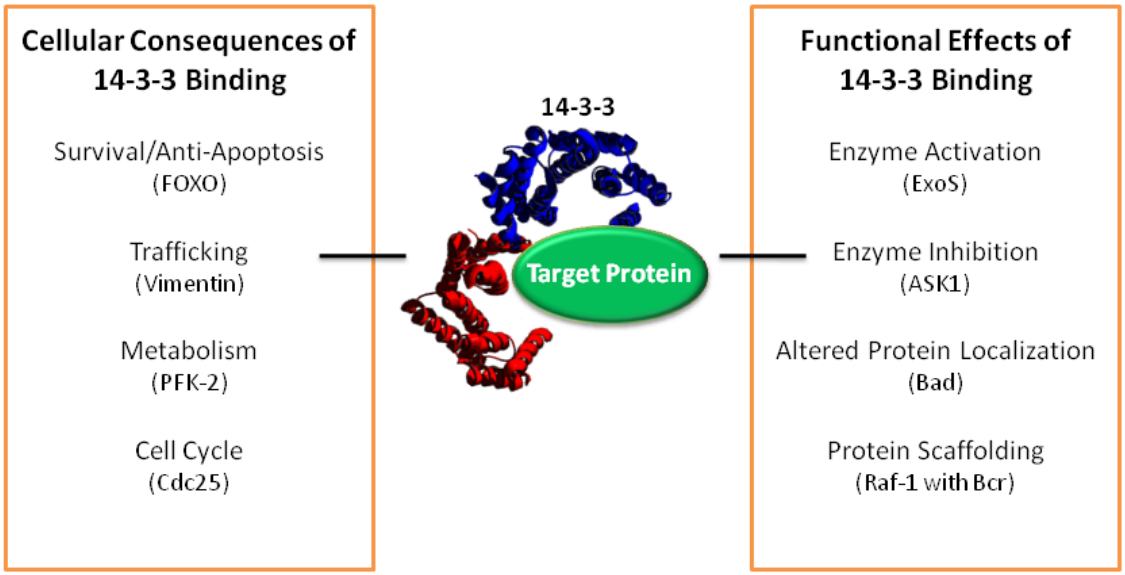
dimeric structure of the protein. This residue is conserved among all mammalian 14-3-3 isoforms except 14-3-3τ and 14-3-3σ, suggesting this is a common mode of regulation among isoforms. The kinase responsible for this phosphorylation was first suggested to be the sphingosine-dependent kinase-1 (226). Subsequent reports have also identified MAPKAPK2 (227), Akt (228), and PKA (229) as potential Ser 58 kinases. However, the cellular events leading to phosphorylation and dephosphorylation of 14-3-3 at this residue remain to be defined.

Another phosphorylated residue identified to regulate 14-3-3 interactions is Ser 184. This is a surface residue located in helix α8, near the top of the amphipathic groove (230). Phosphorylation of Ser 184 has been confirmed in both 14-3-3ζ and 14-3-3β, and seems to decrease the affinity of 14-3-3 for its client proteins (230, 231). The pro-apoptotic protein JNK has been found to phosphorylate Ser 184 (232). Consistent with a role in cell death, JNK phosphorylation of 14-3-3ζ leads to dissociation of the pro-apoptotic protein Bad from the 14-3-3 amphipathic groove. This liberation of Bad allows it to translocate to the mitochondria, where it can bind to and sequester Bcl-2, inducing mitochondrial permeabilization and triggering the apoptotic machinery (233).

v.) Consequences of 14-3-3 Binding

Decades of research have defined multiple consequences which 14-3-3 binding exerts upon client proteins (Figure 1-6). Through binding to proteins involved in the regulation of diverse metabolic and signaling pathways, 14-3-3 plays a number of important roles in the control of diverse physiological processes, including cell metabolism, cell cycle progression, molecular trafficking, cell survival, development, and

Figure 1-6. Consequences of 14-3-3 binding. 14-3-3 exerts a multitude of cellular effects through interactions with a diverse set of client proteins. 14-3-3 directly effects target protein function through multiple mechanisms, including alterations in enzymatic activity (either through enhancement or diminishment), altered protein localization, or inducing protein scaffold formation. By altering target protein function, 14-3-3 directs a wide range of cellular consequences, many of which are important in cell fate. A major role of 14-3-3 proteins is to maintain cellular survival signaling, and to inhibit pro-apoptotic signal transmission. However, 14-3-3 also alters protein trafficking, cellular metabolism, and induces changes in the cellular replication cycle. Examples of 14-3-3 target binding proteins are given in parentheses.


Cellular Consequences of 14-3-3 Binding
Survival/Anti-Apoptosis (FOXO)
Trafficking (Vimentin)
Metabolism (PFK-2)
Cell Cycle (Cdc25)
14-3-3
Target Protein
Functional Effects of 14-3-3 Binding
Enzyme Activation (ExoS)
Enzyme Inhibition (ASK1)
Altered Protein Localization (Bad)
Protein Scaffolding (Raf-1 with Bcr)

cell transformation. At the molecular level, 14-3-3 may function as a molecular cofactor, a sequestration molecule, a scaffolding dimer, and/or a signal transmitter. Importantly, these functions are not mutually exclusive.

One of the first functional effects attributed to 14-3-3 was as an enzyme cofactor. For example, 14-3-3 was rediscovered by its ability to activate both the tyrosine and tryptophan hydroxylases in the presence of CaMKII (175). Later, it was also shown to be the critical cofactor required for protein kinase C regulation and the activation of the ADP-ribosyltransferase activity of ExoS (234). However, its role as an enzyme cofactor is not always activating. For instance, binding of 14-3-3 to ASK1 leads to a decrease in ASK1 enzymatic activity (53, 54). In a general term, 14-3-3 binding may induce a conformational change of its client protein, leading to alterations in its enzymatic function.

A second general consequence of 14-3-3 binding is the subcellular change in client protein localization. For example, in response to cellular survival signals, 14-3-3 binds to phosphorylated Bad and sequesters it away from the mitochondrial membrane; this inhibits the interaction of Bad with Bcl-2 at the mitochondria and blocks the pro-apoptotic function of Bad (206). Another type of altered protein localization is cytoplasmic/nuclear shuttling, which may be attributed to 14-3-3-induced blockage or exposure of nuclear import/export signal sequences on client proteins. For example, interaction of 14-3-3 with Cdc25 blocks its translocation into the nucleus (235). Similarly, 14-3-3 binding induces the cytosolic retention of a number of other client proteins, such as histone deacetylase, YAP, and the forkhead transcription factors (207, 236, 237). 14-3-3 has also been shown to control molecular shuttling between the

cytoplasm and plasma membrane and between the ER and plasma membrane (238, 239). Alteration of subcellular localization appears to be a broad function of 14-3-3 binding with many of its client proteins, which could be due to its ability to either induce a conformational change of client proteins or to physically block or expose functional elements in client proteins, such as an organelle-targeting sequence.

A third main mechanism by which 14-3-3 exerts control over client proteins is through scaffolding of target proteins. The ability of 14-3-3 to simultaneously bind two unique targets is a result of its dimeric structure. It has been reported that Bcr and Raf-1 exist in complex together only in the presence of 14-3-3 (240). Subsequently, 14-3-3 binding was also found to be a critical mediator of the interaction between protein kinase C ζ and Raf-1, and between GSK3β and the Tau protein (241, 242).

The roles of 14-3-3 are likely not limited to those described here. Indeed, a major focus of this dissertation is to characterize a newly defined consequence of 14-3-3 function, namely as a signal "transmitter", whereby 14-3-3 relays signaling events from one transduction pathway to another.

vi.) Inhibiting 14-3-3 Client Protein Interactions

Through binding with numerous client proteins, 14-3-3 controls a wide range of physiological processes. Dysfunction of 14-3-3 has been implicated in a variety of diseases, and therefore 14-3-3 represents an attractive therapeutic target. Thus, chemical tools are important for pharmacological probing of 14-3-3 function under *in vivo* conditions and for potential 14-3-3-targeted drug discovery. Although targeting protein-protein interactions is known to be difficult, mutational analysis has shown that a single

charge-reversal mutation, Lys 49 to Glu, is sufficient to disrupt interactions between 14-3-3 and client proteins (243). This demonstrates, in principle, the feasibility of using a small molecule modulator to control client protein interactions within the conserved amphipathic groove. In support of the strategy to identify 14-3-3 modulators, a small molecule natural compound, fusicoccin, has been found to bind 14-3-3 in complex with the plant H+-ATPase (244). The fungal phytotoxin fusicoccin, a diterpene glucoside, causes wilting of plants by permanently activating the H+-ATPase, an action which requires 14-3-3 (245). In fact, fusicoccin binds to 14-3-3, filling a gap that remains in the 14-3-3 amphipathic groove upon binding to the C-terminal motif of H+-ATPase, and in this way stabilizes the interaction between the two proteins, thus allowing permanent ATPase activation (223, 244). While this example illustrates a naturally occurring target-specific 14-3-3 activator, efforts to identify 14-3-3 inhibitors are also in progress.

As a first step to target 14-3-3, high-affinity peptide antagonists were identified by screening phage display libraries (246). One peptide, termed R18, was found to bind multiple 14-3-3 isoforms with an estimated K_D value of 7-9 $\times$ 10^{-8} M. Further experiments showed that R18 interfered in the association of 14-3-3 with many client proteins, including Raf-1, ASK1 and ExoS, suggesting it competed for binding within the amphipathic groove (53, 189, 217, 247). This was confirmed when the co-crystal structure of R18 in complex with 14-3-3[illegible] was solved (246). Interestingly, R18 does not contain a phosphorylation modification, but instead contains a WLDLE motif. Together, these data suggest that R18 may be a general antagonist of 14-3-3 proteins, and targeting the conserved amphipathic groove of 14-3-3 could be a general approach to modulate 14-3-3 protein function in diverse signaling pathways. Peptide dimerization may lead to

increased affinity for 14-3-3, likely because of bidentate interaction with the 14-3-3 dimer (205). Due to this effect, the R18 peptide was further developed into difopein (dimeric fourteen-three-three peptide inhibitor) by connecting two R18 coding sequences with a short peptide linker (247). As another advantage, difopein was generated in a mammalian expression vector, allowing *in vivo* monitoring of 14-3-3 interactions. Together, R18 and difopein have been widely used to probe the function of 14-3-3 interaction with client proteins, both in various biological systems and in proteomics-based approaches (194, 248, 249).

Other examples of 14-3-3 peptide antagonists have also been shown to interfere with client protein binding. These peptides are generally derived from physiological 14-3-3 binding proteins, such as Raf-1 and Bad. A cell-permeable 14-3-3 inhibitory peptide was created by linking the peptide to a penetratin sequence to enable greater cell permeability (214). However, difficult delivery of a peptide inhibitor into cells or animals, as well as the instability of these peptides *in vivo*, poses significant obstacles for mechanistic and therapeutic exploration with these tools. Non-peptide small molecule inhibitors of 14-3-3 proteins would greatly facilitate such research.

V.) Scope of this Dissertation

This dissertation aims to understand how the newly identified member of the ASK1 signalosome, ASK2, regulates ASK1 function. I propose that ASK2 is a critical determinant of the inhibitory effect of 14-3-3 on ASK1, via Ser 967 phosphorylation. I demonstrate that ASK2 is a novel 14-3-3 interacting partner, and characterize the

biochemical features of this interaction. I further show that ASK1, ASK2, and 14-3-3 exist in a ternary complex, which is dynamically regulated by phosphorylation. Significantly, ASK2 interaction with 14-3-3 is required for 14-3-3 to be recruited to the ASK1 complex, where it is then inhibited by the 14-3-3 protein. I then go on to reveal the specific characteristics of the interaction between the ASK1 and ASK2 protein. This thesis provides a mechanism for a novel source of regulation of ASK1, and defines a new type of 14-3-3-mediated effect, namely as a transmitter of a signaling relay. Through my research, strategies to inhibit the ASK1 interaction with ASK2, and thus with 14-3-3, may provide a novel target in diseases which are triggered by defects in the apoptotic machinery.

Chapter 2

Materials and Methods

I. General Methods

i.) Reagents

Calf intestinal phosphatase (CIP) was obtained from New England Biolabs (Ipswich, MA, USA) and supplied in solution. Okadaic acid and cyclosporine A were from Sigma (St. Louis, MO, USA). Okadaic acid was diluted in ethanol, while cyclosporine A was diluted in methanol. Platinum Pfx DNA polymerase, deoxynucleotide triphosphates (dNTPs), $MgSO_4$, and Enhancer solution were purchased from Invitrogen (Carlsbad, CA, USA), and supplied in solution. Primers were ordered from Operon (Huntsville, AL, USA) and diluted in PCR-grade water to a final concentration of 1 μg/μl. FuGene6 was from Roche Applied Science (Indianapolis, IN, USA), and supplied ready-to-use. Protein A, Protein G, and glutathione S-transferase (GST) conjugated beads were from GE Healthcare (Piscataway, NJ, USA); prior to use they were washed three times with lysis buffer (see section II-ii) and resuspended in lysis buffer to a 50% final solution. Hexahistidine (6xHis) conjugated beads were from Novagen (Gibbstown, NJ, USA); before use, they were first charged with 1X charging buffer (100 mM $NiSO_4$) and washed with 1X binding buffer (500 mM NaCl, 20 mM Tris pH 8, 5 mM imidazole) before resuspending in binding buffer to a final 50% solution. West Pico and West Dura enhanced chemiluminescent detection reagents were purchased in solution from Pierce Biotechnology (Rockford, IL, USA). Anti-HA, anti-ASK1, anti-GST, anti-14-3-3 K19, anti-His, anti-Raf-1 primary antibodies, and all corresponding horseradish peroxidase-conjugated secondary antibodies (anti-mouse and anti-rabbit) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-pSer 967 ASK1 and

anti-pThr 183/Tyr 185 JNK primary antibodies were from Cell Signaling Technology (Danvers, MA, USA); the anti-Flag primary antibody was from Sigma; and the anti-ASK2 primary antibody was from Abgent (San Diego, CA, USA). All cell culture reagents were purchased ready-to-use from Mediatech, Inc. (Manassas, VA, USA).

ii.) Cell Culture and DNA Transfection

COS7 and HeLa cells were maintained in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were grown in T-75 or T-175 flasks to approximately 80% confluence at 37°C with 5% CO_2 in a humidified incubator.

For DNA transfection, COS7 cells were seeded in 6 well plates at a density of 3 x 10^5 cells per well (2 ml media per well), and HeLa cells were seeded at a density of 20 x 10^6 cells per 150 mm dish (20 ml media per dish). The following day, cells were transfected using FuGene6, according to the manufacturer's instructions. Briefly, FuGene6 was diluted into serum-free DMEM in separate tubes for each transfection and incubated at room temperature for 5 min. DNA was added to each tube, to a final ratio of 3 μl FuGene6 to 2 μg DNA. After incubation at room temperature for an additional 15 min, the DNA solution was added drop-wise to cells, and immediately and gently mixed. Cells were cultured under indicated conditions for approximately 40 hrs, until lysis.

iii.) Plasmids and Generation of Mutants

For protein expression in mammalian cells, expression vectors for each indicated gene, both wild-type (WT) and mutants, were used. All plasmid DNA was amplified in

DH5α *Escherichia coli* (*E. coli*), generated in our laboratory, and purified using Hi-Speed Midi-Prep or Maxi-Prep Kits purchased from Qiagen (Valencia, CA, USA), to obtain large amounts of transfection-quality DNA. In general, the protocol was followed according to the manufacturer's instructions. Briefly, a 3 ml culture of Luria-Bertani (LB) broth was inoculated with a single colony of plasmid-expressing cells, and grown at 37°C for 8 hrs with shaking (250 rpm). This culture was used to inoculate (1:1000) a larger culture of either 50 ml LB or 150 ml LB, for either Midi-Prep or Maxi-Prep, respectively. All bacterial cultures were maintained under selective pressure with the appropriate antibiotic, either ampicillen or kanamycin, diluted to a final concentration of 1:1000. Large bacterial cultures were grown overnight at 37°C with shaking (250 rpm). The following morning, cells were harvested by centrifugation at 6000 rpm at 4°C for 15 min. The bacterial pellet was resuspended in ice-cold buffer P1 (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 μg/ml RNase A). Bacterial cells were then lysed in buffer P2 (200 mM NaOH, 1% SDS) with gentle inversion and incubation at room temperature for 5 min. The lysate was neutralized in buffer P3 (3M potassium acetate pH 5.5) and poured into a cartridge where it was allowed to incubate for 10 min, during which time proteins, genomic DNA, and detergent were precipitated from solution. The lysate was then filtered out of the cartridge into a pre-equilibrated QIA-Tip, and contaminants were removed with a buffer QC (1 M NaCl, 50 mM MOPS pH 7.0, 15% isopropanol) wash. DNA was eluted with buffer QF (1.25 M NaCl, 50 mM Tris-HCl pH 8.5, 15% isopropanol) into polycarbonate centrifuge tubes. DNA was precipitated by adding 0.7 volumes of room temperature isopropanol and incubating for 5 min. The DNA was concentrated by sequential passes through a QIA-Precipitator attached to a syringe, and

washed with 70% ethanol. DNA was finally eluted with PCR-grade sterile water. The concentration and quality of the DNA was determined by spectrophotometry, and confirmed by agarose gel electrophoresis.

The ASK1 constructs pcDNA3-HA-ASK1 WT and its mutant pcDNA3-HA-ASK1 S967A were previously described (15, 53). Similarly, each of the pDEST26-His-14-3-3 isoforms were also previously described (88) The ASK2-KC gene was generated from cDNA isolated from a heart cDNA library by PCR. The ASK2 full-length gene was amplified using a human cDNA clone as template, which was purchased from OriGene Technologies, Inc. (Rockville, MD, USA). This template contained all but 39 bases at the extreme 5' end of the gene, which were added with a primer by PCR. Both ASK2 DNAs were cloned into the pDEST27 GST mammalian expression vector (Invitrogen) using the Gateway homologous recombination technology, according to the manufacturer's protocol. The primers for generation of each of these constructs are listed in Table 2-1.

Point mutations were generated into the pDEST27-GST-ASK2 constructs using site-directed mutagenesis. Briefly, PCR was performed using 2X Platinum Pfx buffer, 400 μM dNTPs, 1 mM $MgSO_4$, 250 ng of each primer, 100 ng of template DNA, and 1 μl of Platinum Pfx polymerase, either with or without 1X Enhancer solution. A general PCR protocol was followed (94°C for 5 min; 25 cycles of 94°C for 15 sec, 55°C for 30 sec, 68°C for 9 min; 72°C for 5 min), although adjustments to time and temperature were made as needed. PCR products visualized by agarose gel analysis were incubated overnight with *Dpn1* restriction enzyme (Invitrogen) to remove template DNA, and transformed into DH5α *E. coli* cells. DNA was extracted from single colonies using a

Table 2-1. List of primers used to generate ASK2 constructs. Primers used for the generation of ASK2-WT and ASK2-KC constructs, along with their associated point mutations, truncations, and peptides.

ASK2 Construct	Primer(s)
General ASK2 Constructs	
ASK2-WT	*5' to 3':* GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGCGGGGCCG TGTCCCCGGTCCGGGGCGGAGCGCGCCGGCAGCTGCTGGCAGG ACCCG *3' to 5':* TAGTCAAAGATGTGGGCGACTCTGGGTCGAAAGAACATGTTTCA CCAGGGG
ASK2-KC	*5' to 3':* GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGTTGGAGTTTG ATTATGAGTACACG *3' to 5':* TAGTCAAAGATGTGGGCGACTCTGGGTCGAAAGAACATGTTTCA CCAGGGG
***ASK2 Serine to Alanine Mutations**[Φ]*	
ASK2 S912A	*5' to 3':* CCCTTCCTGCAGCCTGGGAAAAGAGCTCGCAGCCCCAGCTCCCC
ASK2 S914A	*5' to 3':* CCTGCAGCCTGGGAAAAGGAGCCGGGCGCCCAGCTCCCC
ASK2 S916A	*5' to 3':* GCCGCAGCCCCGCTAGCCCACGACATGCTCCACGGCCC
ASK2 S917A	*5' to 3':* GCCGCAGCCCCAGTGCACCACGACATGCTCCACGGCCC
ASK2 S964A	*5' to 3':* CCGAAGCGCTGCCTCGCTTATGGGGGTACCAGCCAGCTCCGG

ASK2 Construct	Primer(s)
	***ASK2 Truncations**$^{\Phi}$*
ASK2 N937X	*5' to 3':* CCCACTCCTTCAGCCTAGTCGACCACCGAGTCTCAGACATTCCCG
ASK2 V973X	*5' to 3':* GGGGGCACCAGCCAGCTCCGCTAGCCCGAGGAGCCTGCGGCC
ASK2 C1042X	*5' to 3':* GGAAGAGCTGCTGCGTTAACTCGGGGCACACATCC
ASK2 R1065X	*5' to 3':* CGGGCGCTGCAAGGCTAGCTGAGGGCCCAGGGC
ASK2 R1097X	*5' to 3':* CCTCCGCAAGCGCCAGATCTAACCACACTGGATGTTCG
ASK2 P1159X	*5' to 3':* CCCGAGCAGGGCTGAGCTCCTCTGATGGTGCAGCTGAGCC
ASK2 Q1188X	*5' to 3':* GGCGGGGAAGGAACGGGAGTACTAAGCTTTGGTGCAGCGGGCTC
ASK2 L1257X	*5' to 3':* GCCACTCGAGATGACTAGATCTACACCCGCATCAGGGG
	***ASK2 Peptides**†*
ASK2 R1097 to P1288	*5' to 3':* GGGGACAAGTTTGTACAAAAAAGCAGGCTTACGTCCACACTGGATGTTCG *3' to 5':* GGTCAGTGGAGACCTGGG**ACT**CTGGGTCGAAAGAACATGTTTCACCAGGGG
ASK2 P1159 to Q1188	*5' to 3':* GGGGACAAGTTTGTACAAAAAAGCAGGCTTACCCGCTCCTCTGATGGTG *3' to 5':* CCCTTCCTTGCCCTCATGGTC**ACT**CTGGGTCGAAAGAACATGTTTCACCAGGGG

ASK2 Construct	Primer(s)
	ASK2 Peptides[†] (continued)
ASK2 R1170 to Q1188	*5' to 3':* GGGGACAAGTTTGTACAAAAAAGCAGGCTTAAGGGCAGAGACTGATCGG *3' to 5':* CCCTTCCTTGCCCTCATGGTC**ACT**CTGGGTCGAAAGAACATGTTTCACCAGGGG
ASK2 R1170 to P1288	*5' to 3':* GGGGACAAGTTTGTACAAAAAAGCAGGCTTAAGGGCAGAGACTGATCGG *3' to 5':* GGTCAGTGGAGACCTGGG**ACT**CTGGGTCGAAAGAACATGTTTCACCAGGGG
ASK2 P1130 to M1163	*5' to 3':* GGGGACAAGTTTGTACAAAAAAGCAGGCTTACCGAGGTCAGAGGAGCTGAG *3' to 5':* CCGGGGCGAGGAGACTAC**ACT**CTGGGTCGAAAGAACATGTTTCACCAGGGG
ASK2 P1159 to P1288	*5' to 3':* GGGGACAAGTTTGTACAAAAAAGCAGGCTTACCCGCTCCTCTGATGGTG *3' to 5':* GGTCAGTGGAGACCTGGG**ACT**CTGGGTCGAAAGAACATGTTTCACCAGGGG
ASK2 Q1188 to P1288	*5' to 3':* GGGGACAAGTTTGTACAAAAAAGCAGGCTTACAGGCCCTGGTGCAGCGG *3' to 5':* GGTCAGTGGAGACCTGGG**ACT**CTGGGTCGAAAGAACATGTTTCACCAGGGG

***** 3' to 5' primers are exact reverse complement of what is shown.
[Φ] Introduced mutations indicated by underline.
[†] Introduced stop codons indicated in bold.

Mini-Prep Kit (Qiagen) and verified for the correct mutation by restriction enzyme analysis. All primers for mutagenesis are listed in Table 2-1.

ASK2 peptides were generated using PCR, as described above. Correct PCR products were purified using either a PCR purification kit (Qiagen) or a gel extraction kit (Qiagen). Purified DNA was then inserted by Gateway homologous recombination technology into the pDONR201 and pDEST27-GST vectors, using the BP and LR enzymes, respectively (Invitrogen). Correct DNA was verified using a Mini-Prep Kit (Qiagen) and restriction enzyme analysis. All primers for peptide generation are listed in Table 2-1.

iv.) SDS-PAGE and Immunoblotting

All proteins were diluted with 6X sodium dodecyl sulfate (SDS) sample buffer (350 mM Tris-Cl pH 6.8, 10% SDS, 30% glycerol, 9.3% DTT) and boiled for 5 min. Proteins were separated and resolved using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) methodology (250). Either 10% or 12.5% polyacrylamide gels were made using a Mighty Small™ SE 245 dual gel caster (GE Healthcare). Following electrophoresis, proteins were transferred to nitrocellulose membrane using a GENIE® electrophoresis transfer apparatus from Idea Scientific (Minneapolis, MN, USA). Transfer occurred for 3 hrs in pre-chilled transfer buffer (20 mM Tris-Cl, 200 mM glycine, 15% methanol).

Proteins were detected by Western blot using standard techniques. All Western blot solutions were diluted in 1X TBS-T buffer (20 mM Tris pH 7.6, 500 mM NaCl, 0.05% Tween-20). The nitrocellulose blot was incubated for 1 hr in 5% nonfat dried

milk to block. This was followed by an overnight incubation at 4°C with the primary antibody, diluted in 5% milk to a final concentration of 1:500 to 1:5000. The following day, the membrane was washed three times with 1X TBS-T for 5 min each wash. The corresponding horseradish peroxidase-conjugated secondary antibodies were then added to each membrane, diluted 1:5000 in 1X TBS-T. Blots were incubated in secondary antibody for 1 to 2 hrs at room temperature, and washed three times with 1X TBS-T for 10 min each. The cross-reacting proteins were visualized using the enhanced chemiluminescent reagents West Pico or West Dura and a Kodak IS2000 Imaging System from Carestream Health, Inc. (Rochester, NY, USA).

II.) Interaction Assays

i.) Hexahistidine (6xHis) Pulldown

Cells were lysed 40 hours post-transfection in 200 µl of 6xHis pulldown lysis buffer (1% NP-40, 137 mM NaCl, 1 mM $MgCl_2$, 40 mM Tris-Cl pH 8, 60 mM imidazole, 5 mM $Na_4P_2O_7$, 5 mM NaF, 2 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mg/L aprotinin, 10 mg/L leupeptin). Lysates were then cleared by high-speed centrifugation (13,200 rpm) at 4ºC for 10 min. The clarified cell lysate was incubated and rotated with 25 µl Ni^{2+}-charged hexahistidine resin for 2 hours at 4ºC. Following this, the resin was washed twice with washing buffer (500 mM NaCl, 20 mM Tris-Cl, 60 mM imidazole) and 1 time with binding buffer (500 mM NaCl, 20 mM Tris-Cl, 5 mM imidazole). Bound proteins were released from the resin by boiling in 20 µl 6X SDS sample buffer. Interacting proteins were detected by SDS-PAGE, as described

in section I-iv.

ii.) Glutathione S-Transferase (GST) Pulldown

Cells were lysed 40 hours after transfection in 200 µl of lysis buffer (1% NP-40, 150 mM NaCl, 10 mM HEPES, 5 mM $Na_4P_2O_7$, 5 mM NaF, 2 mM Na_3VO_4, 1 mM PMSF, 10 mg/L aprotinin, 10 mg/L leupeptin). Cell lysates were cleared using high-speed centrifugation (13,200 rpm) at 4°C for 10 min. Prepared glutathione-bound Sepharose (25 µl) was added to cleared cell lysates and rotated for 2 hours at 4˚C. The Sepharose was subsequently washed three times with lysis buffer, and boiled in 20 µl 6X SDS sample buffer. Bound proteins were separated and visualized by SDS-PAGE, as described in section I-iv.

iii.) Co-Immunoprecipitation (co-IP)

After 40 hours of transfection, cells were lysed in lysis buffer (1% NP-40, 150 mM NaCl, 10 mM HEPES, 5 mM $Na_4P_2O_7$, 5 mM NaF, 2 mM Na_3VO_4, 1 mM PMSF, 10 mg/L aprotinin, 10 mg/L leupeptin). Either 200 µl (6 well plate) or 1 ml (150 mm dish) volume was used. Lysates were cleared with high-speed centrifugation (13,200 rpm) at 4°C for 10 min. Cleared lysates were incubated with 25 µl or 50 µl Protein A Sepharose for the large or small volumes, respectively, and anti-HA or anti-Flag antibodies for 2 hours at 4˚C. After incubation, Sepharose was washed three times with lysis buffer, and boiled in 20 µl 6X SDS sample buffer. Proteins were resolved by SDS-PAGE, as described in section I-iv.

III.) Enzymatic Assays

i.) Phosphatase Assay

Cells were lysed 40 hours post-transfection in 200 µl 1% NP-40 lysis buffer without phosphatase inhibitors (buffers indicated in section II, without added $Na_4P_2O_7$, NaF, Na_3VO_4). Lysates were cleared by centrifugation at maximal speed (13,200 rpm) at 4°C for 10 min. Following this, NEB Buffer #3 (New England Biolabs) was added to achieve a final concentration of 1X. The lysate was then divided into two samples. Phosphatase inhibitors ($Na_4P_2O_7$, NaF, Na_3VO_4) were added to the first "plus inhibitors" sample to a final concentration of 50 mM each. An equal volume of lysis buffer was added to the second "minus inhibitors" sample to balance the lysate volume. CIP was added to each sample and lysates were incubated at 37ºC, or alternatively, no CIP was added, and lysates were incubated at 37°C. At each indicated time point, equal amounts of lysate were removed and added to stopper tubes containing a 20 mM final concentration of each phosphatase inhibitor ($Na_4P_2O_7$, NaF, Na_3VO_4). These treated lysates were then subjected to 6xHis pulldown or anti-HA co-IP, as indicated.

ii.) Kinase Assay

Cells were lysed in 200 µl 1% NP-40 co-IP lysis buffer, as described in section II-iii. Lysates were cleared by maximal speed centrifugation (13,200 rpm) at 4°C for 10 min. The cleared lysates were added to 25 µl of glutathione-bound or Protein A-bound Sepharose, and rotated at 4°C for 2 hrs. Following incubation, the Sepharose was washed three times with 1% NP-40 co-IP lysis buffer. This was followed by one wash with 1X

kinase buffer (20 mM Tris-Cl, 20 mM Na-β–Glycerophosphate, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM DTT, 1 mM PMSF). All liquid volume was aspirated from the resin, then 15 μl of kinase reaction buffer was added (1X kinase buffer, 15 μM ATP, 1 μg per reaction myelin basic protein (MBP), 2 μCi per reaction γ^{32}P-ATP) to each sample. Samples were incubated at 30°C for 30 min, and kinase reactions were then terminated with the addition of 6 μl 6X SDS buffer and boiling. Proteins were resolved by SDS-PAGE, as described in section I-iv, and visualized by staining with Coomassie solution (50% methanol, 10% acetic acid, 0.05% Coomassie Brilliant Blue R-250) followed by destaining (7% acetic acid, 5% methanol). Auto- and transphosphorylation was visualized by a Typhoon PhosphorImager (GE Healthcare).

Chapter 3

Dual Engagement of 14-3-3 Proteins Controls Signal Relay from ASK2 to the ASK1 Signalosome

I. 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 (26, 251). Among the MAPK cascades, those mediated by the MAP3K Apoptosis Signal-Regulating Kinase 1 (ASK1) are critical determinants of cell fate (15, 16, 252). For example, ASK1 relays signals important in the transmission of death receptor-induced and ER-induced stress (77, 132). Recently, the functional role of ASK1 has broadened to include the regulation of innate immunity, reactive oxygen species (ROS) response, and various human diseases such as cardiac hypertrophy and remodeling, insulin resistance, and neurodegeneration (152, 153, 161, 163). Because of its importance as a central mediator of diverse developmental and stress signals, ASK1 is tightly controlled. The newly defined ASK1 signalosome reflects the numerous phosphorylation and protein interaction events critical to maintain ASK1 regulation (252). One important ASK1 regulatory protein is 14-3-3, a phosphoserine/threonine-recognition protein (68, 215, 222, 224, 253, 254). Binding of 14-3-3 to ASK1 through a phosphorylated Ser 967 motif (RSI**pS**LP) decreases ASK1 kinase activity and inhibits ASK1-induced apoptosis (53, 54, 70). Recently another MAP3K, ASK2, was shown to bind to and activate ASK1 (67, 71, 72). Thus, dissection of ASK1-associated protein interactions may offer opportunities to gain further understanding of general mechanisms controlling signal transmission through MAP3Ks.

14-3-3 is a regulatory protein important in maintaining a multitude of cellular processes, including cell cycle control, cell proliferation, and inhibition of apoptosis (68,

255). 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 via a phosphorylated motif. The canonical 14-3-3 recognition motif has been identified as RSX**pS**XP, in which phosphorylation of the second serine is critical for 14-3-3 recognition and inhibition (204, 205). Other examples of proteins which inhibit ASK1 function via interaction include thioredoxin, p21, and Raf-1 (101, 104, 106). Conversely, there are several examples of proteins which, upon binding, exert a positive regulation on ASK1, leading to activation of the protein kinase. Some ASK1-activating proteins identified to date include Daxx, TRAF2, and AIP1 (70, 77, 85). Because of the evidence that protein-protein interaction is an important mode of ASK1 regulation, the identification of new ASK1 binding partners is essential to further define the mechanisms by which ASK1 responds to stress signaling.

ASK2, a protein kinase with a predicted molecular weight of 112 kDa, was first identified as MAP3K6 due to the similarities in both domain structure (N-terminus, kinase domain, and C-terminus) and homology it shares with other MAP3Ks (72). Within the kinase domain, MAP3K6 is 82% homologous to ASK1 at the amino acid level. Because of its similarity to ASK1, MAP3K6 has been designated ASK2, and is now considered to be a member of the ASK family (252). Recently, ASK2 was shown to be an ASK1-binding protein that exerts a positive regulation on ASK1 activity. One report detailed a heteromeric complex formed between ASK1 and ASK2, in which ASK2 may be required for H_2O_2-induced ASK1 activation of JNK (67). Within this same complex, ASK2 was also shown to phosphorylate ASK1 at Thr 838, leading to activation of ASK1 enzymatic activity. Additionally, ASK2 protein stability seems to be dependent

on the presence of ASK1, as ASK2 is extremely unstable in ASK1-deficient cells. The existence of this heteromeric complex in cells was confirmed in a second report, which additionally showed that reduction of ASK2 protein expression together with serum-starvation led to the activation of stress-induced apoptotic pathways culminating in caspase-3 activation and poly(ADP-ribose) polymerase (PARP) cleavage (71).

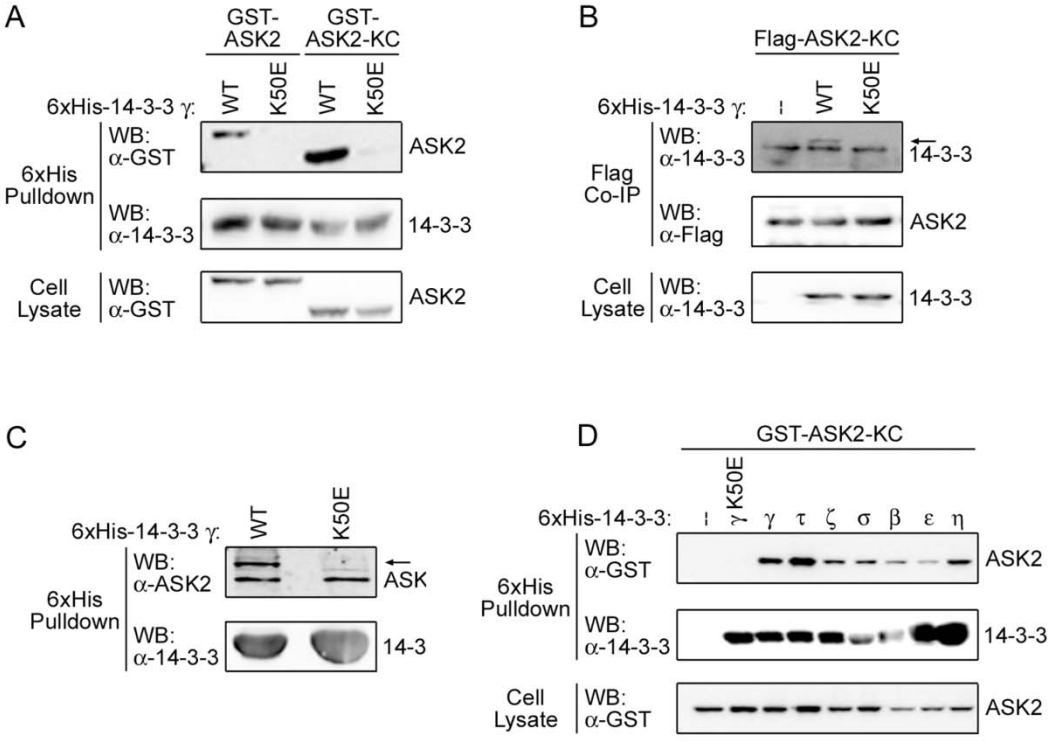
Here we describe a novel mechanism by which ASK2 suppresses ASK1 function, through the induced recruitment of 14-3-3 proteins. Our work establishes a new signaling complex consisting of ASK2, ASK1, and 14-3-3, suggests an inhibitory function of ASK2 to ASK1, and reveals a dynamic signal-relay function for the family of 14-3-3 proteins.

II. Results

i.) 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 Ser 916 (RSP**pS**SP) in the C-terminus of ASK2. This motif fits well with the defined consensus 14-3-3 binding motif, RSX**pS**XP, and raised the possibility of ASK2 as a new 14-3-3 target protein (204, 205). To test if ASK2 interacts with 14-3-3, we carried out a series of affinity pulldown assays using Ni^{2+}-charged resin to isolate hexahistidine (6xHis) tagged 14-3-3γ protein complexes from COS7 cell lysates. 14-3-3γ K50E, a client protein binding-defective mutant, was used as a control (256, 257). As revealed by Western blot, GST-ASK2 was found within the 14-3-3γ WT complex, but absent in the K50E complex (Figure 3-1A), suggesting a

Figure 3-1. Interaction of ASK2 with 14-3-3 proteins. ***(A)*** *ASK2 is in complex with 14-3-3γ.* COS7 cells were co-transfected with either GST-ASK2 or GST-ASK2-KC and 6xHis-14-3-3γ WT or K50E. The 14-3-3 protein complex was isolated 40 hrs 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-IP.* 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 detection of 14-3-3 by Western blot. ***(C)*** *14-3-3 interacts with endogenous ASK2.* COS7 cell lysates with over-expressed 6xHis-14-3-3γ WT or K50E were subjected to affinity pulldown assay as in (A), and the presence of endogenous levels of ASK2 protein was monitored by a specific antibody. ***(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).


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
ASK
WB: α-14-3-3
14-3
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

specific interaction of ASK2 with 14-3-3γ. Consistent with a potential role of ASK2 Ser 916 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 3-1A). To confirm this interaction, a reciprocal experiment was also 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 3-1B). The interaction assays described here only test the interaction between exogenously overexpressed proteins. Because we were concerned that the observed interaction could be a result of non-specific binding induced by artifacts of overexpression, we attempted to recapitulate the results with endogenous ASK2. In further support of these results, 14-3-3γ was also able to pull down endogenous ASK2. After pulldown of 6xHis-14-3-3γ WT or K50E from COS7 cell lysates, we probed the resulting interacting proteins with an antibody generated against ASK2. Indeed, a band correlating with the predicted molecular weight of ASK2 appeared in the 14-3-3γ WT pulldown, but not the control 14-3-3γ K50E pulldown (Figure 3-1C). 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.

ii.) ASK2 Interacts with 14-3-3 Through the Amphipathic Groove

There are seven 14-3-3 isoforms in mammalian cells, denoted with Greek letters (γ, τ, ζ, σ, β, ε, η). 14-3-3 interaction with ASK2 is not unique to the 14-3-3γ isoform, as ASK2 was found in complex with all seven 14-3-3 isoforms (Figure 3-1D). These data

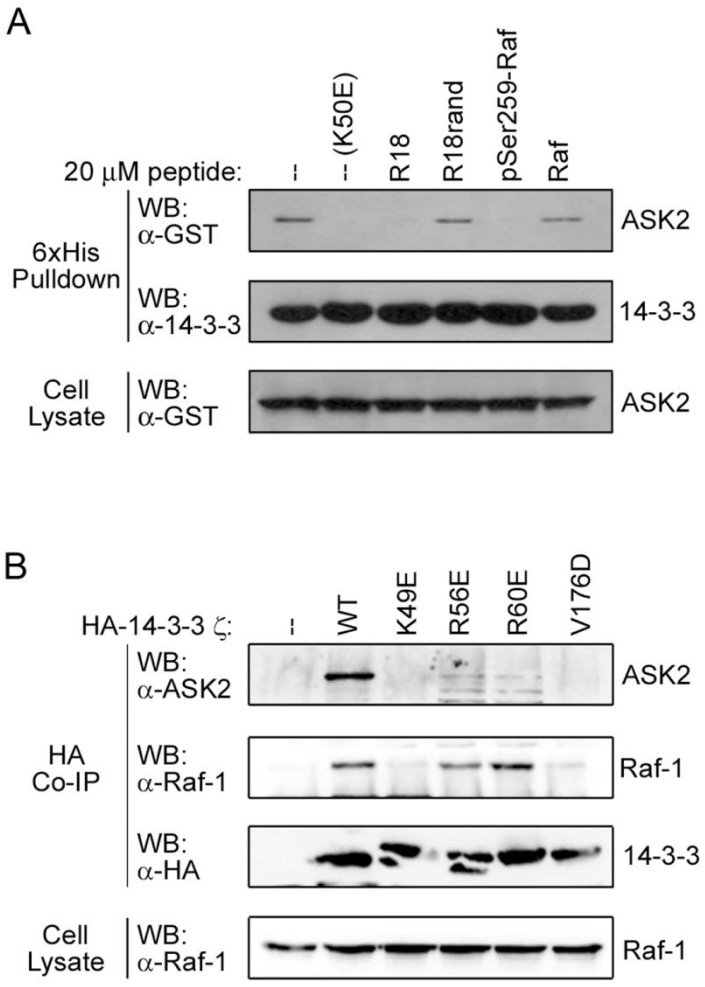
imply that ASK2 may use a common binding site within 14-3-3 proteins, such as the conserved amphipathic groove within the 14-3-3 structure (180, 181, 243). To test this possibility, we employed two approaches: peptide competition assay and mutational analysis. Indeed, two well-defined 14-3-3 groove-binding peptides (189, 204, 246), a phosphorylated Raf peptide (pSer259-Raf) and an unphosphorylated antagonist peptide (R18), effectively competed with the ASK2 and 14-3-3γ interaction (Figure 3-2A). 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 (K49E, R56E, R60E, V176D) within the groove (243, 256) greatly diminished interaction with ASK2 (Figure 3-2B). As these data show, the amphipathic groove of 14-3-3 proteins is utilized as an ASK2 docking site, allowing common and specific binding between 14-3-3 and ASK2.

iii.) ASK2 Requires Phosphorylation for 14-3-3 Binding

Many protein-protein interactions are mediated by reversible phosphorylation events. This is true for 14-3-3-ligand interactions, which except in rare cases, only occur when a consensus binding motif is phosphorylated within the target protein. This allows 14-3-3 interaction with client proteins to be reversibly and dynamically controlled. Such is the case for ASK1, where phosphorylation of Ser 967 is diminished by phosphatases, putatively in the PP1/PP2A family, and enhanced by an unidentified kinase (53, 54). We sought to determine if this was also true for ASK2.

We found that incubation of cell lysates at 37˚C led to a decrease in ASK2

Figure 3-2. ASK2 interacts via the 14-3-3 amphipathic groove. ***(A)*** *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 Figure 3-1A, either in the presence or absence of antagonistic peptides (pSer259Raf and R18). Non-phosphorylated Raf peptide (Raf) or a randomized R18 peptide (R18rand) were used as controls. ***(B)*** *14-3-3 charge reversal amphipathic groove mutations inhibit ASK2 binding.* COS7 cells were transfected with pcDNA or HA-14-3-3ζ constructs, either WT or with the indicated charge reversal mutations. Following transfection, cell lysates were subjected to co-IP with anti-HA antibody, and the resulting 14-3-3/ASK2 complexes were resolved and visualized by Western blot.


A
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
B
HA-14-3-3 ζ:
--
WT
K49E
R56E
R60E
V176D
HA Co-IP
WB: α-ASK2
ASK2
WB: α-Raf-1
Raf-1
WB: α-HA
14-3-3
Cell Lysate
WB: α-Raf-1
Raf-1

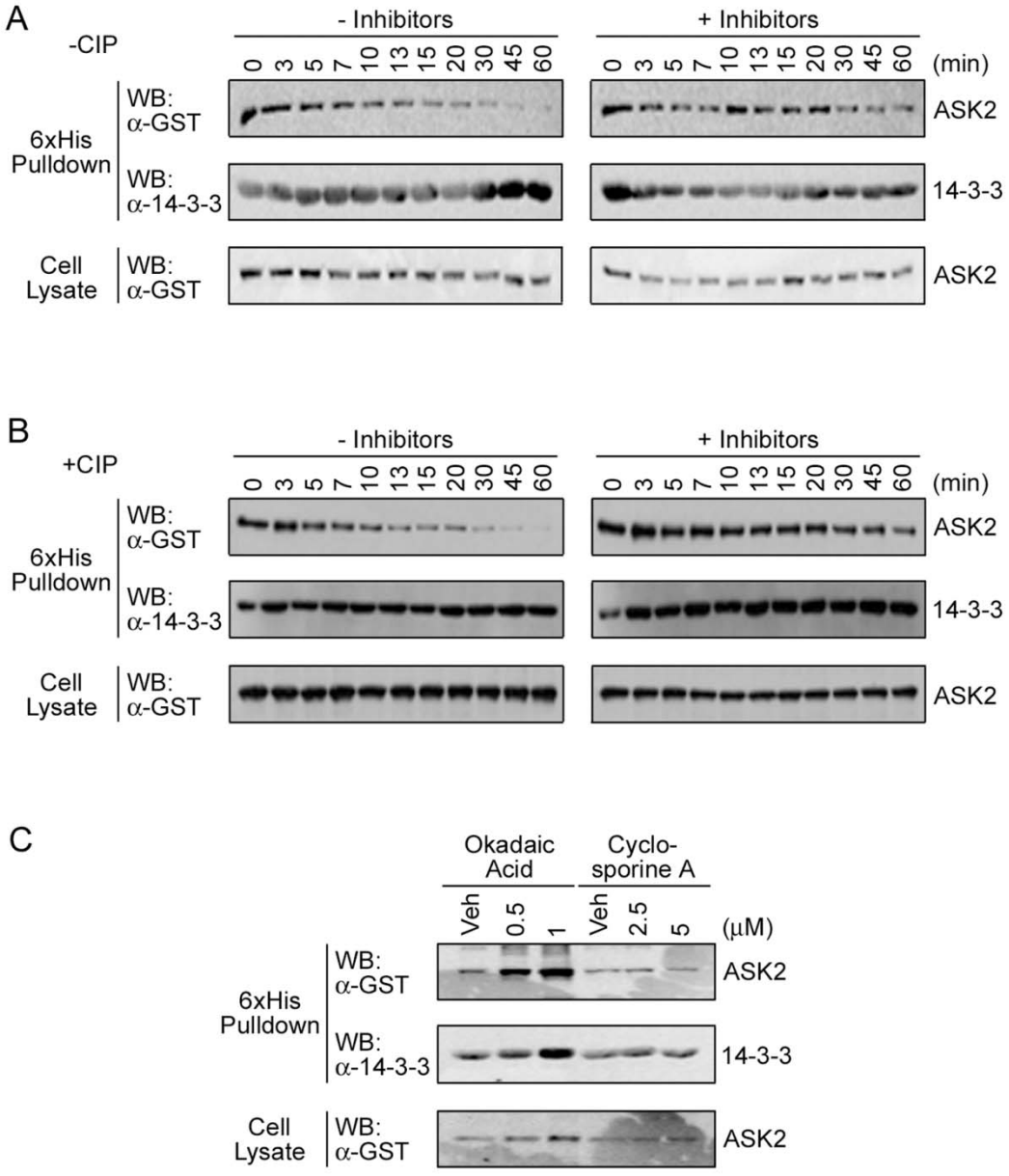
interaction with 14-3-3γ (Figure 3-3A). This decrease was likely due to the increased action of an endogenous protein phosphatase(s), as the presence of a consensus motif (RSP**pS**SP) surrounding Ser 916 of ASK2 predicts a phosphorylation requirement for recognition by 14-3-3. In support of this notion, the inclusion of general phosphatase inhibitors during this incubation prevented the dissociation (Figure 3-3A). The phosphorylation-dependence of the interaction was further confirmed in a separate assay, in which the presence of calf intestinal phosphatase (CIP) was shown to accelerate the dissociation of ASK2 from 14-3-3 (Figure 3-3B). Again, phosphatase inhibitors prevented this dissociation, signifying the specificity of action of phosphatases within this experiment.

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, while cyclosporine A is specific for inhibition of PP2B, or calcineurin (258). 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 3-3C). From these data, we conclude that ASK2 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.

iv.) 14-3-3 Binds ASK2 Through a Novel Ser 964-Mediated Motif

The phosphorylation-dependence of the ASK2/14-3-3 interaction, as well as the presence of a putative 14-3-3 binding motif surrounding Ser 916 within the ASK2

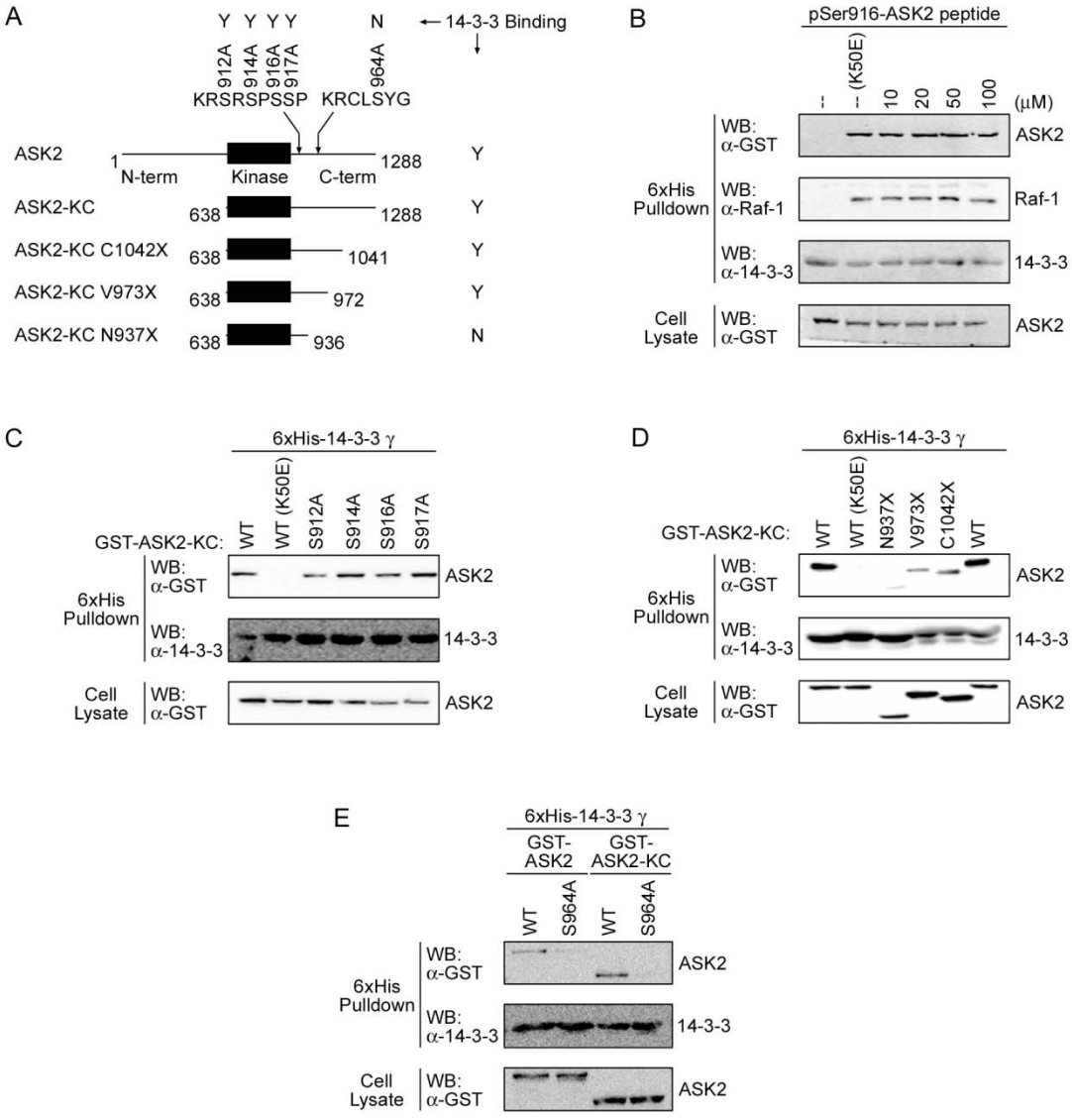
Figure 3-3. ASK2 interaction with 14-3-3 requires phosphorylation. ***(A)*** *Endogenous phosphatases control the ASK2/14-3-3 interaction.* Cell lysates with co-expressed GST-ASK2-KC and 6xHis-14-3-3γ were divided into two samples. Samples were incubated at 37ºC either in the presence or absence of 50 mM phosphatase inhibitors ($Na_4P_2O_7$, NaF, Na_3VO_4). Lysate was removed at indicated times and subjected to affinity pulldown and Western blot as described in Figure 3-1A. ***(B)*** *Phosphatase inhibition increases the amount of ASK2 interacting with 14-3-3.* Cell lysates with co-expressed GST-ASK2-KC and 6xHis-14-3-3γ were divided into two samples and treated as described in Figure 3-3A, except CIP was added to each lysate prior to incubation at 37°C. ***(C)*** *An inhibitor of the PP2A family blocks ASK2 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, or vehicle (ethanol or methanol, respectively), for 1 hour. Cells were then lysed and used in an affinity pulldown assay as described in Figure 3-1A.


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

sequence (RSP**pS**SP), both predict the importance of Ser 916 as the critical phosphoacceptor for mediating 14-3-3 interaction (Figure 3-4A). To test if the Ser 916-containing motif is indeed an essential determinant of 14-3-3 interaction, we performed a competitive binding assay using a phosphorylated peptide representing the residues surrounding Ser 916 (pSer916-ASK2). Unexpectedly, this peptide failed to compete with ASK2 for 14-3-3 binding, even at concentrations up to 100 μM (Figure 3-4B). 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 if ASK2 Ser 916 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 3-4C). We also mutated the serines within and around this predicted 14-3-3 binding motif in ASK2, generating GST-ASK2 S912A, S914A, and S917A. However, none of these mutations showed any effect on the ASK2/14-3-3 association, either (Figure 3-4C). 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 within ASK2 other than the Ser 916-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 3-4A). Using these truncations, we localized the binding site within the ASK2 sequence between Asn 937 and Val 973 (Figure 3-4D). Examination of the amino acid sequence within this region revealed that the most likely site for 14-3-3 recognition was a motif

Figure 3-4. Determination of the 14-3-3 binding site within ASK2. ***(A)*** *Schematic of predicted 14-3-3 recognition motifs within ASK2.* Amino acid sequences surrounding tested 14-3-3 binding motifs within ASK2 were shown, along with truncations and Ser to Ala mutations used in Figure 3-4C through E. Binding results are summarized as positive (Y) or negative (N). ***(B)*** *Competition with a pSer916-ASK2 peptide has no effect on the interaction of ASK2 with 14-3-3.* Experiments were performed as in Figure 3-2A, and the 14-3-3/Raf-1 interaction was used as a control. The sequence for the pSer916-ASK2 peptide was NH_2-CQPGKRSRSPpSSPRH-COOH. ***(C)*** *Mutations in the Ser 916 motif of ASK2 fail to disrupt 14-3-3 binding.* Point mutations were generated as shown, and presence of mutated ASK2 in the 14-3-3 complex was determined as in Figure 3-1A. ***(D)*** *Mapping of the 14-3-3 binding site within ASK2.* ASK2 truncations containing the 14-3-3 recognition motif were identified in an affinity pulldown assay and Western blot, as described in Figure 3-1A. ***(E)*** *ASK2 S964A abolishes binding to 14-3-3.* COS7 cells were co-transfected with 6xHis-14-3-3γ and GST-ASK2 or GST-ASK2-KC WT or S964A mutants. After transfection, cells were lysed and used in the 14-3-3 affinity pulldown assay, described in Figure 3-1A.


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)
6xHis Pulldown
WB: α-GST
ASK2
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-KC:
WT
WT (K50E)
N937X
V973X
C1042X
WT
6xHis Pulldown
WB: α-GST
ASK2
WB: α-14-3-3
14-3-3
Cell Lysate
WB: α-GST
ASK2
E
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

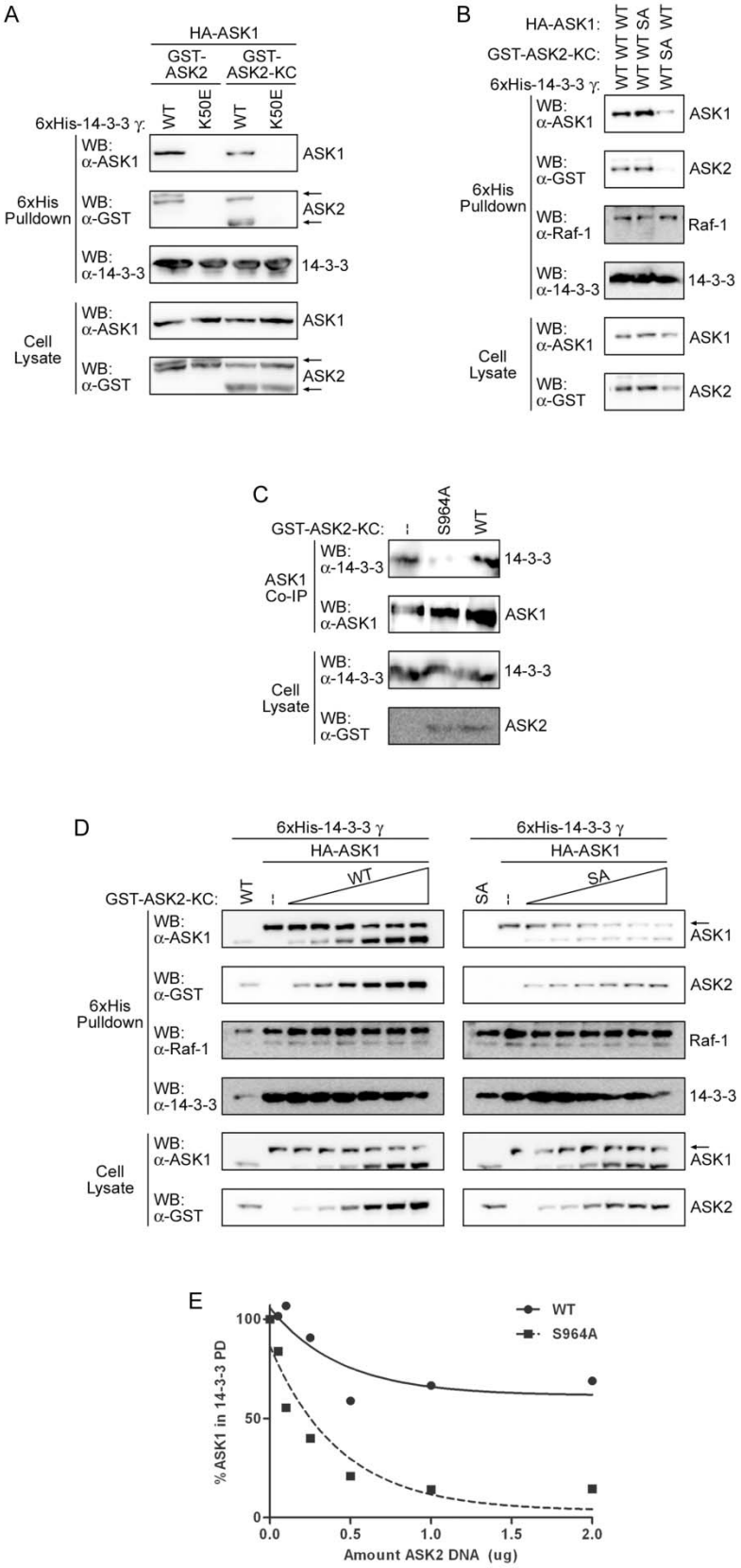
(RCL**pS**YG) surrounding Ser 964 (Figure 3-4A). We then mutated the Ser 964 residue to alanine (S964A), and used this mutant in an affinity pulldown assay. The single ASK2 S964A point mutation alone was sufficient to diminish ASK2 interaction with 14-3-3 (Figure 3-4E), suggesting that phosphorylation at Ser 964 plays an essential role in 14-3-3 binding. Thus, this study identifies Ser 964 as the primary 14-3-3 recognition site within the ASK2 protein, and defines a novel, non-canonical 14-3-3 motif which may be present in other 14-3-3 client proteins.

v.) 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 (Figure 3-1) and ASK1 (53). 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 3-5A). This complex occurred independently of the expression of either the full-length or KC versions of GST-ASK2. Neither ASK1 nor ASK2 were 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 complexes. We therefore set out to test the specificity and functional significance of this ternary complex.

vi.) ASK2 Interaction with 14-3-3 Dictates ASK1 Presence in the 14-3-3 Complex

Figure 3-5. Association of ASK2, 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 GST-ASK2 were co-transfected into COS7 cells with either 6xHis-14-3-3γ WT or K50E. Cell lysates were used in a 14-3-3 affinity pulldown assay, and the presence of ASK1 and ASK2 were 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, 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 were detected with respective antibodies by Western blot. ***(C)*** *ASK2 S964A mutation diminishes 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-IP was detected with a pan anti-14-3-3 antibody by Western blot. ***(D)*** *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 3-1A. ***(E)*** *Quantification of data from (D).* 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.


A
HA-ASK1
GST-ASK2
GST-ASK2-KC
6xHis-14-3-3 γ:
WT
K50E
WT
K50E
6xHis Pulldown
WB: α-ASK1
ASK1
WB: α-GST
ASK2
WB: α-14-3-3
14-3-3
Cell Lysate
WB: α-ASK1
ASK1
WB: α-GST
ASK2
B
HA-ASK1:
WT WT
WT WT
SA WT
GST-ASK2-KC:
6xHis-14-3-3 γ:
6xHis Pulldown
WB: α-ASK1
ASK1
WB: α-GST
ASK2
WB: α-Raf-1
Raf-1
WB: α-14-3-3
14-3-3
Cell Lysate
WB: α-ASK1
ASK1
WB: α-GST
ASK2
C
GST-ASK2-KC:
S964A
WT
ASK1 Co-IP
WB: α-14-3-3
14-3-3
WB: α-ASK1
ASK1
Cell Lysate
WB: α-14-3-3
14-3-3
WB: α-GST
ASK2
D
6xHis-14-3-3 γ
HA-ASK1
6xHis-14-3-3 γ
HA-ASK1
GST-ASK2-KC:
WT
WT
SA
SA
6xHis Pulldown
WB: α-ASK1
ASK1
WB: α-GST
ASK2
WB: α-Raf-1
Raf-1
WB: α-14-3-3
14-3-3
Cell Lysate
WB: α-ASK1
ASK1
WB: α-GST
ASK2
E
WT
S964A
% ASK1 in 14-3-3 PD
100
50
0
0.0
0.5
1.0
1.5
2.0
Amount ASK2 DNA (ug)

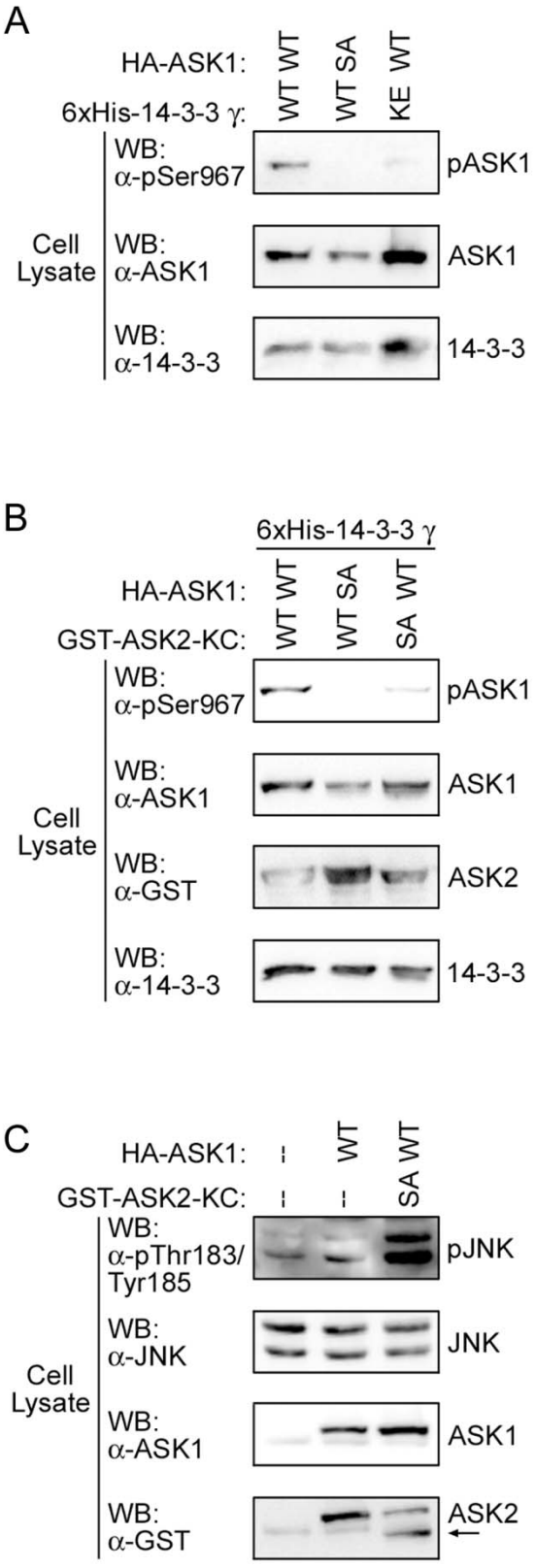
The interaction of 14-3-3 with both ASK2 and ASK1 are dynamically regulated by phosphorylation through Ser 964 of ASK2 (Figure 3-4E) and Ser 967 of ASK1 (53). Point mutation of these sites to alanine allows the generation of ASK1 or ASK2 proteins that are specifically defective in 14-3-3 binding, permitting the examination of 14-3-3 contribution to the ASK1/ASK2 complex. We utilized 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. ASK2/14-3-3 interaction appears to be independent of the ASK1/14-3-3 interaction, as overexpression of neither ASK1 WT nor S967A induced any discernable change in ASK2/14-3-3 binding (Figure 3-5B). However, the ability of ASK2 to bind 14-3-3 showed a dramatic impact on the 14-3-3 interaction with ASK1. As previously shown, ASK1 WT is associated with 14-3-3 while ASK1 S967A has diminished 14-3-3 binding (53). 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 3-5B). One explanation of 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 by the ASK2/14-3-3 interaction. 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 3-5B). Importantly, the effect of ASK2 S964A could be recapitulated with endogenous 14-3-3/ASK1 interaction, as well (Figure 3-5C). To further validate the ASK2 effect, dose-response experiments were carried out. Indeed, 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 3-5D and E).

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 which 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 3-5B and D). These data suggest that the ASK2/ASK1/14-3-3 ternary complex is distinct from the 14-3-3/Raf-1 complex. Together, these results show that ASK2, ASK1, and 14-3-3 form a specific and unique ternary complex, where ASK2 interaction with 14-3-3 determines the extent of ASK1 binding to 14-3-3.

vii.) ASK2 Interaction with 14-3-3 Controls ASK1 Function

Previous studies have demonstrated that 14-3-3 binding suppresses ASK1 activity by maintaining Ser 967 in a phosphorylated state, inhibiting ASK1-mediated JNK pathway activation (53, 70, 259). We hypothesized that 14-3-3 actually helps to maintain ASK1 in a Ser 967-phosphorylated state by protecting the site from dephosphorylation by endogenous phosphatases. To test this, we co-expressed HA-ASK1 WT with either 6xHis-14-3-3γ WT or K50E. Because 14-3-3 forms heteromeric dimers within the cell, the K50E mutant has been speculated to act in a dominant negative manner through dimerization with endogenous, wild-type 14-3-3 monomers (53). Indeed, co-expression of the K50E mutant, but not WT, version of 6xHis-14-3-3γ led to a marked decrease in phosphorylation at Ser 967, detected with a phospho-specific antibody (Figure 3-6A). This indicates that when 14-3-3 is incapable of interacting with ASK1, the protein becomes susceptible to dephosphorylation. We reasoned that ASK2 may exert control over ASK1 by dictating the action of 14-3-3 within this ternary complex. To probe the

Figure 3-6. Effect of ASK2 association with 14-3-3 on ASK1 function. ***(A)*** *Ligand-binding mutant of 14-3-3 exhibits a dominant negative effect on ASK1 phosphorylation at Ser 967.* COS7 cells transfected with HA-ASK1 WT and either 6xHis-14-3-3γ WT or K50E were lysed and separated by SDS-PAGE. Western blot was performed using the specified antibodies. ***(B)*** *ASK2 interaction with 14-3-3 controls the phosphorylation state of ASK1 at Ser 967.* COS7 cells were co-transfected with HA-ASK1 (WT or S967A), GST-ASK2-KC (WT or S964A), and 6xHis-14-3-3γ. Phosphorylation status of ASK1 at Ser 967 was determined by Western blot with an anti-pS967 specific antibody. ***(C)*** *Expression of ASK2 S964A enhances ASK1-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.


A
HA-ASK1:
6xHis-14-3-3 γ:
WT WT
WT SA
KE WT
Cell Lysate
WB: α-pSer967
WB: α-ASK1
WB: α-14-3-3
pASK1
ASK1
14-3-3
B
6xHis-14-3-3 γ
HA-ASK1:
GST-ASK2-KC:
WT WT
WT SA
SA WT
Cell Lysate
WB: α-pSer967
WB: α-ASK1
WB: α-GST
WB: α-14-3-3
pASK1
ASK1
ASK2
14-3-3
C
HA-ASK1:
GST-ASK2-KC:
WT
SA WT
Cell Lysate
WB: α-pThr183/ Tyr185
WB: α-JNK
WB: α-ASK1
WB: α-GST
pJNK
JNK
ASK1
ASK2

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 Ser 967. Indeed, co-expression of ASK2 S964A resulted in a decrease in ASK1 Ser 967 phosphorylation, an effect not observed with ASK2 WT expression (Figure 3-6B).

As a biological readout of ASK1 activity, we monitored JNK activation. As we have previously shown, dephosphorylation of ASK1 at Ser 967 is correlated with an increase in ASK1 kinase activity and ASK1-induced apoptotic signaling (53, 54). After co-expression of ASK1 with ASK2 and 14-3-3, JNK activation was determined by Western blot with an antibody directed against phosphorylated Thr 183/Tyr 185 of JNK (Figure 3-6C). Co-expression of ASK2-KC S964A with ASK1 dramatically increased the activation state of JNK, compared to ASK1 expression with control pcDNA. These results indicate that ASK2 interaction with 14-3-3 is critical to allow 14-3-3 to maintain ASK1 Ser 967 phosphorylation and suppression of ASK1 function, including reducing JNK-mediated ASK1 signaling.

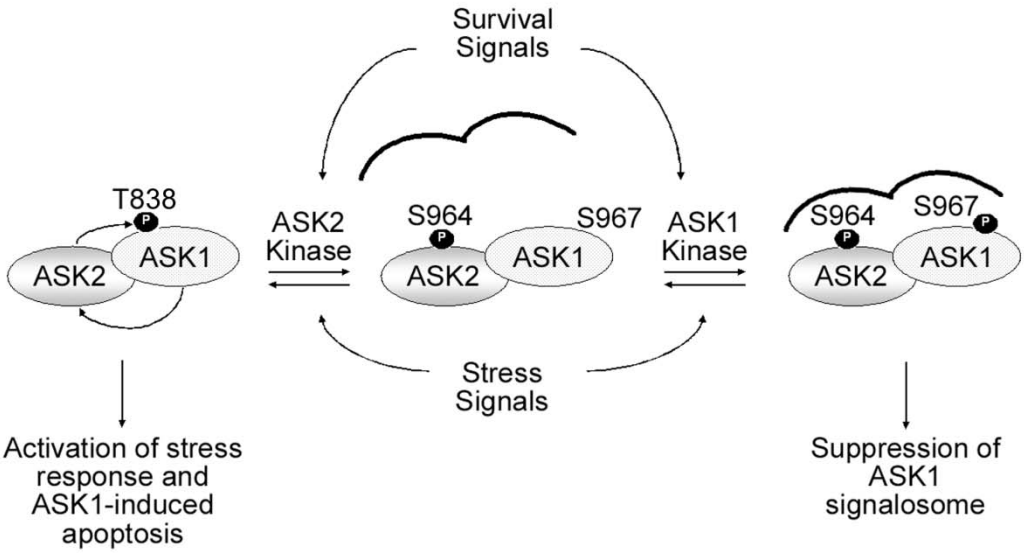
III.) Discussion

ASK1 is an essential regulator of apoptotic stress stimuli, and tight control of ASK1 activation is necessary to prevent aberrant or deregulated cell death. One main source of inhibition of the pro-apoptotic kinase activity of ASK1 occurs via Ser 967 phosphorylation and subsequent 14-3-3 binding. Here, we reveal a novel mechanism of ASK1 regulation, whereby ASK2 dictates the presence of 14-3-3 in the ASK1 signalosome. We found that (i) ASK2 specifically interacts with all seven human 14-3-3

isoforms (Figure 3-1); (ii) ASK2 interaction occurs through the conserved amphipathic groove of 14-3-3 (Figure 3-2); (iii) this interaction is reversibly regulated by phosphorylation (Figure 3-3); (iv) phosphorylation at Ser 964 of ASK2 is critical for 14-3-3 binding (Figure 3-4); (v) ASK1, ASK2, and 14-3-3 exist in a dynamically regulated protein complex dependent on ASK2 Ser 964 phosphorylation (Figure 3-5); and (vi) ASK2 binding with 14-3-3 is necessary in order for 14-3-3 to associate with ASK1 and maintain ASK1 in a Ser 967 phosphorylated and inhibited state (Figure 3-6).

Together, our data suggests a novel mechanism of ASK1 signalosome regulation, through ASK2-mediated recruitment of 14-3-3 proteins (Figure 3-7). Our proposed model predicts an ASK2 kinase that phosphorylates Ser 964 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 via phosphorylated Ser 967. Conversely, signals that promote dephosphorylation of ASK2 at Ser 964 are expected to disengage 14-3-3 from ASK2, triggering 14-3-3 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 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 (Figure 3-5), 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

Figure 3-7. Working model for ASK2-directed ASK1 association with 14-3-3. Under stress conditions, ASK1 and ASK2 exist in a heteromeric complex, and are dephosphorylated at Ser 967 and Ser 964, respectively. In this complex, ASK2 facilitates ASK1 activation through phosphorylation of Thr 838, while ASK1 exhibits positive feedback regulation on ASK2 through stabilization of the ASK2 protein (67). These activities culminate in the activation of ASK1 downstream signals, leading to ASK1-induced apoptosis. Conversely, under conditions of cell survival, ASK2 Ser 964 is phosphorylated through a pro-survival kinase signaling cascade, generating a high affinity 14-3-3 docking site. Upon phosphorylation at Ser 964, ASK2 recruits 14-3-3 to the ASK2/ASK1 complex, leading to interaction with ASK1 through phosphorylated Ser 967 and subsequent suppression of the ASK1 signalosome.


Survival
Signals
T838
P
ASK2
ASK1
ASK2
Kinase
S964
P
S967
ASK2
ASK1
ASK1
Kinase
S964
P
S967
P
ASK2
ASK1
Stress
Signals
Activation of stress
response and
ASK1-induced
apoptosis
Suppression of
ASK1
signalosome

general mechanism by which 14-3-3 regulates signal-relay among MAP3Ks to ensure signaling integrity in various physiological processes.

It is evident that phosphorylation is an extremely important source of ASK1 regulation, and thus inhibition of stress-induced cell death. Phosphorylation of several residues has been shown to be important in controlling ASK1 enzymatic activity. Thr 838 lies within the activation loop of the kinase domain, and phosphorylation at this site is correlated with an increase in ASK1 activity (20, 61). Conversely, phosphorylation at Ser 83 is responsive to Akt stimulation, and in fact direct phosphorylation of Ser 83 by Akt inhibits the pro-apoptotic ability of ASK1 (47). It was also found that ASK1 Ser 1034, a site that is highly phosphorylated under normal cell survival conditions, negatively regulates ASK1 activity (46). Phosphorylation of Ser 967 was the first characterized ASK1 phosphorylation event, and phosphorylation at this residue is critical for inducing 14-3-3 binding, leading to a decrease in ASK1 activity (53). Here, we add a novel level to this signaling event, whereby ASK2 recruits 14-3-3 to ASK1, inducing binding to ASK1 Ser 967.

The relatively recent discovery of ASK2 means that very little function has been ascribed to the ASK2 protein. Recently, a model has emerged whereby ASK2 exists in a heteromeric complex with ASK1, with reciprocal functionality (67). ASK1 interaction with ASK2 may serve to stabilize the ASK2 protein, maintaining it in an active conformation, whereby it can go on to activate downstream MAPKs such as JNK. On the other hand, ASK2 can activate ASK1 through direct phosphorylation of the critical Thr 838 in the activation loop. This may increase the complexity of the already existing model of ASK1 Thr 838 phosphorylation, in which it is proposed that activated ASK1

dimerizes and induces Thr 838 phosphorylation through trans-autophosphorylation (20). This model may now be modified to show that ASK2 can participate in this activation of ASK1 via protein-protein interaction. In this way, Takeda and colleagues described an ASK1-activating function of ASK2. Here, we propose an additional mechanism of ASK2-induced-ASK1 activation, through dissociation of ASK1 from 14-3-3. This is mediated first by dephosphorylation of ASK2 at Ser 964, which limits the amount of ASK2 interacting with 14-3-3, and directs the absence of 14-3-3 within the ASK1 signalosome.

This work predicts a novel functional effect exerted by 14-3-3, as a relay of signal transmission. In this manner, 14-3-3 is responsive to one signaling pathway, namely that one leading to ASK2 Ser 964 phosphorylation, and responds to this pathway by exerting an effect on another protein, namely ASK1 mediated by Ser 967 phosphorylation. The existence of this model predicts that the kinase responsible for phosphorylating ASK2 at Ser 964 is a both a direct and indirect key mediator of not one, but two, proteins. This predicted kinase would directly lead to 14-3-3 association with ASK2 via Ser 964 phosphorylation, and indirectly lead to 14-3-3 association with ASK1 via 14-3-3-maintained Ser 967 phosphorylation.

As this work shows, ASK2 interaction with 14-3-3 is a critical determinant of the presence of 14-3-3 within the ASK1 signalosome. Therefore, this predicts that targeted disruption of this ternary complex may be a useful tool to monitor these signal transduction pathways. Thus, understanding the biochemical characteristics of the ASK1 interaction with ASK2 may have significant implications for the development of novel inhibitors of this interaction, and may lead to an increased understanding of the impact of

activation of the ASK1 signalosome.

Chapter 4

Structure-Function Relationships Within the ASK1/ASK2 Heteromeric Complex

I. Introduction

As described in Chapter 3, ASK2 plays a critical role in determining the inhibitory presence of 14-3-3 within the ASK1 signalosome. Through phosphorylation at Ser 964, ASK2 interacts with 14-3-3 in an amphipathic groove-mediated manner. By binding to ASK2, this allows 14-3-3 to dually recognize and interact with ASK1 at Ser 967. This model predicts that ASK1 and ASK2 exist in a heteromeric complex, and that interaction between these two proteins is necessary for the dual recognition of 14-3-3 proteins (Figure 3-7). In fact, it is also possible that 14-3-3 additionally serves as an adaptor protein, linking these two MAP3Ks together. Therefore, identification of the structure-function relationships and mechanisms responsible for regulating ASK1 interaction with ASK2 has profound implications not only for understanding mechanisms of ASK1 activation and suppression, but also for a greater insight into the function of ASK2.

Several examples exist showing that MAP3Ks interact together. For example, the Raf-1 and ASK1 kinases were found to interact with each other through co-IP analysis (104). This interaction was mapped to the N-terminal regulatory region within ASK1, indicating that Raf-1 may play a role in ASK1 regulation. Notably, both of these kinases each also interact with 14-3-3 (53, 257), and therefore Chen and colleagues tested if 14-3-3 binding impacted the interaction between Raf-1 and ASK1, which they found it did not (104). A functional effect of this interaction was postulated when it was shown that overexpression of Raf-1 could inhibit ASK1-induced apoptosis, which was dependent on the ability of Raf-1 to interact with ASK1. However, it was established that neither the catalytic activity nor the activation of the MEK-ERK cascade was necessary for these

effects, suggesting a unique function for Raf-1. This interaction was later shown to be physiologically relevant, when Alavi and colleagues found that growth factor treatment of endothelial cells prevented doxorubicin-induced apoptosis through induction of the Raf-1 interaction with ASK1 (126). In another example of MAP3K interaction two Raf family members, Raf-1 and B-Raf, were shown to heterodimerize (260). This heterodimerization was induced by activation of the upstream GTPase Ras. It was hypothesized that Ras induced conformational changes within both Raf-1 and B-Raf, which led to the exposure of 14-3-3 recognition motifs in the C-termini of each kinase. This complex formation was later confirmed to be enhanced by the binding of 14-3-3 (261). Thus, interaction between MAP3Ks could be a common mechanism by which multiple signaling pathways can integrate diverse upstream stimuli to common downstream effectors.

The fact that 14-3-3 is a dimeric protein raises the possibility that 14-3-3 can serve an adaptor function whereby it can scaffold together two client binding proteins. Indeed, it has been reported that 14-3-3 induces the interaction of Raf-1 and the Bcr kinase (240). In fact, these two kinases did not interact in a yeast two-hybrid assay until co-expression of the 14-3-3 protein. 14-3-3 expression also increases the association of GSK3β with the tau protein in brain extracts (262), allowing GSK3β to phosphorylate the tau protein (263). Hyperphosphorylated tau has been implicated in the neurofibrillary tangles associated with Alzheimer's disease (264). 14-3-3 also couples Raf-1 with protein kinase C ζ (PKCζ). In fact, this interaction has a negative feedback component, whereby activated PKCζ phosphorylates 14-3-3, resulting in reduced association of the ternary complex (241). Together, these examples provide strong evidence that coupling

two proteins together is a general mechanistic feature of 14-3-3 proteins.

Both ASK1 and ASK2 are MAP3Ks, and both have been established to interact with 14-3-3 [Chapter 3; (53)]. In light of the important functional effects which ASK2 exerts upon ASK1, we sought to determine the biochemical characteristics of the interaction between these two proteins. We first established the role of phosphorylation and 14-3-3 binding on the heteromeric complex between ASK1 and ASK2. We then mapped the interacting regions within the two proteins. Finally, we showed that disruption of the ASK1 interaction with ASK2 resulted in a functional change in the ASK1 signalosome. Thus, targeting the ASK1/ASK2 interaction interface may prove to be a valuable tool both for understanding the ASK1 and ASK2 signalosomes, and for developing novel modulators of ASK1-induced signaling.

II.) Results

i.) ASK1 Interacts with ASK2

We began our exploration of the predicted interaction between ASK1 and ASK2 by first establishing whether these two proteins existed in a heteromeric complex within cells. This was especially important, in light of the observation that protein-protein interaction is a key mechanism of ASK1 regulation. To determine if ASK2 associated with ASK1 under normal cell survival conditions, we performed both a GST pulldown and a co-IP with an anti-HA antibody using lysate from COS7 cells transfected with HA-ASK1 and GST-ASK2. After the resulting protein complexes were isolated and detected by Western blot, ASK1 and ASK2 were indeed found in complex together (Figure 4-1),

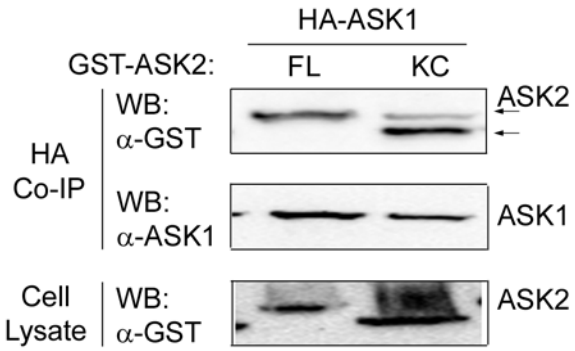
confirming previous results showing a heteromeric complex formed between the two proteins (67, 71). We additionally tested ASK1 interaction with an N-terminal truncated version of ASK2 (GST-ASK2-KC), which we had previously generated for mechanistic studies of the ASK2 interaction with 14-3-3. This truncated version of ASK2 was also found to associate with ASK1, allowing us to begin to narrow down the ASK1 binding region to within the ASK2 kinase and C-terminus domains (Figure 4-1). Because of its robust interaction with ASK1, we continued to use the ASK2-KC truncation to simply interpretation of subsequent experiments.

ii.) ASK1/ASK2 Interaction is Independent of 14-3-3 Binding

The heteromeric nature of the ASK1/ASK2 complex creates the possibility that a scaffolding protein enhances or allows binding to occur. 14-3-3, a candidate adaptor previously found to interact with both ASK1 and ASK2 [Chapter 3; (53)], is a dimeric protein which has been speculated to have a scaffolding role in certain circumstances. Therefore, we initiated our characterization of the ASK1/ASK2 heterodimer by first determining if 14-3-3 proteins were a necessary scaffold for the formation of this complex.

We tested the impact of 14-3-3 interaction on the formation of the ASK1/ASK2 heterodimer in two ways. First, we performed a binding assay to monitor ASK1/ASK2 interaction in the presence or absence of the competitive 14-3-3 binding peptide R18 (246). Lysates co-expressing HA-ASK1, GST-ASK2-KC, and 6xHis-14-3-3γ were generated from transfected COS7 cells, and used in a co-IP assay with an anti-HA antibody. Increasing concentrations of R18, or the appropriate vehicle control, were also

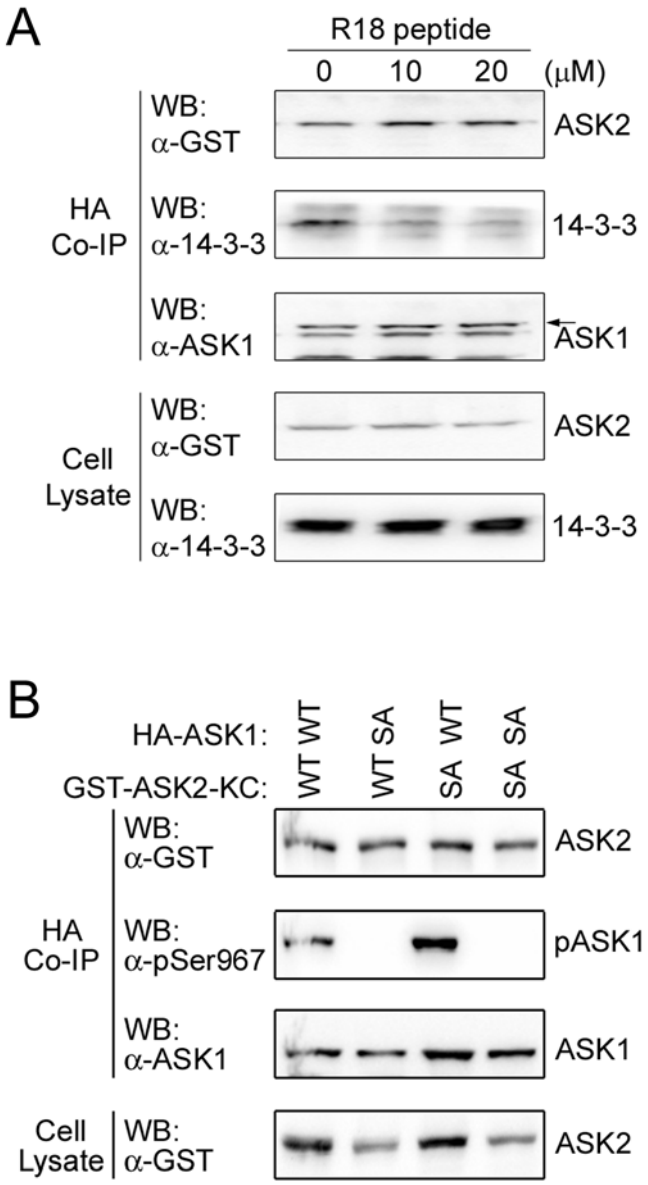
Figure 4-1. ASK2 and ASK1 exist in a heteromeric complex. COS7 cells were transfected with HA-ASK1 and either GST-ASK2 full-length (FL) or GST-ASK2-KC. Approximately 40 hrs post-transfection, cells were lysed and cleared lysates were used in a co-IP assay with an antibody directed against the HA epitope. The resulting protein complexes were resolved by SDS-PAGE and visualized by Western blot.


HA-ASK1
GST-ASK2:
FL
KC
HA
Co-IP
WB:
α-GST
ASK2
WB:
α-ASK1
ASK1
Cell
Lysate
WB:
α-GST
ASK2

included in each co-IP. After separating the resulting ASK1-complexes by SDS-PAGE, the amount of ASK2 bound to ASK1 was determined by Western blot. Inclusion of R18, even up to 20 μM, seemed to have no effect on the formation of the ASK1/ASK2 heteromeric complex (Figure 4-2A). The antagonistic properties of the R18 peptide were confirmed in this assay by the detection of decreasing amounts of 14-3-3 bound to the ASK1/ASK2 complex. The lack of effect of R18 on the ASK1/ASK2 complex led us to believe that 14-3-3 is not important for the association of these two kinases. However, it was apparent that within this assay, R18 did not completely abrogate 14-3-3 association with the ASK1 complex. Therefore, to more directly test the importance of 14-3-3 on ASK1/ASK2 complex formation, we utilized 14-3-3 binding-deficient mutants of each protein (ASK1 S967A and ASK2 S964A). These constructs contain alanine point mutations at critical serine residues within their 14-3-3 recognition motifs, which have been shown to abolish interaction to 14-3-3 proteins [Figure 3-4E; (53)]. Various combinations of the WT and SA versions of HA-ASK1 and GST-ASK2-KC were co-expressed in COS7 cells, and the resulting heteromeric complexes were isolated by co-IP with an anti-HA antibody. None of the WT or SA combinations affected the binding between the two proteins (Figure 4-2B). Significantly, the heteromeric complex formed between ASK1 S967A and ASK2 S964A, in which neither protein bound 14-3-3, was not diminished compared to the complex formed between the WT versions. Together, these data indicate that 14-3-3 is not an important component of the ASK1/ASK2 interaction, and therefore does not have a scaffolding role within this heteromeric complex.

iii.) Phosphorylation Does Not Mediate ASK1/ASK2 Binding

Figure 4-2. 14-3-3 is not necessary for ASK1/ASK2 interaction. ***(A)*** *R18 does not disrupt the ASK1/ASK2 interaction.* COS7 cell lysates expressing GST-ASK2-KC, HA-ASK1, and 6xHis-14-3-3γ were subjected to co-IP with an anti-HA antibody as described in Figure 4-1. Prior to co-IP, the indicated concentrations of R18, or its vehicle DMSO, were added to each sample. ***(B)*** *14-3-3 binding mutations have no effect on the ASK1/ASK2 heteromeric complex.* The WT and SA versions of HA-ASK1 and GST-ASK2-KC were transfected into COS7 cells as indicated. Transfected lysates were subjected to co-IP with an anti-HA antibody as described in Figure 4-1.

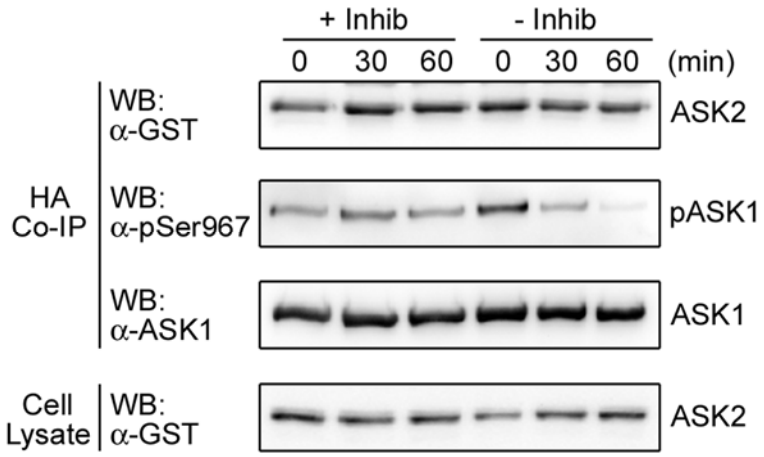

A
R18 peptide
0
10
20
(μM)
HA Co-IP
WB: α-GST
ASK2
WB: α-14-3-3
14-3-3
WB: α-ASK1
ASK1
Cell Lysate
WB: α-GST
ASK2
WB: α-14-3-3
14-3-3
B
HA-ASK1:
WT WT
WT SA
SA WT
SA SA
GST-ASK2-KC:
HA Co-IP
WB: α-GST
ASK2
WB: α-pSer967
pASK1
WB: α-ASK1
ASK1
Cell Lysate
WB: α-GST
ASK2

We were intrigued by our finding that 14-3-3 was not important for the interaction between ASK1 and ASK2, and therefore sought to determine other regulatory mechanisms which controlled this interaction. Phosphorylation is an important post-translational modification which has been shown to mediate multiple protein-protein interactions. For example, phosphorylation of MDM2 on various proposed sites inhibits association with the tumor suppressor p53 (265). ASK1 and ASK2 are both phosphorylated, suggesting that their interaction may be mediated by this modification. In order to test if phosphorylation was an important regulator of ASK1/ASK2 binding, we performed an *in vitro* phosphatase assay using cell lysates co-expressing HA-ASK1 and GST-ASK2-KC. Cell lysates, prepared using lysis buffer containing no phosphatase inhibitors, were incubated with calf intestinal phosphatase (CIP) at 37˚C. Samples containing general phosphatase inhibitors ($Na_4P_2O_7$, NaF, Na_3VO_4) were included as controls. At the indicated times, treated lysate was removed and subjected to a co-IP using an anti-HA antibody. The resulting ASK1/ASK2 protein complexes were visualized by Western blot. We found that the CIP treatment was effective, evidenced by a decreased signal using the anti-pSer 967 ASK1 antibody, which was inhibited with the addition of phosphatase inhibitors (Figure 4-3). However, there was no change in the proportion of ASK2 interacting with ASK1 in either case, suggesting that although both of these proteins undergo phosphorylation modifications, it is not an important regulatory component of the ASK1 interaction with ASK2.

iv.) The C-terminus of ASK1 is Essential for ASK2 Interaction

ASK1-interacting proteins have been shown to bind to ASK1 through diverse

Figure 4-3. Phosphorylation is not essential for ASK1/ASK2 interaction. Both GST-ASK2-KC and HA-ASK1 were transfected into COS7 cells. Approximately 40 hrs post-transfection, cells were lysed in 1%NP-40 lysis buffer containing no phosphatase inhibitors. Cell lysates were then divided into two samples. CIP was added to each, and samples were incubated at 37ºC either in the presence or absence of 50 mM phosphatase inhibitors ($Na_4P_2O_7$, NaF, Na_3VO_4). Lysate was removed at indicated times and subjected to co-IP with an anti-HA antibody and Western blot as described in Figure 4-1.

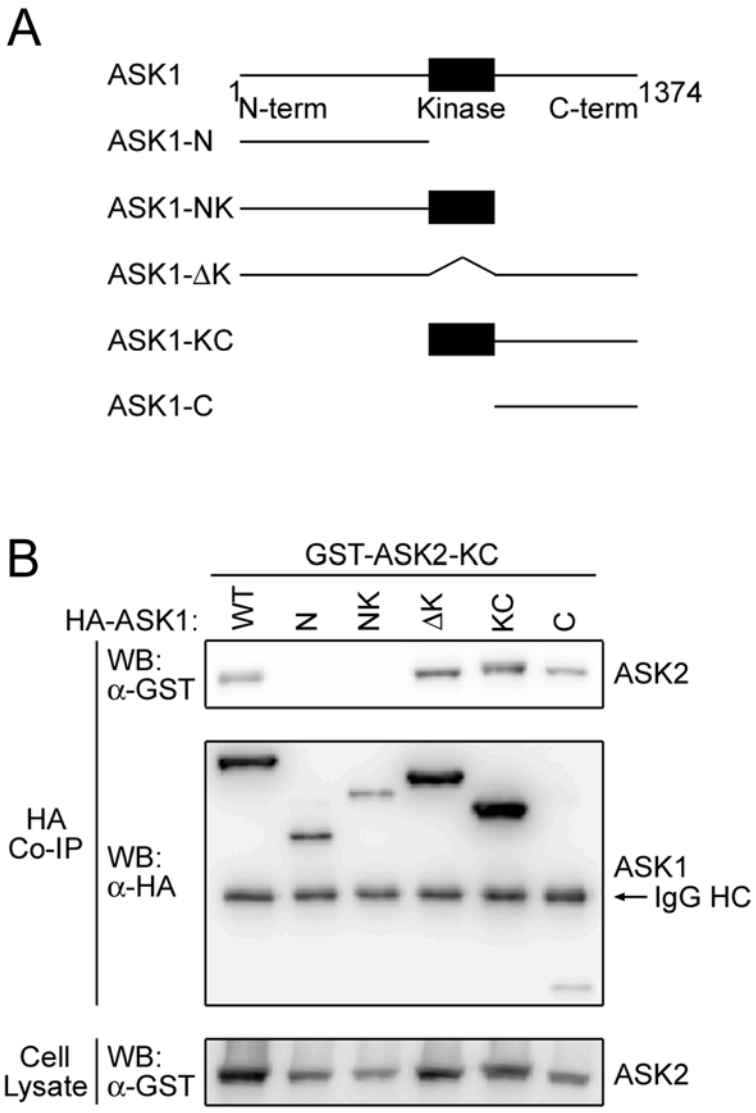

+ Inhib
- Inhib
0
30
60
0
30
60
(min)
HA Co-IP
WB: α-GST
ASK2
WB: α-pSer967
pASK1
WB: α-ASK1
ASK1
Cell Lysate
WB: α-GST
ASK2

regions within the protein. Trx and the TRAF2 and TRAF6 proteins bind to the N-terminus of ASK1 (87, 106), while Raf-1, AIP1, and 14-3-3 interact with the C-terminus (53, 70, 104). To begin to understand the function that ASK2 binding can exert on ASK1, we sought to identify the region within ASK1 where ASK2 binds. To first determine this, we utilized HA-ASK1 truncations, representing the N-terminus (HA-ASK1-N), the N-terminus plus the kinase domain (HA-ASK1-NK), the protein lacking the kinase domain (HA-ASK1-ΔK), the kinase domain plus the C-terminus (HA-ASK1-KC), and the C-terminus alone (HA-ASK1-C), depicted in Figure 4-4A. Each of these HA-ASK1 constructs was co-expressed in COS7 cells with GST-ASK2-KC. Following cell lysis, the ASK1 truncations were isolated by co-IP using an anti-HA antibody, and the resulting interacting ASK2 was detected by Western blot. ASK2 was only found in complex with ASK1 constructs which contained the C-terminus, including the WT, ΔK, KC, and C truncations (Figure 4-4B). Interestingly, although HA-ASK1-C was expressed to only a minimal degree in comparison to the other ASK1 truncations, a similar proportion of ASK2 was found in complex with it, indicating a high affinity interaction. Interaction with the HA-ASK1-C truncation also showed that the C-terminus of ASK1 was sufficient for ASK2 interaction. The lack of any interaction of ASK2 with the HA-ASK1-NK truncation allowed us also to find that the presence of the C-terminus was necessary for ASK2 binding. Thus, we concluded that the ASK1 C-terminus was both necessary and sufficient for interaction with ASK2.

v.) The C-terminus of ASK2 is Essential for ASK1 Interaction

We next performed a reciprocal set of experiments to determine the binding

Figure 4-4. ASK2 interacts with the ASK1 C-terminus. ***(A)*** *Schematic of ASK1 constructs*. Depiction of HA-ASK1 constructs used in Figure 4-4B. ***(B)*** *The ASK1 C-terminus is necessary and sufficient for binding to ASK2.* HA-ASK1 constructs were co-expressed in COS7 cells with GST-ASK2-KC as indicated, and binding with ASK2 was determined through anti-HA co-IP and Western blot, as described in Figure 4-1.


A
ASK1
1
N-term
Kinase
C-term
1374
ASK1-N
ASK1-NK
ASK1-ΔK
ASK1-KC
ASK1-C
B
GST-ASK2-KC
HA-ASK1:
WT
N
NK
ΔK
KC
C
WB:
α-GST
ASK2
HA
Co-IP
WB:
α-HA
ASK1
← IgG HC
Cell
Lysate
WB:
α-GST
ASK2

region within ASK2 important for ASK1 interaction. Because we had already determined that ASK2-KC was sufficient for ASK1 binding (Figure 4-1), we initially focused our attention on the C-terminus of ASK2. We first generated GST-ASK2-KC truncations of decreasing size, depicted in Figure 4-5A, which were co-expressed in COS7 cells with HA-ASK1. Lysates generated from these cells were then used in a GST pulldown, after which interacting ASK1 was visualized by Western blot (Figure 4-5B). Using this technique, we determined that the ASK1 binding region on ASK2 was located within the approximately 90 amino acid residues between Arg 1097 and Gln 1188, as ASK2 truncations up to R1097X were unable to bind ASK1, while the Q1188X truncation restored binding. We then further narrowed down the binding region with an additional ASK2 truncation generated within this region, GST-ASK2-KC P1159X (Figure 4-5C). Because this truncation also abolished binding with ASK1, we concluded that the ASK1 binding region within ASK2 was located within the 29 amino acids between Pro 1159 and Gln 1188.

vi.) Development of an ASK2 Peptide Capable of Binding ASK1

To more fully define the exact binding interface of ASK1 within ASK2, we generated various peptides representing the amino acid sequences surrounding the potential ASK1 binding region, largely located between Arg 1097 and Pro 1288 (Figure 4-6A). Each of these GST-ASK2 peptides were co-transfected into COS7 cells with HA-ASK1, and binding to ASK1 was determined with a GST pulldown. Western blot showed that a peptide containing ASK2 amino acids R1170 to P1288 was sufficient for binding ASK1 (Figure 4-6B). In fact, this ASK2 peptide interacted with ASK1 to the

Figure 4-5. ASK1 interacts with the ASK2 C-terminus. ***(A)*** *Schematic of ASK2 truncations.* Depiction of GST-ASK2-KC truncations generated for use in Figure 4-5B. ***(B)*** *The ASK1 binding site lies within the C-terminus of ASK2.* GST-ASK2-KC truncations were co-expressed with HA-ASK1 in COS7 cells. 40 hrs post-transfection, cells were subjected to GST pulldown with glutathione-conjugated Sepharose. The resulting interacting proteins were separated by SDS-PAGE and visualized by Western blot. ***(C)*** *Residues 1159 to 1188 of ASK2 contain the ASK1 binding site.* HA-ASK1 was co-transfected with the GST-ASK2 P1159X truncation, and interaction was determined by GST pulldown as in Figure 4-5B.

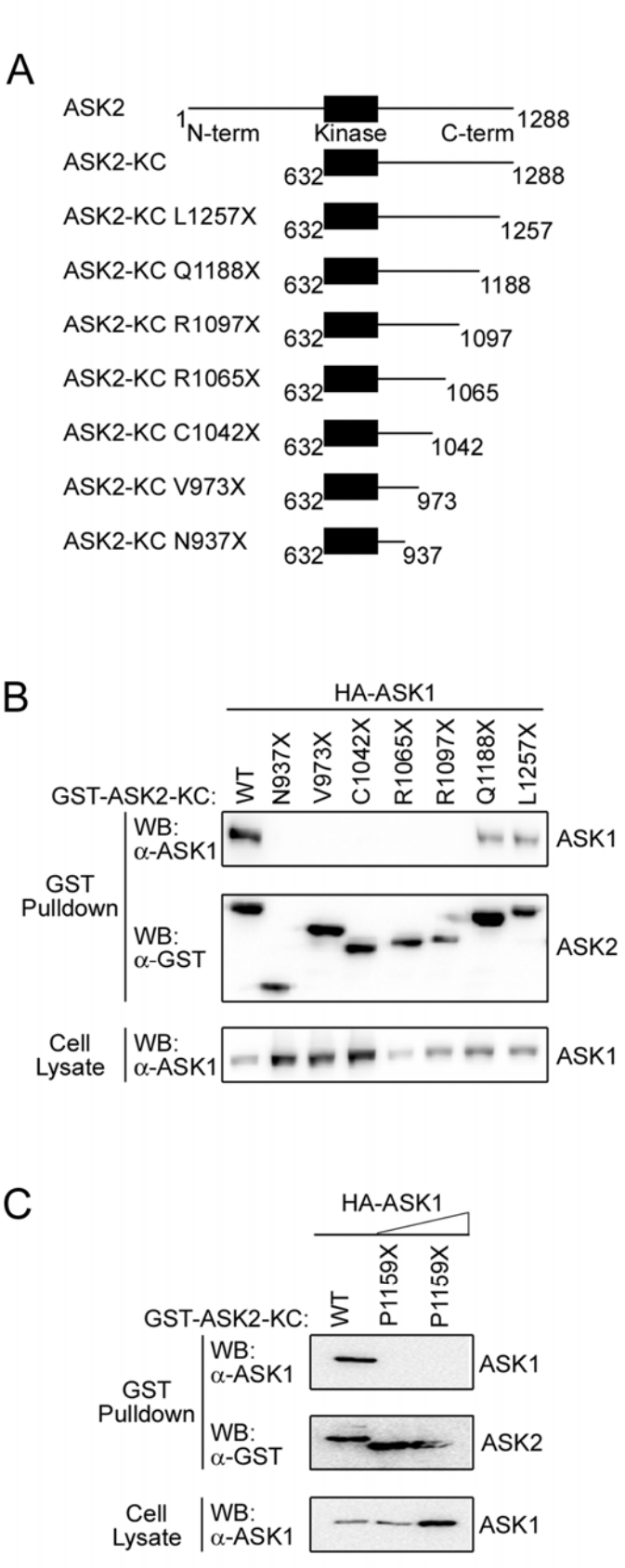
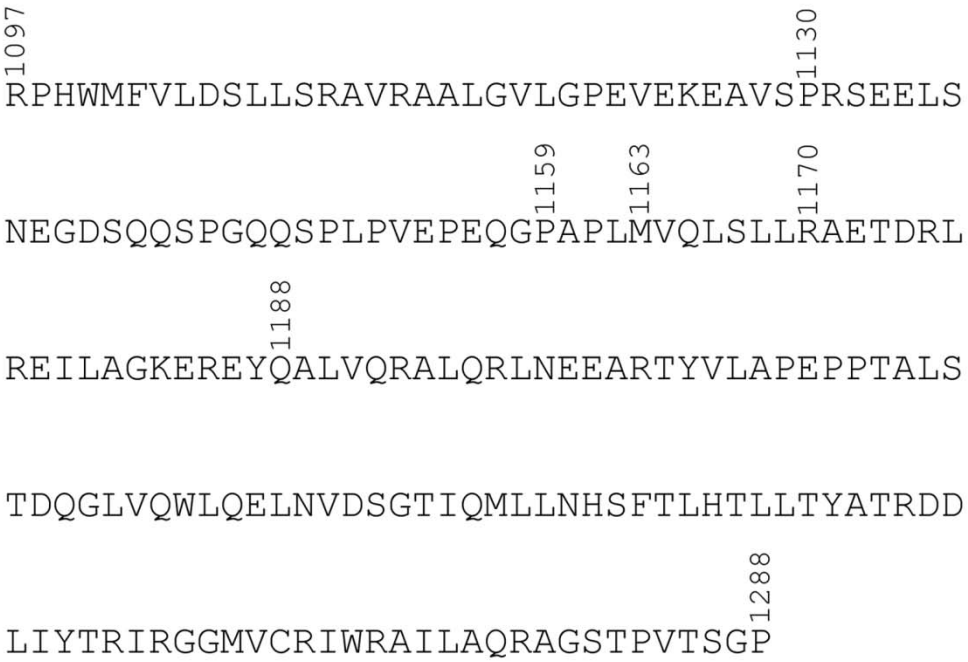
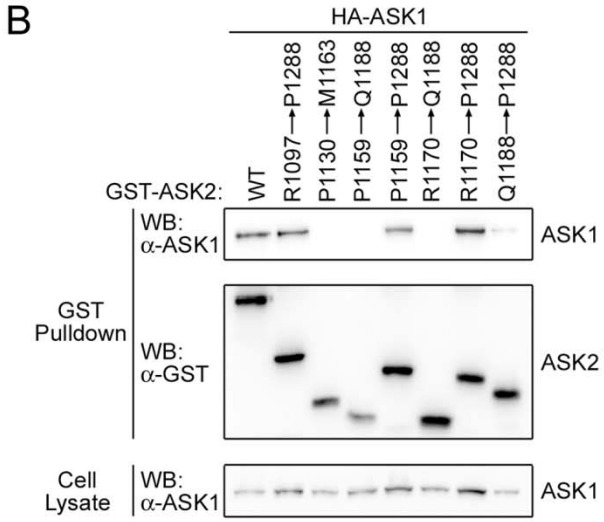

A
ASK2
1
N-term
Kinase
C-term
1288
ASK2-KC
632
1288
ASK2-KC L1257X
632
1257
ASK2-KC Q1188X
632
1188
ASK2-KC R1097X
632
1097
ASK2-KC R1065X
632
1065
ASK2-KC C1042X
632
1042
ASK2-KC V973X
632
973
ASK2-KC N937X
632
937
B
HA-ASK1
WT
N937X
V973X
C1042X
R1065X
R1097X
Q1188X
L1257X
GST-ASK2-KC:
GST Pulldown
WB: α-ASK1
ASK1
WB: α-GST
ASK2
Cell Lysate
WB: α-ASK1
ASK1
C
HA-ASK1
WT
P1159X
P1159X
GST-ASK2-KC:
GST Pulldown
WB: α-ASK1
ASK1
WB: α-GST
ASK2
Cell Lysate
WB: α-ASK1
ASK1

Figure 4-6. Development of an ASK2 peptidomimetic. ***(A)*** *ASK2 C-terminal sequence.* The ASK2 sequence containing the proposed ASK1 binding site is depicted. Residues used to generate the peptides used in Figure 4-6B are indicated. ***(B)*** *Peptides representing the ASK2 sequence from R1097 to P1288 and from R1170 to P1288 bind to ASK1.* COS7 cells were transfected with the indicated GST-ASK2 peptides and HA-ASK1. 40 hrs post-transfection, cells were lysed and subjected to GST pulldown and Western blot as described in Figure 4-5.

A


1097
1130
RPHWMFVLDSLLSRAVRAALGVLGPEVEKEAVSPRSEELS
1159
1163
1170
NEGDSQQSPGQQSPLPVEPEQGPAPLMVQLSLLRAETDRL
1188
REILAGKEREYQALVQRALQRLNEEARTYVLAPEPPTALS
TDQGLVQWLQELNVDSGTIQMLLNHSFTLHTLLTYATRDD
1288
LIYTRIRGGMVCRIWRAILAQRAGSTPVTSGP

B


HA-ASK1
WT
R1097→P1288
P1130→M1163
P1159→Q1188
P1159→P1288
R1170→Q1188
R1170→P1288
Q1188→P1288
GST-ASK2:
WB:
α-ASK1
ASK1
GST
Pulldown
WB:
α-GST
ASK2
Cell
Lysate
WB:
α-ASK1
ASK1

same degree as WT, suggesting that it contained the primary interaction region. Surprisingly, an ASK2 peptide containing residues R1170 to Q1188 was not sufficient for ASK1 binding. This was completely unexpected, given our previous results showing that an ASK2 truncation ending at Q1188 was sufficient for ASK1 binding (Figure 4-5B). This led us to wonder if the amino acid residues immediately surrounding Q1188 were involved in ASK1 binding, and that perhaps the ASK2-KC Q1188X truncation offered more protein structural support than the R1170 to Q1188 peptide, allowing ASK1 to interact. Supporting this notion, a peptide representing ASK2 residues Q1188 to P1288 weakly interacted with ASK1 (Figure 4-6B). Thus, together with our truncation data, we concluded that the region within ASK2 important for ASK1 binding was restricted to the residues C-terminal to Arg 1170. We further surmised that the region immediately surrounding Gln 1188 were the critical residues necessary for mediating this interaction.

vii.) Inhibition of the ASK1/ASK2 Complex Diminishes the Presence of 14-3-3 in the ASK1 Signalosome

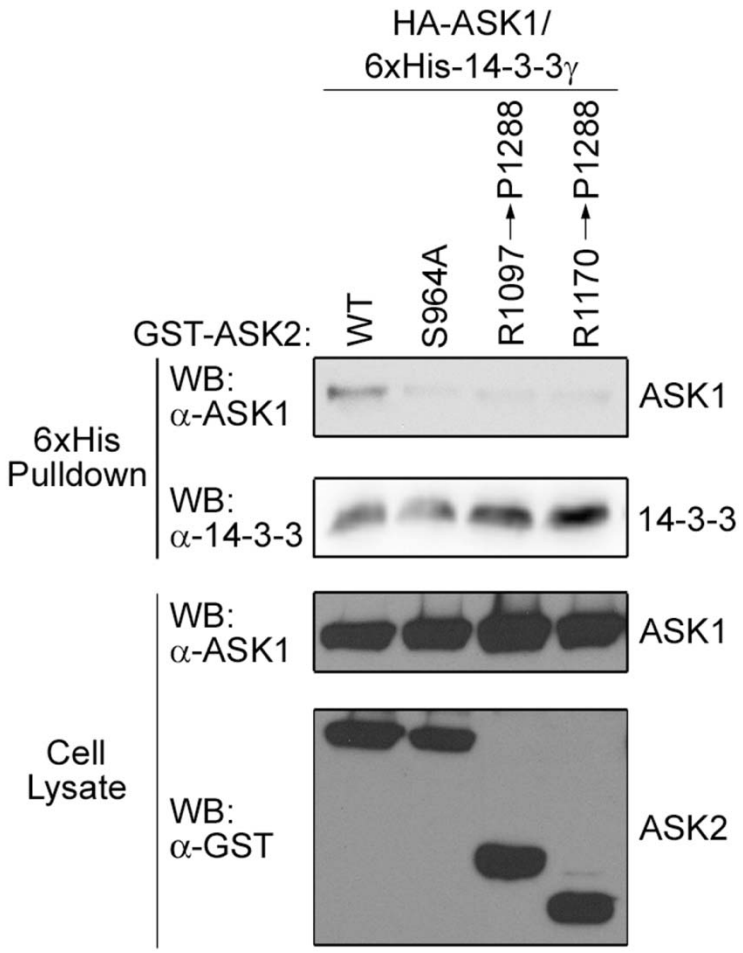
After determining the binding region of ASK1 on ASK2, we were interested in the functional effect of inhibiting this interaction. Recently, we discovered that one critical role which ASK2 exerts on the ASK1 signalosome is to determine its interaction with 14-3-3 (Chapter 3). In this model, ASK2 interaction with 14-3-3 through Ser 964 is first necessary in order to direct the presence of 14-3-3 within the ASK1 signalosome. This model predicts that ASK1 and ASK2 must also physically interact, in order for 14-3-3 to dually engage the two proteins. We tested this prediction by using the ASK2 peptidomimetics created in section II-vi, which we showed could interact with ASK1 to

the same degree as ASK2 WT (Figure 4-6B). We used the peptides GST-ASK2 R1097 to P1288 and GST-ASK2 R1170 to P1288; both of which do not contain a putative 14-3-3 recognition sequence, and therefore are insufficient to confer binding to 14-3-3. Both GST-ASK2 peptides were co-expressed with HA-ASK1 and 6xHis-14-3-3γ in COS7 cells. A 6xHis affinity pulldown assay was used to isolate 14-3-3 protein complexes, and the amount of ASK1 complexed with 14-3-3 was determined by Western blot. Indeed, both ASK2 peptidomimetics dramatically decreased the amount of ASK1 found to interact with 14-3-3 (Figure 4-7). Importantly, this decrease in binding occurred to the same level as that induced by GST-ASK2 S964A, which we used as a control to monitor the ability to dissociate the ASK1/14-3-3 complex. These results allowed us to validate our predicted model, showing that formation of an ASK1/ASK2 heteromeric complex is necessary in order for 14-3-3 to interact with ASK1. Taken together, we conclude that inhibition of the ASK1/ASK2 heteromeric complex leads to dissociation of 14-3-3 from the ASK1 signalosome.

III.) Discussion

Regulation of the ASK1 signalosome occurs through various mechanisms, which can be largely divided into either protein phosphorylation or protein-protein interaction events. ASK2, a protein which exists in a heteromeric complex with ASK1, exerts multiple layers of regulation upon ASK1. ASK2 has been shown to activate ASK1 through phosphorylation, and is required for ASK1-induced JNK activation following oxidative stress (67). Recently, we found a kinase-independent role of ASK2, in which phosphorylation at Ser 964 induces 14-3-3 binding, in turn allowing 14-3-3 to interact

Figure 4-7. Disruption of the ASK1/ASK2 complex dissociates 14-3-3. COS7 cells were transfected with 6xHis-14-3-3γ, HA-ASK1, and the indicated GST-ASK2-KC constructs (WT or S964A) or GST-ASK2 peptides. Cell lysates were used in a 6xHis affinity assay, and the resulting 14-3-3 complexes were resolved by SDS-PAGE. Proteins contained within the 14-3-3 complexes were visualized by Western blot.


HA-ASK1/
6xHis-14-3-3γ
GST-ASK2:
WT
S964A
R1097→P1288
R1170→P1288
6xHis
Pulldown
WB:
α-ASK1
ASK1
WB:
α-14-3-3
14-3-3
Cell
Lysate
WB:
α-ASK1
ASK1
WB:
α-GST
ASK2

within the ASK1 signalosome (Chapter 3). Here, we further extend this model, showing that ASK2 interaction with ASK1 is critical for 14-3-3 to have this functional effect (Figure 4-7). Additionally, through a rationally designed approach using peptidyl mimicry, we identified the residues within ASK1 and ASK2 necessary to allow formation of the heteromeric complex (Figures 4-4 through 4-6).

A wide number of protein-protein interactions are under investigation as targets for small molecule development. However, small molecule drug design is hampered by several physical limitations of protein interactions (266, 267). For example, unlike enzymatic targets, protein interaction interfaces often do not offer deep pockets within which a small molecule can bind. Additionally, many proteins interact through extensive binding regions, creating the need for large, poorly druggable molecules to disrupt the interaction. Therefore, pinpointing small, critical regions within these interfaces that determine a particular protein-protein interaction is essential for the development of viable small molecule inhibitors of that interaction. This concept was illustrated with the rational development of sheperdin, a cell-permeable peptidomimetic which blocks the interaction between survivin and heat-shock protein 90 (Hsp90) (268). Hsp90 is a molecular chaperone which facilitates the stability of survivin, a member of the inhibitor of apoptosis (IAP) protein family that is upregulated in multiple tumor types (269, 270). Sheperdin is a small, 9 amino acid long survivin peptide with a high affinity (K_D ~80 nM) for Hsp90. Its potential for development as an inhibitor of the survivin interaction with Hsp90 was illustrated both with *in vitro* binding assays and *in vivo* inhibition of animal tumor growth. Here, we sought to similarly narrow the region within ASK2 necessary for ASK1 binding.

Inhibiting the ASK1/ASK2 heteromeric complex represents an exciting new target for cancer drug discovery, in which enhancing cell death would be beneficial. We show that inhibition of the ASK1/ASK2 complex with an ASK2 peptidomimetic results in dissociation of 14-3-3 from the ASK1 signalosome. This would presumably lead to activation of downstream ASK1-induced apoptotic pathways such as those leading to JNK and p38 (15), although this remains to be verified.

The ASK2 peptidomimetic also provides validation for the targeting of 14-3-3/client protein interactions (246, 247). Although in this case the dissociation of ASK1 from 14-3-3 is an indirect result of the disruption of the ASK1/ASK2 interaction, the effect remains the same. Therefore, this creates the possibility of a multi-modal approach for targeting the ASK1 signalosome with small molecule inhibitors capable of disrupting multiple protein interactions, thereby leading to an additive or synergistic response.

This work details the fundamental biochemical characteristics involved in the formation of the ASK1/ASK2 heteromeric complex. We show that (i) ASK1 and ASK2 exist in a heteromeric complex within cells (Figure 4-1); (ii) this heteromeric complex is not formed as a result of a scaffolding role of 14-3-3 (Figure 4-2); (iii) the formation of this heteromeric complex is not dependent on phosphorylation (Figure 4-3); (iv) ASK2 binds to the C-terminal portion of ASK1 (Figure 4-4); (v) the residues C-terminal to Arg 1170 in ASK2 are necessary and sufficient for ASK1 interaction (Figures 4-5 and 4-6); and (vi) disruption of the ASK1/ASK2 heteromeric complex with an ASK2 peptidomimetic leads to dissociation of 14-3-3 from the ASK1 signalosome. Thus, this work further supports our model in which ASK2 has a direct and causal role in regulation of ASK1, namely through directing 14-3-3 association with ASK1. ASK2 represents a

novel target for drug discovery, and targeting the ASK1/ASK2 interaction may represent an exciting strategy for mediating the ASK1-induced apoptotic machinery in cells.

Chapter 5

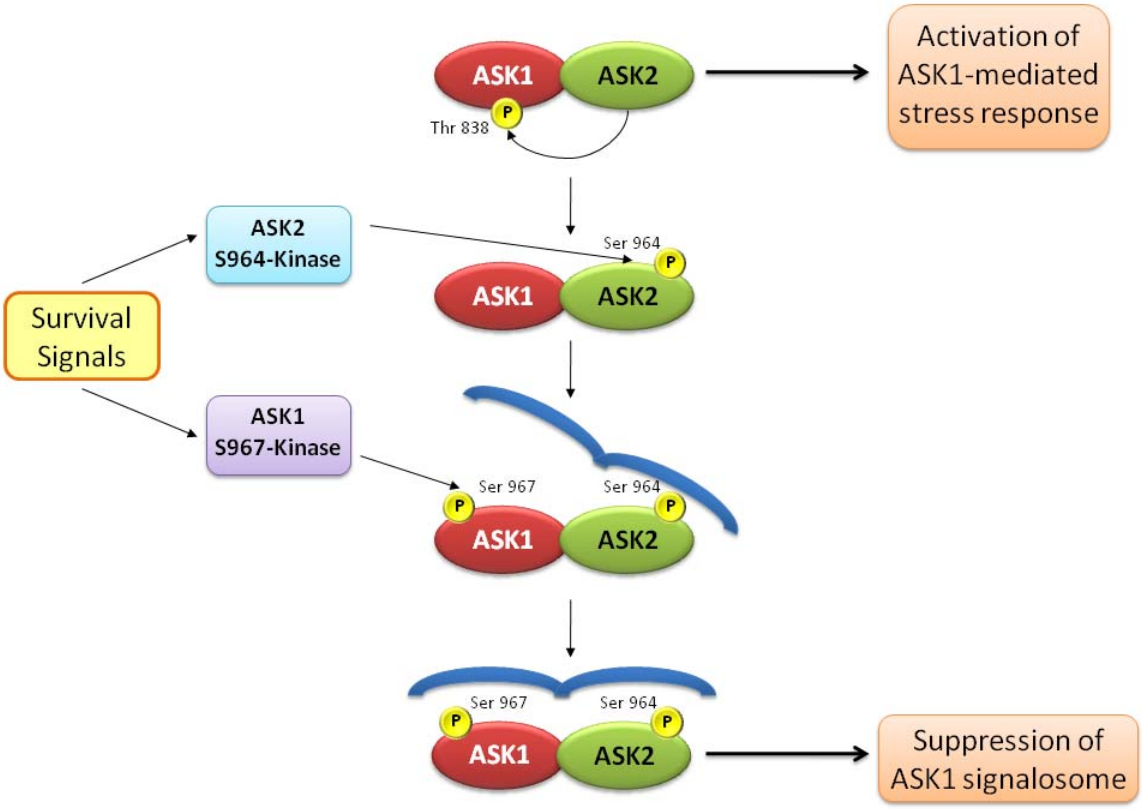
Conclusions and Future Directions

I. Conclusions

My research has established ASK2 as a key mediator within the ASK1 signalosome. I propose a model whereby ASK2 interaction with 14-3-3 determines the presence of 14-3-3 within the ASK1 signalosome (Figure 5-1). Significantly, this model predicts a novel functional effect of 14-3-3, in which this phosphoserine-binding protein integrates dual signaling cascades by simultaneously responding to ASK2 Ser 964 and ASK1 Ser 967 phosphorylation. This concomitant regulation of ASK1 and ASK2 by 14-3-3 is dependent on the heteroligomerization of the two kinases, as disruption of this interaction leads to dissociation of 14-3-3 from the ASK1 signalosome. Thus, 14-3-3 dually engages phosphorylated ASK1 and ASK2, integrating pro-survival and pro-apoptotic signaling cascades through a newly defined functional mechanism.

Before I completed this body of work, the cellular function of the ASK2 kinase was largely unknown. It was first identified only as the ASK1-interacting protein MAP3K6, whose catalytic activity did not seem to trigger activation of the ASK1 downstream targets JNK and p38 (72). Subsequently, during the course of this research, Takeda and colleagues identified ASK2 as a novel component of the ASK1 signalosome (67). In this report, ASK2 was shown to functionally interact with ASK2, with diverse results. For instance, ASK2 resulted in an increase in ASK1 phosphorylation at Thr 838, a site well characterized for its association with increased ASK1 catalytic activity (20, 61). It was also shown that ASK1 concomitantly results in an increase in ASK2 catalytic activity, through an undefined phosphorylation-independent mechanism. Finally, the ASK2 protein itself was shown to be highly labile in the setting of ASK1 knockout MEFs, suggesting that ASK1 interaction helped to stabilize the ASK2 protein. Indeed,

Figure 5-1. Proposed model of ASK2-directed 14-3-3 regulation of ASK1. ASK1 and ASK2 exist in a complex in which ASK2 promotes ASK1 phosphorylation at Thr 838. This leads to ASK1 activation, and an ASK1-mediated stress response. Survival signals, including serum, growth factors, and other pro-survival stimuli, triggers activation of a kinase responsible for ASK2 phosphorylation at Ser 964. Phosphorylation at Ser 964 results in binding between ASK2 and 14-3-3. Either concurrently or subsequently, pro-survival stimuli also result in activation of the kinase responsible for ASK1 phosphorylation at Ser 967. It is important to note that a single or multiple kinases may control the ASK1 and ASK2 phosphorylation events. ASK2-mediated recruitment of 14-3-3 to the ASK1 signalosome, coupled with phosphorylation of ASK1 at Ser 967, results in 14-3-3 suppression of the ASK1 signalosome.


ASK1
ASK2
P
Thr 838
Activation of ASK1-mediated stress response
ASK2 S964-Kinase
Survival Signals
Ser 964
P
ASK1
ASK2
ASK1 S967-Kinase
Ser 967
Ser 964
P
P
ASK1
ASK2
Ser 967
Ser 964
P
P
ASK1
ASK2
Suppression of ASK1 signalosome

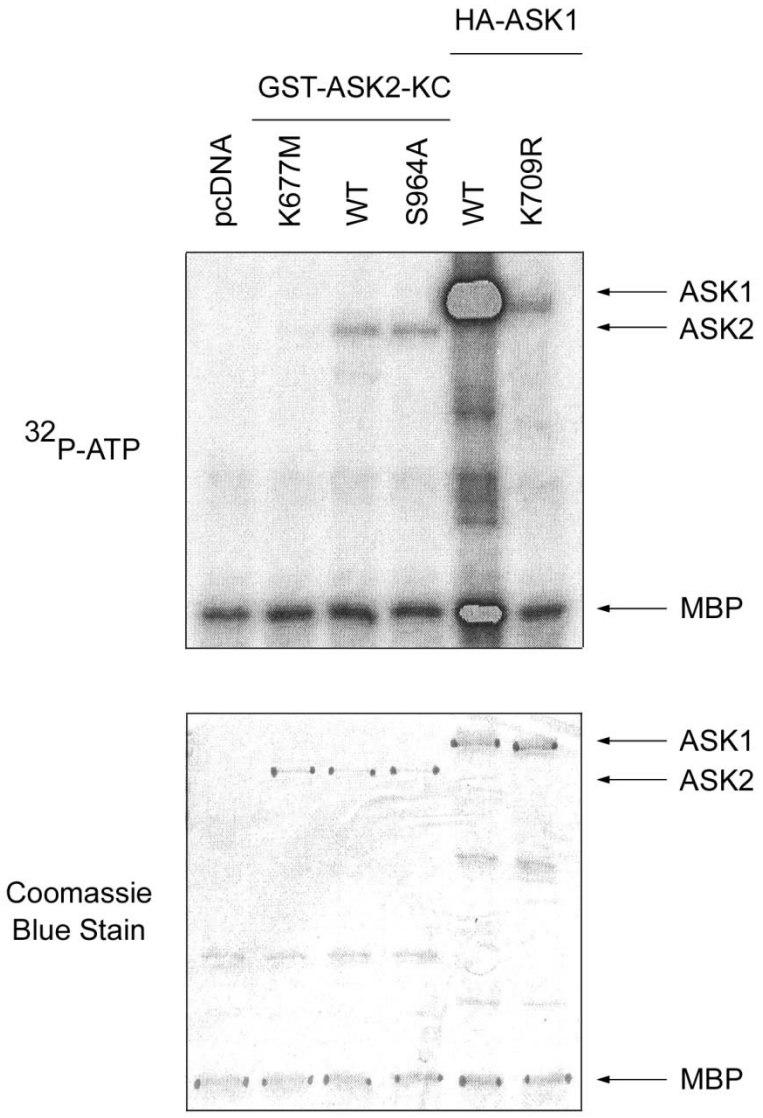
treatment of these ASK1 knockout cells with inhibitors of the proteasome returned the ASK2 protein level to that noted in ASK1 wild-type cells. These diverse functions of ASK2 were the first evidence of a functional role of ASK2 within the ASK1 signalosome. In fact, it was proposed that ASK2 may function as a functionally similar MAP3K. However, despite the high amino acid sequence similarity between the catalytic domains of the two proteins, they display substantial differences within their individual N- and C-terminal domains. Significantly, it is these domains within ASK1 that are key for mediating the numerous interacting proteins important in the tight regulation of ASK1 activity. Therefore, it is possible that ASK2 is differentially regulated, through a set of binding proteins which are both shared with ASK1 and unique to ASK2.

Our interest in ASK2 began when we noticed a putative 14-3-3 recognition motif within the C-terminus of ASK2. Significantly, this putative motif revolved around Ser 916 which was located in a homologous location, slightly C-terminal to the kinase domain, of the 14-3-3 binding site surrounding Ser 967 within ASK1. Given the importance of 14-3-3 in suppression of the kinase activity of ASK1, we first sought to determine if 14-3-3 indeed interacted with ASK2. Conclusively, we showed that ASK2 bound to all seven human 14-3-3 isoforms. We deemed this interaction to be specific, as a charge-reversal point mutation, K50E, of 14-3-3γ displayed no binding to ASK2. We went on to show that ASK2 interacted within the conserved amphipathic groove of 14-3-3, a region known to mediate the majority of 14-3-3 client protein interactions. We identified several residues within this groove, namely Lys 49, Arg 56, Arg 60, and Val 176, which were essential to maintain this interaction. This indicated that ASK2 forms extensive interactions with the 14-3-3 protein upon binding.

The presence of the putative 14-3-3 binding motif surrounding Ser 916 provided evidence that ASK2 association with 14-3-3 was induced through phosphorylation of the ASK2 protein. Indeed, *in vitro* and *in vivo* phosphatase assays confirmed that this interaction was reversibly regulated by phosphorylation and dephosphorylation. We further traced the phosphatase responsible for this regulation to a member of the PP1/PP2A family of phosphatases. Surprisingly, we found that although Ser 916 fell within a highly conserved, canonical "perfect" 14-3-3 recognition motif, this site had no role in determining ASK2 interaction with 14-3-3. We confirmed this both through a peptide competition assay and site-directed mutagenesis. We then took a more global approach to identify the ASK2 amino acid(s) responsible for mediating 14-3-3 binding, by creating truncations of the ASK2 protein. This allowed us to narrow down the binding site to a non-canonical motif surrounding Ser 964. Importantly, this motif may represent a novel, high-affinity 14-3-3 recognition motif sequence present in other 14-3-3 binding partners, as the ASK2 interaction with 14-3-3 was highly stable, even in the presence of high-salt washing conditions.

We used this newly generated 14-3-3-binding deficient mutant of ASK2 (ASK2 S964A) to further characterize the ASK2 protein. Importantly, an *in vitro* kinase assay which monitored both autophosphorylation and transphosphorylation of ASK2 showed no discernable difference in the catalytic activity of the WT or S964A protein (Figure 5-2). This suggested that unlike ASK1, 14-3-3 did not exert a suppressive effect on the kinase activity of ASK2 upon binding. This caused us to look to other functional effects of 14-3-3 binding on ASK2. Because 14-3-3 interacted with both ASK1 and ASK2, we tested and confirmed that the three proteins existed in a complex together in cells.

Figure 5-2. 14-3-3 does not suppress ASK2 kinase activity. COS7 cells were transfected with pcDNA, the GST-ASK2-KC constructs (WT, S964A, or K677M), or the HA-ASK1 constructs (WT or K709R), as indicated. Approximately 40 hrs post-transfection, cells were lysed and subjected to either GST pulldown (pcDNA or GST-ASK2 samples; described in Figure 4-5) or co-IP with an anti-HA antibody (HA-ASK1 samples; described in Figure 4-1). Following co-IP, the protein-bead complexes were washed and used in an *in vitro* kinase assay with ^{32}P-ATP. Kinase reactions were carried out at 30°C for 30 min, and terminated with the addition of 6X SDS buffer. Proteins were resolved by SDS-PAGE, stained by Coomassie staining solution, and visualized using a Typhoon imager. Myelin basic protein (MBP) was included as a control to monitor transphosphorylation. The kinase dead versions of each protein (GST-ASK2-KC K677M and HA-ASK1 K709R) were included as controls.


HA-ASK1
GST-ASK2-KC
pcDNA
K677M
WT
S964A
WT
K709R
32P-ATP
ASK1
ASK2
MBP
Coomassie
Blue Stain
ASK1
ASK2
MBP

We demonstrated that expression of the ASK2 S964A mutant protein altered the amount of ASK1 in the 14-3-3 complex, an effect which remained true when we looked at the inverse ASK1 complex. Importantly, this phenomenon was specific only for the ASK1 and ASK2 proteins, as another MAP3K, Raf-1, was unaffected by the expression of either ASK1 S967A or ASK2 S964A. This suggested a novel, dual mechanism of 14-3-3-client protein recognition, unique to ASK1 and ASK2.

We next showed the functional effect of ASK2-mediated dissociation of 14-3-3 from the ASK1 signalosome. We found that co-expression of ASK2 S964A, and subsequent dissociation of 14-3-3 from ASK1, led to a decrease in ASK1 Ser 967 phosphorylation. This was likely due to a loss of the protective effect which 14-3-3 maintains against phosphatase action on its client proteins. The decrease in ASK1 Ser 967 was then found to be associated with an increase in the activation of a downstream ASK1 target, JNK.

The important function of ASK2 on determining the interaction between ASK1 and 14-3-3 suggested that ASK2 and ASK1 interacted. We showed that these two proteins were found to bind together in cells, an interaction which was mediated neither by phosphorylation nor by 14-3-3 scaffolding. We then mapped the binding domains for this interaction to the C-terminus of each protein. A peptidomimetic generated which represented this region within ASK2 was sufficient to bind ASK1. Significantly, we used this peptidomimetic to test and confirm our model, which proposed that ASK2 interaction with ASK1 was necessary to allow 14-3-3 to associate in the ASK1 signalosome.

The findings of this research provides evidence for a novel function of the MAP3K ASK2, namely as a suppressor of ASK1 function via recruitment of 14-3-3

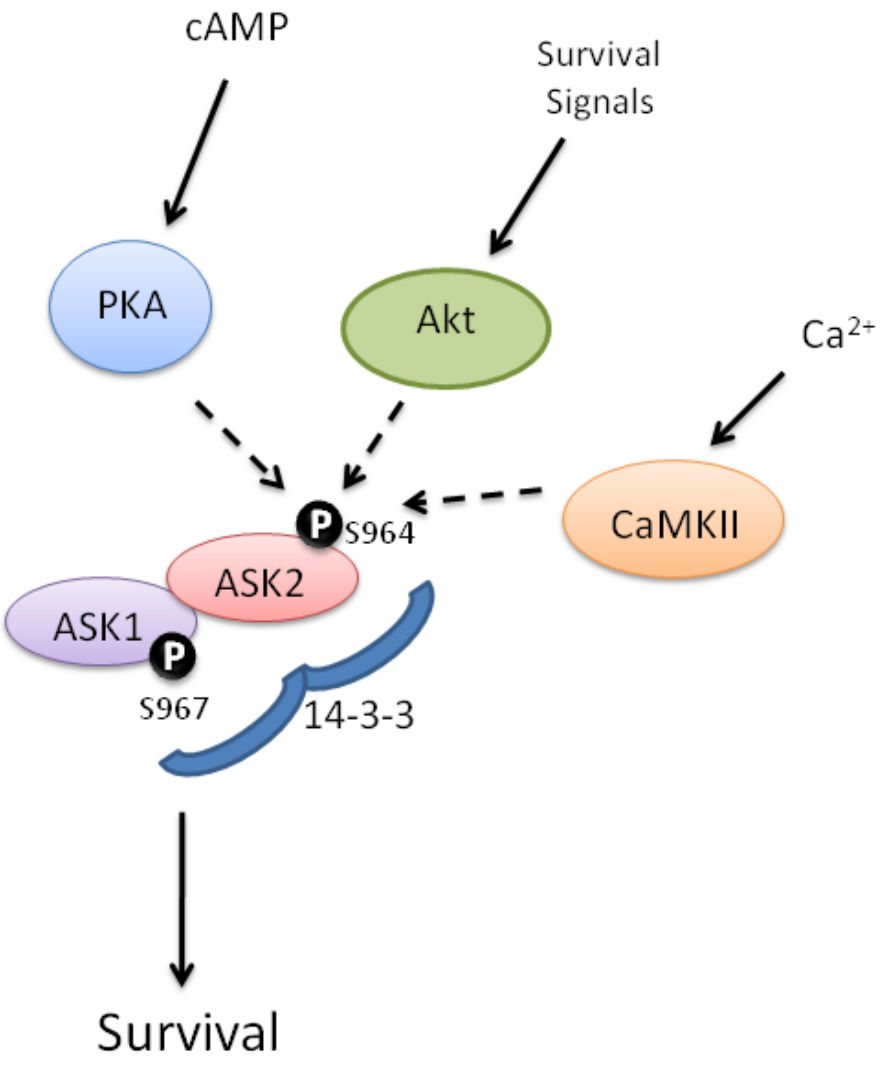
proteins. Additionally, we provide evidence of a previously unidentified consequence of 14-3-3 binding, namely as a biological "sensor" which transmits a signal relay from one signaling pathway to a second pathway, in this instance, through phosphorylation of ASK2 Ser 964.

II. Future Directions

The discovery that phosphorylation of ASK2 at Ser 964 is a key mediator for both ASK1 and ASK2 predicts a kinase responsible for this phosphorylation. The identification of this kinase is an important next step in understanding the mechanism determining the role of ASK2 in the ASK1 signalosome (Figure 5-3). A large number of 14-3-3 client proteins are substrates of two families of kinases, the AGC and the CaMK families (215). Upon inspection of the sequence surrounding ASK2 Ser 964, we found that it represented a likely recognition site for the kinases Akt, PKA, and CaMKII. This provides a useful tool to begin to explore the importance of these kinases in ASK2 Ser 964 phosphorylation. This can be primarily and most directly accomplished through the use of radioactive *in vitro* kinase assays, with recombinant, active enzymes and a purified ASK2 substrate. Indeed, we have begun this work, and generated ASK2 C-terminal substrate proteins (ASK2-C) in bacterial cells for large-scale purification. The use of a radioactive kinase assay will overcome the need for tools to specifically monitor Ser 964 phosphorylation, such as a phospho-specific antibody. Therefore, the use of an ASK2-C S964A mutant substrate will provide an important and critical control to test for the specificity of phosphorylation by the recombinant protein kinase.

In addition to these *in vitro* assays, which would test if ASK2 is a direct substrate

Figure 5-3. Possible kinases involved in ASK2 Ser 964 phosphorylation. The residues surrounding ASK2 Ser 964 represents a motif recognized by several kinases, including PKA, CaMKII, and Akt. Pharmacological and genetic manipulation of these kinases in cells may reveal their potential role in the induction of ASK2 Ser 964 phosphorylation. *In vitro* kinase assays with the purified recombinant proteins will confirm the direct phosphorylation of these kinases on ASK2.


cAMP
Survival
Signals
PKA
Akt
Ca2+
CaMKII
P
S964
ASK2
ASK1
P
S967
14-3-3
Survival

of these kinases, pharmacological and genetic manipulation of these kinase cascades in an *in vivo* cellular setting will be informative as well. For example, the PI3K-specific inhibitors LY2940092 and wortmannin can result in diminution of the Akt pathway in cells, while a CaMKII-specific inhibitor such as KN-93 can decrease the activity of this kinase. A subsequent decrease in ASK2 Ser 964 phosphorylation can then be monitored through several methods. The generation of a pSer 964-specific antibody will allow for direct monitoring of the signaling events resulting in phosphorylation at this site. Alternatively, 14-3-3 binding to ASK2 could also be used as an indirect readout of ASK2 Ser 964 phosphorylation. Conversely, activators of these kinase cascades can be used to test if ASK2 Ser 964 phosphorylation is increased. For example, growth factors such as insulin and the insulin-like growth factor would allow for an increase in Akt pathway activation, while the Ca^{2+} ionophore ionomycin would lead to an increase in CaMKII activity. Again, ASK2 Ser 964 phosphorylation can be monitored either with an antibody specific for phosphorylation at this site, or by testing for an increase in 14-3-3 interaction. Genetic tools, including kinases with activating or inactivating mutations, can be used to further delineate the exact pathways leading to phosphorylation at this site within ASK2. However, it should be noted that though the motif surrounding ASK2 Ser 964 predicts a role for one or more of these potential kinases, it is possible that other kinases are instead involved. In this case, a more general proteomics-based approach may help to gain information regarding the true ASK2 Ser 964 kinase(s).

This research suggests that ASK2 has a key role in regulation of the ASK1 signalosome. However, this may not be the only, nor the primary, function of this kinase. Significantly, ASK2 seems to be a MAP3K by homology, yet its primary downstream

targets have not been identified. Additionally, no cellular or physiological effect has been attributed to the function of ASK2, except for its modulation of ASK1 pathways. Therefore, the generation of ASK2 knockdown cell lines could provide valuable insight into novel functions of ASK2. Microarray analysis comparing WT and ASK2 knockdown cells may reveal changes in gene expression resulting from activation or inactivation of MAPK cascades downstream of ASK2. Knockdown of the *ASK2* gene in tumor cells could be useful to determine if this protein is important in mechanisms of oncogenesis, apoptosis, or drug resistance. Depending on the cell type in which the ASK2 knockdown is introduced, cellular phenotypic changes may also show an importance of ASK2 in cell growth or motility as well.

Finally, the existence of long N- and C-terminal domains within the ASK2 protein suggests that ASK2 can interact with numerous proteins itself. This predicts the existence of an "ASK2 signalosome" as well. Proteomics analyses may be used to begin to identify novel ASK2-interacting partners. Yeast two-hybrid with a cDNA library, using ASK2 as bait, may yield many new ASK2 binding proteins. Likewise, affinity chromatography with an "ASK2 column" can be used to identify ASK2 protein complexes in cells. Importantly, these ASK2 columns can be used to compare changes in the complement of ASK2 binding proteins under different cellular situations, such as phases of the cell cycle, drug treatment, serum withdrawal, and mitogen activation.

Chapter 6

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