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Mechanism of Oxidative Stress-Induced ASK1-Catalyzed MKK6 Phosphorylation

Emmanuel Sturchler, Daniel Feurstein, Patricia McDonald, and Derek Duckett*

Department of Molecular Therapeutics and Translational Research Institute, Scripps Florida, Jupiter, Florida 33458

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ABSTRACT: Apoptosis signal-regulating kinase 1 (ASK1) is a serine/threonine kinase that responds to a plethora of stress-inducing signals. In turn, activation of ASK1 is associated with a number of human pathological conditions, including neurodegenerative disease, inflammation, and heart failure. In response to oxidative stress, ASK1 activates the cell death-associated p38 MAPK pathway by phosphorylating MKK6. Here, we investigated the regulation of oxidative stress-induced ASK1-catalyzed phosphorylation of MKK6. MKK6 phosphorylation levels increased immediately after H_2O_2 treatment in intact cells and decreased following treatment for 30 min. When expressed in HEK293T cells, ASK1 was reproducibly purified within a high-molecular mass complex (~1500 kDa) known as the ASK1 signalosome. Measurement of the in vitro kinetic parameters revealed that the catalytic efficiency (k_{cat}/K_m) of ASK1 was 4000-fold greater in cells treated with H_2O_2 for 3 min than in untreated cells. Interestingly, although the $K_{m(ATP)}$ values were found to be unchanged, the $K_{m(MKK6)}$ was dramatically decreased (~1000-fold). The increased affinity was specific for MKK6 and short-lived, as the $K_{m(MKK6)}$ returned to basal levels 30 min after treatment. Consistently, endogenous MKK6 was found within the ASK1 signalosome in intact cells and in addition copurified with ASK1 following treatment for 3 min. In contrast, proteins modulating ASK1 activity and degradation were found to interact with the ASK1 signalosome once MKK6 activation was completed. Taken together, these data suggest that oxidative stress rapidly increases ASK1 catalytic efficiency for MKK6 phosphorylation by increasing MKK6 binding affinity within the ASK1 signalosome prior to induction of inactivation and degradation of the complex.

Apoptosis signal-regulating kinase 1 (ASK1,[1] also designated MAP3K5) is a ubiquitously expressed mitogen-activated protein kinase kinase kinase (MAP3K) involved in the modulation of cell survival and inflammation (*1*, *2*). ASK1 is activated in response to a wide variety of environmental and biological stressors, including reactive oxygen species (ROS), endoplasmic reticulum (ER) stress, and calcium overload, as well as by receptor-mediated signals, such as lipopolysaccharides (LPS), Fas ligand, TNFα, and certain G protein-coupled receptor (GPCR) agonists (for recent reviews, see refs (*2*−*6*)). ASK1 relays its response to such stresses through the p38 and JNK MAPK pathways by direct phosphorylation and activation of the dual specificity kinases, MKK4 (serine 257/threonine 261) and MKK6 (serine 207) (*1*, *7*, *8*). A growing body of evidence implicates ASK1 in neuronal cell death mediated by ER stress and ROS production observed in neurodegenerative diseases such as Alzheimer's disease (*9*−*12*), polyglutamine (polyQ) diseases (*13*), amyotrophic lateral sclerosis (*14*), and Parkinson's disease (*15*). In addition, angiotensin-II-induced cardiac hypertrophy and remodeling are severely inhibited in ASK1-deficient mice (*16*). Although these observations have heightened interest in ASK1 as a therapeutic target for the treatment of these pathologies, small molecule ASK1 inhibitors are not well-documented in the literature.

To understand the role of ASK1 in human disease, an improved comprehension of ASK1 regulation is essential. Recently, a conserved docking site, termed DVD, which is distinct from the phosphoacceptor site, was found to be critical for formation of a complex between ASK1 and its substrates, MAP kinase kinases (MKKs) (*17*). In resting cells, ASK1 is associated with scaffold proteins within high-molecular mass complexes (1000−2000 kDa), designated as the ASK1 signalosome (*18*, *19*). Subsequent stress, such as redox imbalance, induces ASK1 autophosphorylation (residue Thr838 located in the activation loop) and multimerization which in turn elicits the recruitment of specific factors involved in the modulation of the ASK1 signalosome activity (*19*−*22*). Activation of the ASK1 signalosome is therefore highly complex, and details concerning regulation of its activity remain to be fully elucidated.

This study was designed to evaluate the molecular mechanism of MKK6 phosphorylation within the ASK1 signalosome, derived from unstressed or H_2O_2-treated cells as a function of time. To monitor the phosphorylation of ASK1 and MKK6, mass spectrometry-based proteomic analysis, native gel electrophoresis, and Western blotting techniques were used. For steady-state kinetic analysis, the incorporation of a ^{33}P-labeled phosphate from [γ-^{33}P]ATP into recombinant MKK6 protein was monitored to determine the differences in substrate binding and maximal turnover between stimulated and nonstimulated forms of the ASK1 signalosome. Major findings of this study include the following. (1) ASK1 migrates as part of a high-molecular weight (HMW) complex not only in intact cells but also following

*To whom correspondence should be addressed. Phone: (561) 228-2224. Fax: (561) 228-3081. E-mail: ducketdr@scripps.edu.

[1]Abbreviations: ASK1, apoptosis signal-regulating kinase 1; MAP(K), mitogen-activated protein (kinase); MAP3K5, mitogen-activated protein kinase kinase kinase 5; MKK, MAP kinase kinase; ROS, reactive oxygen species; ER, endoplasmic reticulum; HMW, high-molecular weight; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

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purification and has an increased level of phosphorylation at residue Thr838 in response to H_2O_2 stimulation. (2) Determination of kinetic parameters revealed a large increase in ASK1 catalytic efficiency for MKK6 phosphorylation (k_{cat}/K_m) immediately after H_2O_2 treatment. (3) This increased efficiency was due to a 1000-fold decrease in the substrate K_m for MKK6, which was reflected in transient association of MKK6 within the ASK1 signalsome in intact cells. (4) Finally, the duration of ASK1 signaling in response to oxidative stress appears to be regulated by the recruitment of multiple proteins modulating ASK1 signalosome levels via a ubiquitin-dependent mechanism.

MATERIALS AND METHODS

Cell Lines and Reagents. Human embryonic kidney cells (HEK293T) were purchased from the American Type Culture Collection (ATCC, Rockville, MD). HEK293T cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (Invitrogen), containing 10% fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin. The myelin basic protein and all the chemicals were purchased from Sigma Aldrich. ASK1, MKK6, and phospho-specific ASK1, MKK6, and p38 antibodies were purchased from Cell Signaling Technology. Mouse monoclonal anti-FLAG (FLAG M2) antibody was purchased from Sigma Aldrich. Rabbit anti-ubiquitin antibody was purchased from Santa Cruz Biotechnology. IRDye-labeled goat anti-mouse and goat anti-rabbit antibodies were purchased from Licor.

Cloning, Expression, and Purification. The human ASK1 cDNA was purchased from Open Biosystems. The coding region of ASK1 was amplified by standard PCR techniques using the oligonucleotide primers 5′-ATG GAT TAC AAG GAT GAC GAC GAT AAG TTA GTT CCT CGT GGT TCT AGC ACG GAG GCG GAC GAG GGC ATC-3′ and 5′-TCA AGT CTG TTT GTT TCG AAA GTC AA-3′. The PCR product was purified using a cleanup kit (Qiagen) according to the manufacturer's instructions and subcloned using standard molecular biology techniques into pcDNA3.3 (Invitrogen, Carlsbad, CA) and its sequence confirmed. The oligonuclotides were designed so that ASK1 was expressed as an N-terminally FLAG-tagged fusion protein. pcDNA3.3-FLAG-ASK1^{K709M} was generated using the pcDNA3.3-FLAG-ASK1 plasmid as a template and Stratagene's QuickChange site-directed mutagenesis kit according to the manufacturer's instructions. ASK1 was expressed in HEK293T cells using a standard transfection protocol. Briefly, cells were transfected 24 h after being plated by calcium phosphate precipitation. Sixteen hours after transfection, the medium was replaced with fresh medium and the mixture cultured at 37 °C in 5% CO_2 for an additional 24 h. For ASK1 purification, cells were washed with ice-cold phosphate-buffered saline (PBS) (pH 7.0) and extracted in ice-cold M-PER lysis buffer (Thermo Scientific, Rockford, IL) containing Complete protease inhibitors (Roche Diagnostic) and phosphatase inhibitor cocktail (Sigma Aldrich, St. Louis, MO). The lysate was incubated on ice for 10 min and centrifuged at 14000*g* for 15 min at 4 °C. The protein was purified from the cleared lysate by anti-FLAG M2 affinity chromatography (Sigma Aldrich). A 1 mL bed volume of FLAG-agarose was equilibrated in buffer A [50 mM Tris-HCl and 150 mM NaCl (pH 7.4)]. The clarified lysate was diluted in buffer A to a concentration of 1 mg/mL and loaded onto the column at a rate of 0.25 mL/min. The column was washed with 20 column volumes of buffer A and eluted using buffer B [0.1 M glycine-HCl (pH 3.5)]. The eluted protein was dialyzed against buffer A and concentrated by filtration to a final concentration of 0.3 mg/mL. DTT and glycerol were added to the purified sample to final concentrations of 2 mM and 10%, respectively, and stored in aliquots at −80 °C.

The human MKK6 cDNA was obtained from Open Biosystems. The coding region of MKK6 was amplified by standard PCR techniques using the oligonucleotides 5′-CACC ATG TCC GGC CTG AAC GAC ATC TTC GAG GCT CAG AAA ATC GAATGG CAC GAA TCT CAG TCG AAA GGC AAG AAG CGA-3′ and 5′-TCA CTT ATC GTC GTC ATC CTT GTA ATC GTC TCC AAG AAT CAG TTT TAC AAA-3′. The MKK6 protein was expressed as a biotinylated N-terminally Avi-tagged protein and a C-terminally FLAG-tagged protein (MKK6-FLAG). The PCR product was cloned into the pENTR/D-topo entry vector (Invitrogen) and subcloned into the pDEST14 destination vector (Invitrogen) using gateway technology. *Escherichia coli* BL21 (AI) cells were cotransformed with the pDEST14-MKK6 construct and the BirA plasmid (GeneCopoeia). A 10 mL overnight culture was used to inoculate 500 mL of LB medium containing 50 μg/mL ampicillin and 33 μg/mL chloramphenicol. Cultures were grown at 37 °C until the OD_{600} reached ~0.5. The temperature was adjusted to 30 °C to allow optimal expression. D-Biotin (Supelco) was added at a final concentration of 50 μM, and expression of MKK6 and BirA was induced for 4 h with 0.2% L-arabinose and 0.5 mM IPTG, respectively. Bacteria were collected by centrifugation, and proteins were extracted with B-PER lysis buffer (Thermo Scientific) containing complete protease inhibitors, incubated on ice for 10 min, and clarified by centrifugation at 4 °C (14000*g* for 15 min). The protein was purified by anti-FLAG M2 affinity chromatography (Sigma Aldrich). A 1 mL bed volume of FLAG-agarose was equilibrated in buffer A [50 mM Tris-HCl and 150 mM NaCl (pH 7.4)]. The clarified lysate was diluted in buffer A to a concentration of 1 mg/mL and loaded onto the column at a rate of 0.25 mL/min. The column was washed with 20 column volumes of buffer A and eluted using buffer B [0.1 M glycine-HCl (pH 3.5)]. The eluted protein was equilibrated with wash buffer. DTT, EGTA, and glycerol were added to the protein sample to final concentrations of 2 mM, 0.1 mM, and 10%, respectively. The purity and integrity of FLAG-ASK1 and Avi-MKK6-FLAG were confirmed by SDS−PAGE (Invitrogen) and mass spectrometry. The protein concentration was measured by use of the Bradford assay (Bio-Rad).

Immunoblotting. HEK293T cells were plated in six-well plates and incubated with 2 mM H_2O_2 for the indicated time(s). After treatment, cells were washed with ice-cold PBS and lysed in 100 μL of ice-cold M-PER lysis buffer (Pierce) containing Complete protease inhibitors (Roche Diagnostic) and phosphatase inhibitor cocktail (Sigma Aldrich), incubated on ice for 10 min, and centrifuged at 14000*g* for 15 min at 4 °C. Protein concentrations of the lysates were measured using the BCA Protein Assay Reagent (Pierce). Thirty micrograms of total protein was added to Laemmli sample buffer containing 4% 2-mercaptoethanol and heated to 90 °C for 5 min. The proteins were resolved via SDS−PAGE (4−12%) and blotted onto nitrocellulose using an iBlot system (Invitrogen). For native gels, 60 μg of total protein was added to NativePAGE loading buffer (Invitrogen). The protein complexes were separated with a 4 to 16% NativePAGE Bis-Tris gel (Invitrogen) and blotted onto a PVDF membrane (Invitrogen).

Immunocomplex Kinase Activity and in Vitro Kinase Assay. To analyze FLAG-ASK1 kinase activity, 1 mg of cleared lysates was incubated with 50 μL of anti-FLAG M2 affinity gel (Sigma Aldrich) for 2 h on a nutator at 4 °C. Immunocomplexes were collected by centrifugation at 1000*g* for 1 min followed by four washes with buffer A. The immunoprecipitates were mixed with 10 μg of MKK6-FLAG suspended in kinase buffer [20 mM Tris-HCl (pH 7.4), 20 mM $MgCl_2$, 1 mM DTT, and 20 μM ATP] supplemented with phosphatase inhibitor cocktail (Sigma Aldrich). The kinase assay of purified FLAG-ASK1 was performed at 23 °C for 1 h in 15 μL of kinase buffer at the following final concentrations: 20 mM Tris-HCl (pH 7.4), 20 mM $MgCl_2$, 1 mM DTT, 20 μM ATP (Sigma Aldrich), 0.25 μM MKK6-FLAG, complemented with phosphatase inhibitor cocktail (Sigma Aldrich), and protease inhibitor (Roche). The reactions were terminated by the addition of Laemmli sample buffer, and the products were resolved by SDS−PAGE (4 to 12%) and blotted onto nitrocellulose using the iBlot system (Invitrogen). The membranes were blocked with Odyssey blocking buffer (Licor) and probed with the indicated primary antibody (1:333) for 45 min using the SNAP i.d. (Millipore) system. After being washed with Odyssey blocking buffer, the blots were incubated for 45 min with the appropriate IRDye-labeled secondary antibody (1:10000, Licor). The bands were visualized using the Odyssey Infrared System (Licor).

MALDI Analysis of Avi-MKK6-FLAG. A 5 μL aliquot of approximately 1 μg/μL Avi-MKK6-FLAG in 100 mM ammonium bicarbonate was diluted with 10 μL of 0.1% TFA. This sample was desalted using a reverse-phase C4 zip-tip as follows. The C4 zip-tip was conditioned using 2 × 10 μL of acetonitrile followed by 3 × 10 μL of 0.1% TFA. We loaded the sample onto the C4 material by pipetting the sample into and out of the zip-tip with 20 repetitions. The C4 material was washed with 5 × 10 μL of 0.1% TFA. We eluted the peptides with 10 μL of 0.1% TFA in 75% acetonitrile and 24.9% water by pipetting the eluent into and out of the zip-tip with 10 repetitions. The eluate was vacuum-dried in a speed-vac concentrator and reconstituted into 2 μL of 0.1% TFA in 75% acetonitrile and 24.9% water. MALDI matrix was prepared fresh and consisted of 10 mg/mL sinapinic acid in 0.1% TFA in 50% acetonitrile and 50% water. One microliter of matrix was added to the 1 μL of Avi-MKK6-FLAG eluate, and 1 μL of the protein/matrix solution was spotted on the MALDI target plate. The solution was allowed to dry at room temperature and was analyzed using a Bruker Microflex mass spectrometer; 100 laser shots were acquired to generate the MALDI spectra.

LC−MS/MS Analysis of Avi-MKK6-FLAG and FLAG-ASK1. In-solution digestion of purified Avi-MKK6-FLAG was performed with 20 ng of trypsin in 100 mM ammonium bicarbonate (pH 8) for approximately 8 h at 37 °C. Trypsin-generated Avi-MKK6-FLAG peptides were next treated with 15 mM DTT for 1 h and were carbamidomethylated with 40 mM iodoacetamide for 1 h at room temperature. In-gel trypsin digestion of Ask1 was performed via standard protocols. Briefly, the Coomassie-stained gel band containing FLAG-ASK1 was cut from an SDS−PAGE gel, and the gel band was washed with 20 mM ammonium bicarbonate in 50% acetonitrile. The gel band was then treated with DTT and iodoacetamide to reduce and carbamidomethylate Cys residues, and the band was dehydrated with 50% acetonitrile and then rehydrated with approximately 50 ng of trypsin in 20 mM ammonium bicarbonate. FLAG-ASK1 was digested for approximately 16 h at room temperature, and peptides were extracted by sequential dehydration and rehydration. Peptides from either in-solution or in-gel digestions were loaded onto a capillary precolumn (100 μm inside diameter) packed with C18 reverse-phase resin (5−15 μm diameter). The precolumn was placed in line with an analytical column (75 μm inside diameter) packed with 8 cm of 5 μm diameter C18 reverse-phase material. Peptides were eluted using nanoflow LC (200 nL/min) with a gradient from 0 to 55% B in solvent A, where solvent A consisted of 0.1 M acetic acid in 1% acetonitrile and 99% water and solvent B consisted of 0.1 M acetic acid in 99% acetontrile and 1% water. Gradient eluting peptides were electrosprayed via a nanoelectrospray ionization source operated at approximately 2 kV into an LTQ-Orbitrap mass spectrometer using data-dependent scanning with dynamic exclusion enabled. Full scan spectra (m/z 300−2000) were collected in the Orbitrap and were used to select the five most abundant ions per full scan for collision-activated dissociation (CAD). The resulting CAD MS/MS spectra were extracted and searched using the Sequest database searching algorithm. Output from the Sequest searches was loaded into Scaffold for statistical analysis via Peptide Prophet and Protein Prophet followed by manual verification of all peptide assignments.

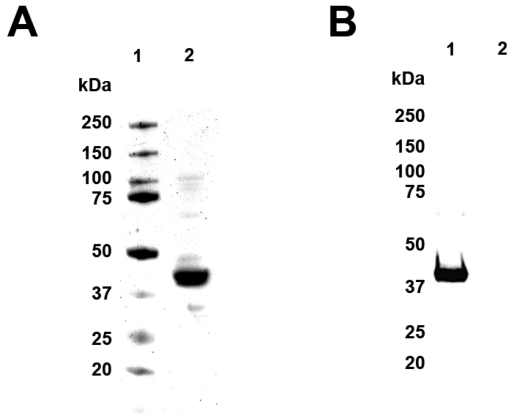

FIGURE 1: Characterization of recombinant MKK6-FLAG. Human MKK6 fused to a FLAG epitope was expressed in *E. coli* and purified using an anti-FLAG M2 affinity column. (A) The purity of MKK6-FLAG recombinant protein (lane 2) was determined with a Coomassie blue-stained SDS−PAGE gel electrophoresed beside a protein molecular weight standard. (B) Western blots of purified MKK6-FLAG protein probed with a specific anti-MKK6 antibody (lane 1). No detectable band was observed using an anti-phospho-MKK6 antibody (lane 2).

Steady-State Kinetics. Initial velocity studies used to determine the steady-state kinetic constants for ATP and MKK6-FLAG were conducted in 20 μL of kinase buffer containing the following final concentrations: 20 mM Tris-HCl (pH 7.4), 20 mM $MgCl_2$, 1 mM DTT, 1 μCi of [γ-^{33}P]ATP (3000 Ci/mmol) (Perkin-Elmer), 5−100 μM ATP (Sigma Aldrich), 0.001−10 μM MKK6-FLAG, complemented with phosphatase inhibitor cocktail (Sigma Aldrich), and Complete protease inhibitors (Roche). The reactions were initiated with 10 nM FLAG-ASK1 (final concentration), and the mixtures were incubated at room temperature for 15 min. Under these conditions, less than 10% of the substrate was converted to product and the reaction was linear. The reactions were quenched with 20 μL of 100 mM EDTA. Twenty microliters of the stopped reaction mixture was spotted in duplicate on an Immobilon-P 96-well plate (Millipore). The samples were vacuum-filtered and washed three times with 200 μL of 75 mM phosphoric acid to remove unincorporated [γ-^{33}P]ATP. After a fourth wash

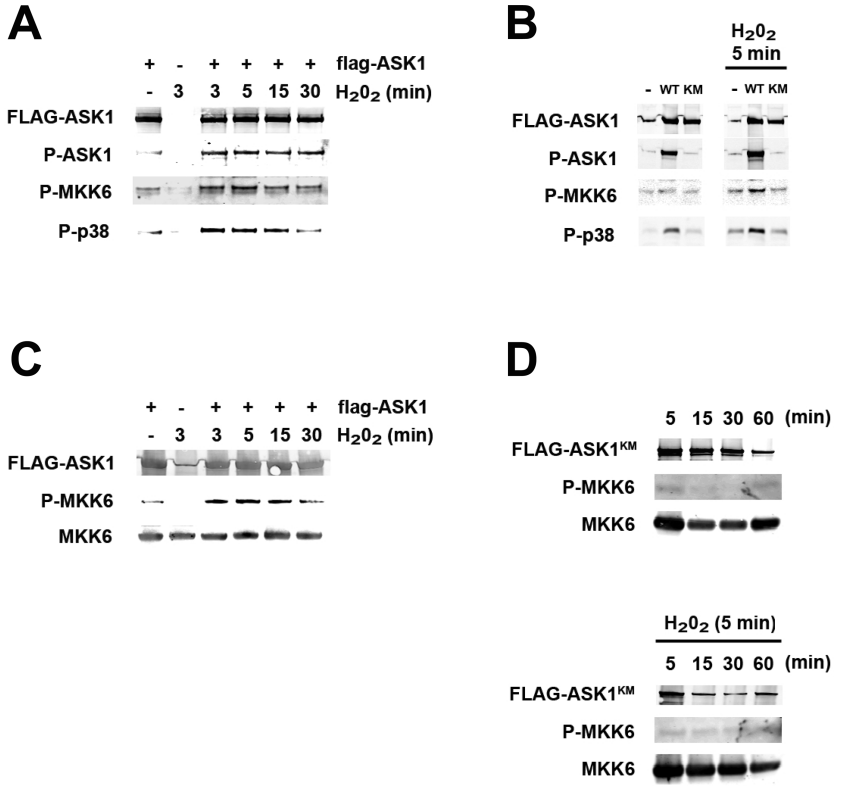

FIGURE 2: Oxidative stress-activated ASK1 phosphorylates MKK6 in HEK293T cells and in vitro. (A) HEK293T cells were transfected with the empty vector pcDNA3 or pcDNA3.3-FLAG-ASK1 and left untreated (−) or exposed (+) to 2 mM H_2O_2 for the indicated period of time. Western blot analysis of cell extracts using specific antibodies to detect total FLAG-ASK1, phosphorylated ASK1 (P-ASK1), phosphorylated MKK6 (P-MKK6), and phosphorylated p38 (P-p38) revealed that H_2O_2-induced activation of ectopically expressed ASK1 leads to MKK6 and p38 phosphorylation. (B) HEK293T cells were transfected with empty vector pcDNA3 (−), pcDNA3.3-FLAG-ASK1 (WT), or pcDNA3.3-FLAG-$ASK1^{KM}$ (KM) and left untreated (left) or exposed to 2 mM H_2O_2 for 5 min (right). Western blot analysis of cell extracts using specific antibodies to detect total FLAG-ASK1, phosphorylated ASK1 (P-ASK1), phosphorylated MKK6 (P-MKK6), and phosphorylated p38 (P-p38) revealed that oxidative stress-induced activation of MKK6 and p38 specifically requires ASK1 kinase activity. (C) HEK293T cells were transfected with the empty vector pcDNA3 or pcDNA3.3-FLAG-ASK1 and left untreated (−) or exposed (+) to 2 mM H_2O_2 for the indicated period of time. Cell lysates were subjected to immunoprecipitation with an anti-FLAG antibody, and the resulting precipitates were examined for ASK1 activity by an immunocomplex kinase assay with MKK6-FLAG as a substrate. MKK6 phosphorylation was detected by Western blotting using a specific anti-phospho-MKK6 antibody (middle). The abundance of FLAG-ASK1 in cell lysates (top) and recombinant MKK6-FLAG (bottom) was examined by Western blot analysis using a specific anti-FLAG antibody. (D) HEK293T cells were transfected with pcDNA3.3-FLAG-$ASK1^{KM}$ and left untreated (top) or exposed to 2 mM H_2O_2 for 5 min (bottom). Cell lysates were subjected to immunoprecipitation with an anti-FLAG antibody, and the resulting precipitates were examined for ASK1 activity by an immunocomplex kinase assay with MKK6-FLAG as a substrate. After incubation for 5, 15, 30, and 60 min, MKK6 phosphorylation was assessed by Western blotting using a specific anti-phospho-MKK6 antibody. The abundance of FLAG-ASK1 in cell lysates and recombinant MKK6-FLAG was examined by Western blot analysis using a specific anti-FLAG antibody.

with H_2O and a final filtration step, 100 μL of Microscint-20 (Packard) was added to each well, and samples were analyzed on a Packard Topcount liquid scintillation counter. Data are the average of three independent experiments. Kinetic data were determined by nonlinear regression analyses.

RESULTS

Expression and Purification of Human MKK6. Full-length human MKK6 fused at the C-terminus to the FLAG epitope (MKK6-FLAG) was expressed in *E. coli*. The recombinant MKK6-FLAG protein was purified by affinity chromatography, and the purity was examined by SDS gel electrophoresis. Coomassie staining and Western blot analysis showed that the purified MKK6-FLAG protein migrated as one major band at 40 kDa (Figure 1A,B), and the molecular mass of the MKK6-FLAG protein was verified by MALDI mass spectrometry (matrix-assisted laser desorption ionization time-of-flight) (Figure S1 of the Supporting Information). Importantly, the purified MKK6-FLAG protein was not phosphorylated at serine 207, as revealed by Western blotting (Figure 1B, lane 2), and mass spectrometry analysis confirmed that less than 1% of the MKK6 protein was phosphorylated (data not shown). The purified MKK6-FLAG was therefore an excellent full-length substrate for subsequent biochemical characterization of ASK1.

Activation of ASK1 in HEK293T Cells. HEK293T cells were transfected with an expression plasmid encoding FLAG-ASK1 and exposed to H_2O_2. In accord with previous reports (*19*, *20*), addition of 2 mM H_2O_2 to transfected HEK293T cells resulted in ASK1 activation as indicated by an increased level of phosphorylation at residue Thr838 (Figure 2A). Phosphorylation occurs within 3 min of exposure to H_2O_2 and remains detectable for up to 30 min. In addition, H_2O_2 treatment also induced a rapid activation of endogenous MKK6 and p38 in FLAG-ASK1-transfected HEK293T cells (Figure 2A). Interestingly, the levels of phosphorylated MKK6 and p38 reached a plateau after 3−5 min and decreased after 15 min. Importantly, oxidative stress (5 min, 2 mM H_2O_2) does not induce ASK1, MKK6, or p38 activation in cells expressing the kinase negative mutant $ASK1^{KM}$, in which Lys709 is substituted with methionine

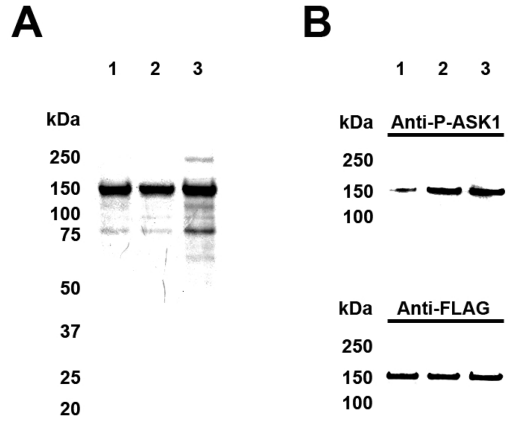

FIGURE 3: Characterization of purified FLAG-ASK1 proteins. (A) SDS−PAGE of FLAG-ASK1 purified from HEK293T cells transfected with pcDNA3.3-FLAG-ASK1 vector and left untreated (NA) or exposed to 2 mM H_2O_2 for 3 min (A3) or 30 min (A30). (B) The phosphorylation status of FLAG-ASK1 purified from HEK293T cells transfected with pcDNA3.3-FLAG-ASK1 and left untreated (NA) or exposed to 2 mM H_2O_2 for 3 min (A3) or 30 min (A30) was examined by Western blot analysis using a specific anti-phospho-ASK1 antibody (P-ASK1). Western blot analysis using a specific anti-FLAG antibody (FLAG-ASK1) was used to demonstrate equivalent loading concentrations of the samples.

(Figure 2B) (*23*). These results, in keeping with previous reports, indicate that H_2O_2 induces ASK1 phosphorylation, leading to strong transient activation of downstream kinases in the p38 MAPK signaling cascade.

We next examined the enzymatic activity of H_2O_2-activated FLAG-ASK1 in vitro by an immunocomplex kinase assay. HEK293T cells were transiently transfected with FLAG-ASK1, incubated in the presence of H_2O_2 or vehicle control, and subjected to immunoprecipitation with an anti-FLAG antibody. The resulting immunoprecipitates were assayed for ASK1 activity using MKK6-FLAG as a substrate. The level of MKK6 phosphorylation was greatly increased at the 3 and 5 min time points and decreased with further incubation with H_2O_2, returning almost to nontreated levels by 30 min (Figure 2C). Importantly, MKK6 was not phosphorylated in immunocomplex kinase assays performed with HEK293T cells transiently transfected with FLAG-ASK1^{KM} either nonstimulated or stimulated with H_2O_2 (Figure 2D, top and bottom panels, respectively). These data are in excellent agreement with the p-MKK6 levels shown in panels A and B of Figure 2 and confirm that MKK6 phosphorylation is specifically catalyzed by FLAG-ASK1.

ASK1 Purification. To investigate the effect of H_2O_2 on ASK1 activity, we isolated FLAG-ASK1 protein from vehicle-treated HEK293T cells (NA-FLAG-ASK1) or from cells treated with 2 mM H_2O_2 for 3 min (A3-FLAG-ASK1) or 30 min (A30-FLAG-ASK1). Figure 3A demonstrates that the FLAG-ASK1 protein affinity purified from control and treated cells migrated in one major band corresponding to the expected molecular mass of the protein. These bands were digested with trypsin and subjected to mass spectrometry analysis. The presence of ASK1 was confirmed in the three different treatments (data not shown). Importantly, Western blot analysis revealed an increase in the level of Thr838 phosphorylation in FLAG-ASK1 purified from H_2O_2-treated cells (Figure 3B).

Steady-State Kinetics. We next investigated the activity of the purified FLAG-ASK1 proteins toward MKK6-FLAG phosphorylation in vitro. The steady-state rate constants for the phosphorylation of MKK6-FLAG catalyzed by ASK1 purified from HEK293T cells treated with H_2O_2 for 3 min (A3-FLAG-ASK1) or 30 min (A30-FLAG-ASK1) compared to untreated cells (NA-FLAG-ASK1) are presented in Table 1. Specifically, the K_m values for ATP were found to be unchanged for ASK1 between preparations derived from untreated cells [$K_{m(ATP)} = 16$ μM] or cells treated with H_2O_2 for 3 min [$K_{m(ATP)} = 18$ μM] or 30 min [$K_{m(ATP)} = 23$ μM]. The V_{max} value for ASK1 derived from untreated cells was 0.2 nM/min (Figure 4A, top). In line with our previous findings, ASK1 derived from cells treated with H_2O_2 for 3 and 30 min had V_{max} values 5- and 3-fold greater than that of untreated cells, respectively. Consistently, the ASK1 derived from cells treated with H_2O_2 for 3 min had a V_{max} 2-fold greater than that of untreated cells when the myelin basic protein (MBP) was used as a substrate. By contrast, the K_m value for MKK6 was decreased by ~1000-fold for ASK1 derived from cells treated for 3 min with H_2O_2 compared to a 30 min treatment or untreated cells (Figure 4A, middle and bottom). Western blot analysis using a specific anti-phospho-MKK6 antibody revealed that ASK1 derived from untreated cells (Figure 4B, NA) phosphorylated MKK6 at a slower rate than ASK1 purified from H_2O_2-treated samples. These results suggest that MKK6 is bound with the highest affinity within the ASK1 complex 3 min after H_2O_2 treatment. Interestingly, the K_m value for MBP, a nonspecific kinase substrate, was not significantly modified by the H_2O_2 treatment [NA-FLAG-ASK1 $K_{m(MBP)} = 0.089$ μM; A3-FLAG-ASK1 $K_{m(MBP)} = 0.087$ μM], suggesting that the oxidative stress-induced changes in affinity are specific for MKK6 (Figure 4C).

Table 1

ASK1	$K_{m(ATP)}$ (μM)	$K_{m(MKK6)}$ (nM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} μM^{-1})
NA	17.5 ± 4.1	21.5 ± 3.4	0.018 ± 0.004	0.87 ± 0.1
A3	18.0 ± 1.8	0.021 ± 0.006	0.084 ± 0.003	4183 ± 830
A30	23.9 ± 1.7	11.5 ± 4.9	0.049 ± 0.002	5.01 ± 1.7

The formation of a high-molecular mass complex is a general feature of endogenous ASK1 (*18*). In this regard, analysis of cell extracts from FLAG-ASK1-expressing cells by native gel electrophoresis and Western blotting using an anti-FLAG antibody revealed the presence of a HMW FLAG-ASK1 complex (~1500 kDa) in stimulated and nonstimulated cells (Figure 5A). A reduction in the intensity of the complex containing FLAG-ASK1 was observed 15 min after H_2O_2 treatment, becoming significantly decreased following 30 min. Interestingly, analysis of cell extracts from FLAG-ASK1-expressing cells by native gel electrophoresis and Western blot analysis revealed the presence of endogenous MKK6 within the HMW complexes only at the earliest time point (Figure 5B). Co-immunoprecipitation experiments performed with cell extracts from FLAG-ASK1-expressing cells confirmed the presence of increased levels of MKK6 within the ASK1 signalosome, 3 min after treatment (Figure 5C). These data suggest that in intact cells MKK6 is recruited to the ASK1 signalosome as few as 3 min after treatment with H_2O_2 (Figure 5B). These data correlate well with the activation states of ASK1 downstream targets MKK6 and p38 (Figure 2) and with the changes we observed in the K_m value for MKK6 in vitro (Table 1). Together, these results indicate that purified FLAG-ASK1 complexes recapitulate ASK1 signalosome activity in intact cells and suggest that regulation of ASK1 signaling is

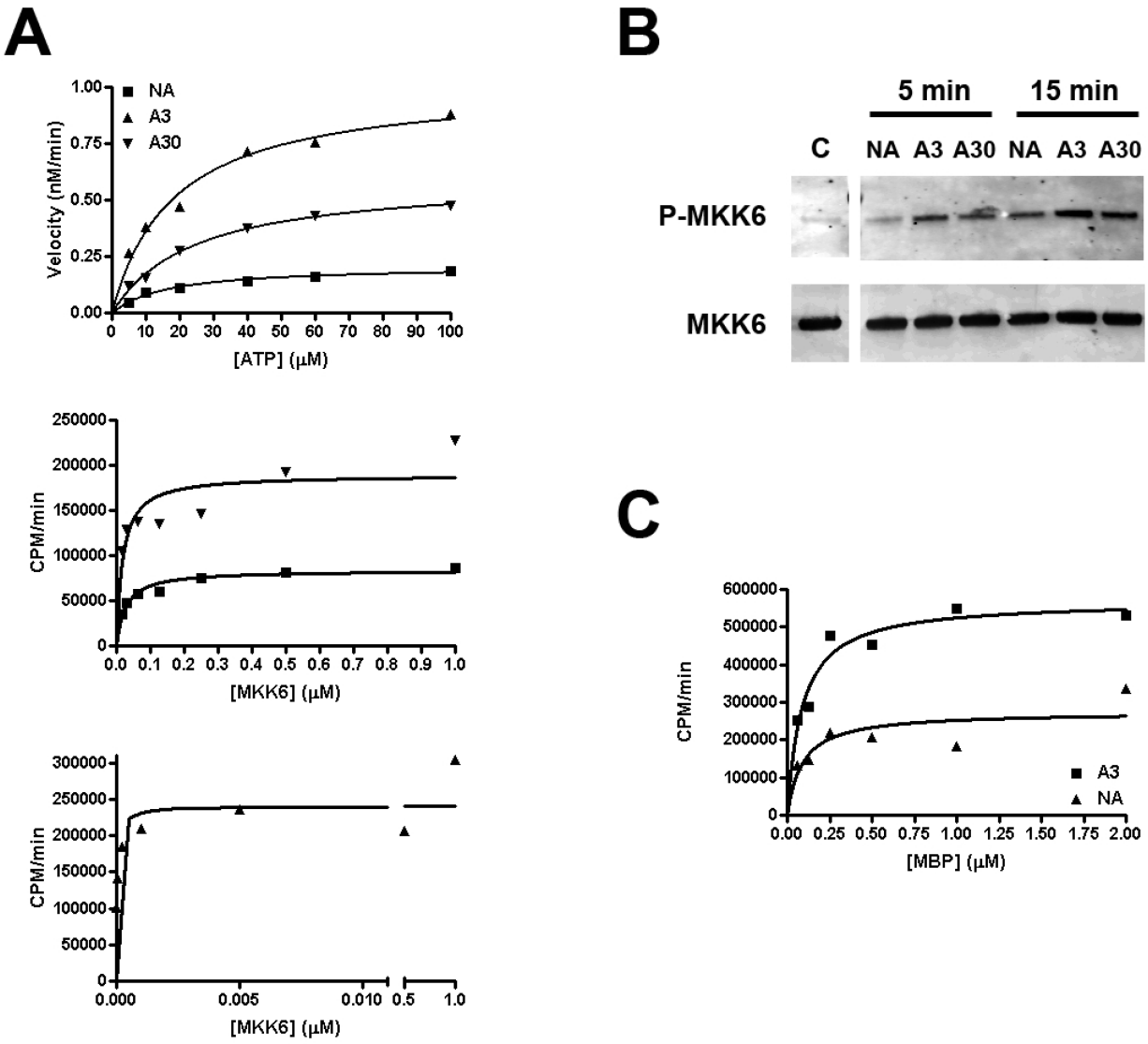

FIGURE 4: In vitro activity of the purified ASK1 proteins. (A) Dependence of initial velocity on ATP concentration (top) and substrate concentration (middle and bottom) in the FLAG-ASK1-catalyzed phosphorylation of MKK6. FLAG-ASK1 was purified from transfected HEK293T cells left untreated (NA) or exposed to 2 mM H_2O_2 for 3 min (A3) or 30 min (A30). The experiments were performed as described in Materials and Methods. (B) In vitro kinase assay, using FLAG-ASK1 purified from transfected HEK293T cells untreated (NA) or exposed to 2 mM H_2O_2 for 3 min (A3) or 30 min (A30) and MKK6-FLAG as the substrate. After incubation at room temperature for 5 and 15 min, MKK6 phosphorylation was assessed by Western blotting using a specific anti-phospho-MKK6 antibody (top). Western blot analysis using the anti-MKK6 antibody was used to demonstrate identical loading concentrations of the samples (bottom). (C) Dependence of initial velocity on substrate concentration in the FLAG-ASK1-catalyzed phosphorylation of MBP. FLAG-ASK1 was purified from transfected HEK293T cells left untreated (NA) or exposed to 2 mM H_2O_2 for 3 min (A3). The experiments were performed as described in Materials and Methods.

driven by modulation of substrate binding interactions within the ASK1 signalosome.

Next we determined whether the ASK1 HMW complex remained intact upon purification. Analysis by a native gel of FLAG-ASK1 purified from HEK293T cells treated with H_2O_2 for 0, 3, or 30 min revealed the presence of HMW FLAG-ASK1 complexes (~1500 kDa) similar to those observed in intact cells (Figure 5D), indicating that ASK1 is purified within a protein complex rather than as a monomer or dimer. This implies that the structural integrity of the ASK1 signalosome might be preserved during the purification step. Further analysis using SDS−PAGE showed four bands that vary significantly between FLAG-ASK1 purified from cells treated with H_2O_2 for 30 min and FLAG-ASK1 purified from the 3 min time point or from untreated cells (Figure 5E). Thus, these extra bands likely comprise proteins that specifically interact with the FLAG-ASK1 signalosome that has completed MKK6 activation. Individual bands were identified using tryptic peptide mass spectral fingerprinting procedures. Several proteins implicated in the modulation of protein degradation were identified, including the 26S proteasome regulatory subunit, ubiquitin-like modifier-activating enzyme 1, and ubiquitin specific protease 9 X-linked (USP9X) (Figure 5E). In addition, the level of ubiquitination of proteins that co-immunoprecipitated with ASK1 increased 30 min after treatment with H_2O_2 (Figure 5F). Furthermore, in contrast to the untreated and early time points, the ASK1 major staining band at the 30 min time point (Figure 5D) was found to contain ubiquitin, suggesting that ASK1 itself is ubiquitinated following activation. These observations are in good agreement with a recent report indicating that oxidative stress-activated ASK1 undergoes ubiquitin-dependent degradation (*24*). Finally, high levels of the heat shock proteins (Hsp70s and Hsp90) known to inhibit ASK1

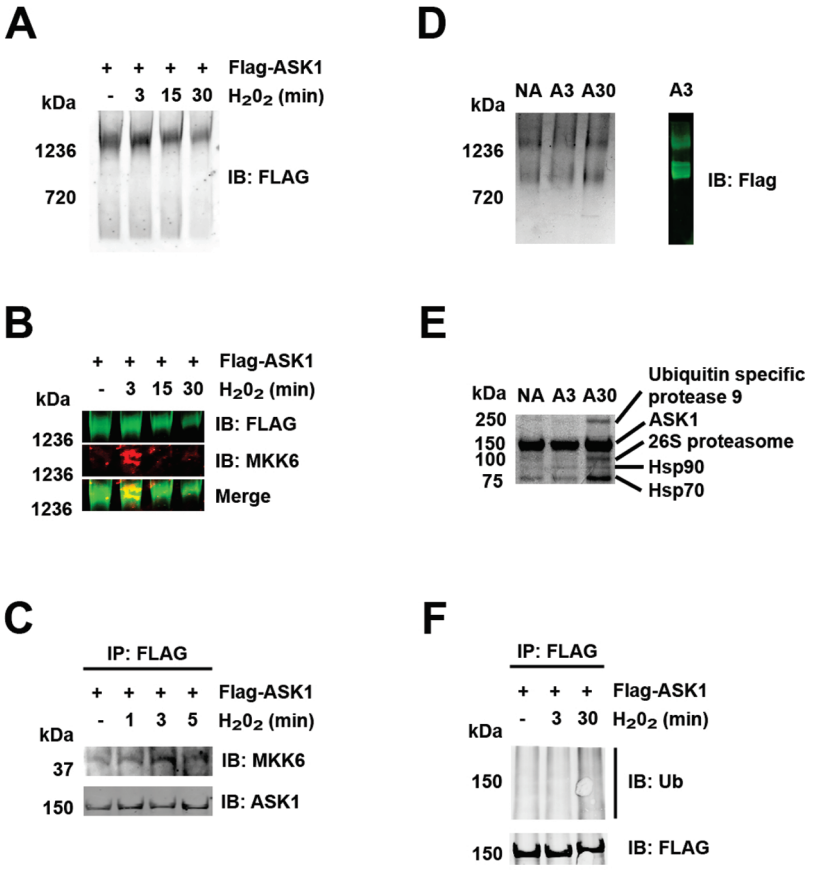

FIGURE 5: (A) HEK293T cells were transfected with pcDNA3.3-FLAG-ASK1 and left untreated (−) or exposed (+) to 2 mM H_2O_2 for the indicated period of time. Cell extracts were fractionated on a native gel and immunoblotted with an anti-FLAG antibody to detect ASK1. (B) HEK293T cells were transfected with pcDNA3.3-FLAG-ASK1 and left untreated (−) or exposed (+) to 2 mM H_2O_2 for the indicated period of time. Cell extracts were fractionated on a native gel and immunoblotted with an anti-FLAG antibody in combination with an anti-MKK6 antibody. (C) HEK293T cells were transfected with pcDNA3.3-FLAG-ASK1 and left untreated (−) or exposed (+) to 2 mM H_2O_2 for the indicated period of time and lysed. The cell extracts were immunoprecipitated with an anti-FLAG antibody followed by immunoblotting (IB) with the indicated antibody. The IB analysis using an anti-ASK1 antibody (bottom) was used to demonstrate equivalent loading concentrations of the samples. (D) Coomassie blue-stained native gel of the purified NA-A3- and A30-FLAG-ASK1. (E) Equal amounts of NA-A3- and A30-FLAG-ASK1 purified proteins were electrophoresed via SDS−PAGE and the protein bands visualized Coomassie blue staining. The identity of the proteins contained in the bands was determined by mass spectrometry. (F) HEK293T cells were transfected with pcDNA3.3-FLAG-ASK1 and left untreated (−) or exposed (+) to 2 mM H_2O_2 for the indicated period of time and lysed. The cell extracts were immunoprecipitated with an anti-FLAG antibody followed by immunoblotting (IB) with the indicated antibody. The IB analysis using anti-FLAG antibody (bottom) was used to demonstrate equivalent loading concentrations of the samples.

activity were also detected in the 30 min time point preparation (Figure 5E).

DISCUSSION

The main objective of this study was to determine the kinetic parameters of ASK1 using the full-length ASK1 protein and its natural substrate, MKK6. Although previous studies have investigated ASK1 activation using immunoprecipitation, SDS−PAGE analysis, and autoradiography, to the best of our knowledge there are no reports defining the steady-state kinetic parameters for ASK1. On the basis of recent findings indicating the importance of ASK1 in neurodegenerative, heart, and inflammatory diseases, a kinetic analysis of ASK1 activation will enhance the understanding of ASK1 in cells and facilitate the development of biochemical assays for the identification of ASK1 inhibitors.

In addition to the interaction between the ASK1 kinase catalytic ATP binding domain and the substrate phospho-acceptor site of MKK6, docking interactions are essential for the activation of MKK6 (*17*) and thus are critical for defining kinetic parameters of the kinase. Therefore, to conduct our kinetic studies, we generated full-length ASK1 and MKK6 proteins. ASK1 was expressed and purified from HEK293T cells for several reasons. First, it enables ASK1 proteins to undergo specific post-transcriptional modifications that are essential for their correct folding and function. Second, in intact cells, ASK1 constitutively forms a HMW complex (~1500 kDa), known as the ASK1 signalosome (*18*). Third, phosphorylation of Thr838 located in the activation loop of ASK1 together with intermolecular rearrangements and the presence of specific accessory proteins within the ASK1 signalosome all contribute to ASK1 activity. In this study, an increased level of phosphorylation at Thr838 in response to H_2O_2 stimulation was observed in intact HEK293T cells, as well as in the immunoprecipitated and purified ASK1 proteins (Figures 2 and 3). Furthermore, FLAG-ASK1 was found to form HMW complexes (~1500 kDa) in intact cells (Figure 5A), and complexes of the same molecular weight were also observed after FLAG-ASK1 purification (Figure 5C). Moreover, mass spectrometry analysis of purified FLAG-ASK1 preparations identified several proteins known to interact with and regulate ASK1 signaling in intact cells. Together, these data suggest that ASK1 signalosomes remain intact

after purification and provide a relevant source of enzyme for characterization.

In this study, we performed kinetic analyses of three distinct complexes containing ASK1: ASK1 derived from untreated cells or ASK1 derived from cells treated with H_2O_2 for 3 or 30 min. In accord with previous reports (*18*) and from our observations in intact cells, we found that FLAG-ASK1 purified from untreated HEK293T cells exhibited kinase activity. Stimulation of the cells with H_2O_2 for 3 or 30 min induced only a modest increase in k_{cat} (5- or 3-fold, respectively). These data are in good agreement with those of Park et al. (*25*), who demonstrated using an immunocomplex kinase assay, a 3.9-fold increase in ASK1 activity immunoprecipitated from NIH 3T3 cells treated with 2 mM H_2O_2 for 20 min. In addition, we observed that treatment with the cellular stressor H_2O_2 strongly increased ASK1 catalytic efficiency (k_{cat}/K_m). This could be achieved either by increasing the k_{cat} for kinase activity or by decreasing the K_m for a specific substrate. Our results showed a dramatic decrease in the A3-FLAG-ASK1 K_m for MKK6 of almost 1000-fold, leading to an increase in k_{cat}/K_m, reflecting a huge increase in substrate specificity. These changes follow closely the increase in the level of ASK1 phosphorylation at Thr838 and correlate with the binding of endogenous MKK6 within the ASK1 signalosome (Figure 5B), as well as with MKK6 phosphorylation (Figures 2 and 4). In contrast, no decrease in $K_{m(MBP)}$ was observed when MBP, a nonspecific kinase substrate, was used. Together, these results suggest that ASK1 signalosome activity may be regulated by the strength of MKK6 docking interactions rather than by an increase in ASK1 catalytic activity. In support of this, Bunkoczi et al. showed that although phosphorylation of Thr838 is essential for ASK1 signaling in vivo, substituting threonine for alanine, to form the nonphosphorylatable ASK1 kinase at this site (ASK1-Thr838Ala), yields the same catalytic activity that the wild-type enzyme has toward MKK6. These results are consistent with the proposal that phosphorylation at Thr838 regulates ASK1 substrate recruitment rather than ASK1 catalytic activity (*26*).

It is established that both the magnitude and duration of MAPK kinase activation are important for determining cellular fate. Thus, the modulation of formation of the ASK1−substrate complex may enable the ASK1 signalosome to be rapidly activated or inactivated toward a specific substrate in a spatial and temporal manner. In this regard, the catalytic efficiency of ASK1 changed significantly over the course of 30 min. Indeed, the k_{cat}/K_m value of FLAG-ASK1-catalyzed MKK6 phosphorylation peaked within 3−15 min of H_2O_2 treatment, returning to untreated levels 30 min post-treatment (Figure 4). These in vitro data recapitulate the phosphorylation kinetics of MKK6 that we observed in intact cells, being activated 3 min after stimulation and downregulated after 30 min. On the other hand, phosphorylation of ASK1 at Thr838 remained constant throughout the treatment period, indicating that Thr838 phosphorylation is not singly responsible for the improved activity of ASK1 toward MKK6 (Figure 2).

Recently, it was shown that recruitment of specific factors, such as heat shock proteins, as well as phosphorylation of ASK1 at Ser83 by Akt or PIM1 (*27*, *28*) suppresses ASK1 signaling. Interestingly, Hsp70 and Hsp90 were found to copurify with FLAG-ASK1 following H_2O_2 treatment for 30 min (Figure 5D), strongly suggesting that they interact with the ASK1 signalosome. Hsp proteins are thought to protect cells against cellular stress, and several reports have shown that Hsp70 and Hsp90 function as endogenous inhibitors of ASK1 (*25*, *29*, *30*). However, whether the recruitment of these proteins to the ASK1 signalosome directly modulates ASK1-MKK6 complex affinity will require further investigation. Moreover, Hsp70 is implicated in ASK1 degradation by recruiting the chaperone and ubiquitin ligase CHIP (C-terminus of Hsp70-interacting protein) (*31*). In this regard, ubiquitin, together with several proteins associated with the process of protein degradation (Figure 5D), was found only in the FLAG-ASK1 signalosome 30 min after H_2O_2 treatment. Interestingly, these observations correlate well with the decrease in the level of the ASK1 HMW complex that we observed in intact cells (Figure 5A). Thus, in agreement with previous reports (*31*, *32*), our data suggest that the duration of ASK1 signaling is regulated by mechanisms modulating the degradation of the signalosome.

In summary, this study describes the purification and characterization of ASK1 HMW complexes before and after treatment with the stressor, H_2O_2. Our results reveal for the first time that H_2O_2 treatment induces rapid ASK1 signalosome activation and MKK6 phosphorylation by increasing the affinity of ASK1 for its substrate rather than by increasing ASK1 catalytic activity. The increased catalytic efficiency was transient, whereas prolonged H_2O_2 treatment led to a decrease in the amount of ASK1 signalosome. Importantly, the results of the in vitro kinetic analysis using purified full-length ASK1 and its acceptor substrate MKK6 correlated exceptionally well with our cell-based observations. The kinetic parameters described herein provide essential information for the development of ATP and substrate competitive inhibitors of the ASK1 signalosome and highlight the importance of using purified full-length ASK1 and MKK6 within the correct scaffolding environment for the development of biochemical assays to identify ASK1 inhibitors.

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SUPPORTING INFORMATION AVAILABLE

Verification of the correct synthesis of FLAG-MKK6 using MALDI-TOF mass spectrometry. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Ichijo, H., Nishida, E., Irie, K., ten Dijke, P., Saitoh, M., Moriguchi, T., Takagi, M., Matsumoto, K., Miyazono, K., and Gotoh, Y. (1997) Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science 275*, 90–94.
2. Matsukawa, J., Matsuzawa, A., Takeda, K., and Ichijo, H. (2004) The ASK1-MAP kinase cascades in mammalian stress response. *J. Biochem. 136*, 261–265.
3. Homma, K., Katagiri, K., Nishitoh, H., and Ichijo, H. (2009) Targeting ASK1 in ER stress-related neurodegenerative diseases. *Expert Opin. Ther. Targets 13*, 653–664.
4. Matsuzawa, A., and Ichijo, H. (2008) Redox control of cell fate by MAP kinase: Physiological roles of ASK1-MAP kinase pathway in stress signaling. *Biochim. Biophys. Acta 1780*, 1325–1336.
5. Sekine, Y., Takeda, K., and Ichijo, H. (2006) The ASK1-MAP kinase signaling in ER stress and neurodegenerative diseases. *Curr. Mol. Med. 6*, 87–97.
6. Takeda, K., Matsuzawa, A., Nishitoh, H., and Ichijo, H. (2003) Roles of MAPKKK ASK1 in stress-induced cell death. *Cell Struct. Funct. 28*, 23–29.
7. Chen, Z., Seimiya, H., Naito, M., Mashima, T., Kizaki, A., Dan, S., Imaizumi, M., Ichijo, H., Miyazono, K., and Tsuruo, T. (1999) ASK1

mediates apoptotic cell death induced by genotoxic stress. *Oncogene 18*, 173–180.
8. McDonald, P. H., Chow, C. W., Miller, W. E., Laporte, S. A., Field, M. E., Lin, F. T., Davis, R. J., and Lefkowitz, R. J. (2000) β-Arrestin 2: A receptor-regulated MAPK scaffold for the activation of JNK3. *Science 290*, 1574–1577.
9. Galvan, V., Banwait, S., Spilman, P., Gorostiza, O. F., Peel, A., Ataie, M., Crippen, D., Huang, W., Sidhu, G., Ichijo, H., and Bredesen, D. E. (2007) Interaction of ASK1 and the β-amyloid precursor protein in a stress-signaling complex. *Neurobiol. Dis. 28*, 65–75.
10. Hashimoto, Y., Niikura, T., Chiba, T., Tsukamoto, E., Kadowaki, H., Nishitoh, H., Yamagishi, Y., Ishizaka, M., Yamada, M., Nawa, M., Terashita, K., Aiso, S., Ichijo, H., and Nishimoto, I. (2003) The cytoplasmic domain of Alzheimer's amyloid-β protein precursor causes sustained apoptosis signal-regulating kinase 1/c-Jun NH2-terminal kinase-mediated neurotoxic signal via dimerization. *J. Pharmacol. Exp. Ther. 306*, 889–902.
11. Kadowaki, H., Nishitoh, H., Urano, F., Sadamitsu, C., Matsuzawa, A., Takeda, K., Masutani, H., Yodoi, J., Urano, Y., Nagano, T., and Ichijo, H. (2005) Amyloid β induces neuronal cell death through ROS-mediated ASK1 activation. *Cell Death Differ. 12*, 19–24.
12. Song, S., Kim, S. Y., Hong, Y. M., Jo, D. G., Lee, J. Y., Shim, S. M., Chung, C. W., Seo, S. J., Yoo, Y. J., Koh, J. Y., Lee, M. C., Yates, A. J., Ichijo, H., and Jung, Y. K. (2003) Essential role of E2-25K/Hip-2 in mediating amyloid-β neurotoxicity. *Mol. Cell 12*, 553–563.
13. Nishitoh, H., Matsuzawa, A., Tobiume, K., Saegusa, K., Takeda, K., Inoue, K., Hori, S., Kakizuka, A., and Ichijo, H. (2002) ASK1 is essential for endoplasmic reticulum stress-induced neuronal cell death triggered by expanded polyglutamine repeats. *Genes Dev. 16*, 1345–1355.
14. Nishitoh, H., Kadowaki, H., Nagai, A., Maruyama, T., Yokota, T., Fukutomi, H., Noguchi, T., Matsuzawa, A., Takeda, K., and Ichijo, H. (2008) ALS-linked mutant SOD1 induces ER stress- and ASK1-dependent motor neuron death by targeting Derlin-1. *Genes Dev. 22*, 1451–1464.
15. Karunakaran, S., Diwakar, L., Saeed, U., Agarwal, V., Ramakrishnan, S., Iyengar, S., and Ravindranath, V. (2007) Activation of apoptosis signal regulating kinase 1 (ASK1) and translocation of death-associated protein, Daxx, in substantia nigra pars compacta in a mouse model of Parkinson's disease: Protection by α-lipoic acid. *FASEB J. 21*, 2226–2236.
16. Izumiya, Y., Kim, S., Izumi, Y., Yoshida, K., Yoshiyama, M., Matsuzawa, A., Ichijo, H., and Iwao, H. (2003) Apoptosis signal-regulating kinase 1 plays a pivotal role in angiotensin II-induced cardiac hypertrophy and remodeling. *Circ. Res. 93*, 874–883.
17. Takekawa, M., Tatebayashi, K., and Saito, H. (2005) Conserved docking site is essential for activation of mammalian MAP kinase kinases by specific MAP kinase kinase kinases. *Mol. Cell 18*, 295–306.
18. Noguchi, T., Takeda, K., Matsuzawa, A., Saegusa, K., Nakano, H., Gohda, J., Inoue, J., and Ichijo, H. (2005) Recruitment of tumor necrosis factor receptor-associated factor family proteins to apoptosis signal-regulating kinase 1 signalosome is essential for oxidative stress-induced cell death. *J. Biol. Chem. 280*, 37033–37040.
19. Tobiume, K., Saitoh, M., and Ichijo, H. (2002) Activation of apoptosis signal-regulating kinase 1 by the stress-induced activating phosphorylation of pre-formed oligomer. *J. Cell. Physiol. 191*, 95–104.
20. Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazono, K., and Ichijo, H. (1998) Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *EMBO J. 17*, 2596–2606.
21. Zhang, R., Al-Lamki, R., Bai, L., Streb, J. W., Miano, J. M., Bradley, J., and Min, W. (2004) Thioredoxin-2 inhibits mitochondria-located ASK1-mediated apoptosis in a JNK-independent manner. *Circ. Res. 94*, 1483–1491.
22. Nadeau, P. J., Charette, S. J., Toledano, M. B., and Landry, J. (2007) Disulfide Bond-mediated multimerization of Ask1 and its reduction by thioredoxin-1 regulate H_2O_2-induced c-Jun NH_2-terminal kinase activation and apoptosis. *Mol. Biol. Cell 18*, 3903–3913.
23. Kanamoto, T., Mota, M., Takeda, K., Rubin, L. L., Miyazono, K., Ichijo, H., and Bazenet, C. E. (2000) Role of apoptosis signal-regulating kinase in regulation of the c-Jun N-terminal kinase pathway and apoptosis in sympathetic neurons. *Mol. Cell. Biol. 20*, 196–204.
24. Nagai, H., Noguchi, T., Homma, K., Katagiri, K., Takeda, K., Matsuzawa, A., and Ichijo, H. (2009) Ubiquitin-like sequence in ASK1 plays critical roles in the recognition and stabilization by USP9X and oxidative stress-induced cell death. *Mol. Cell 36*, 805–818.
25. Park, H. S., Cho, S. G., Kim, C. K., Hwang, H. S., Noh, K. T., Kim, M. S., Huh, S. H., Kim, M. J., Ryoo, K., Kim, E. K., Kang, W. J., Lee, J. S., Seo, J. S., Ko, Y. G., Kim, S., and Choi, E. J. (2002) Heat shock protein hsp72 is a negative regulator of apoptosis signal-regulating kinase 1. *Mol. Cell. Biol. 22*, 7721–7730.
26. Bunkoczi, G., Salah, E., Filippakopoulos, P., Fedorov, O., Muller, S., Sobott, F., Parker, S. A., Zhang, H., Min, W., Turk, B. E., and Knapp, S. (2007) Structural and functional characterization of the human protein kinase ASK1. *Structure 15*, 1215–1226.
27. Gu, J. J., Wang, Z., Reeves, R., and Magnuson, N. S. (2009) PIM1 phosphorylates and negatively regulates ASK1-mediated apoptosis. *Oncogene 28*, 4261–4271.
28. Kim, A. H., Khursigara, G., Sun, X., Franke, T. F., and Chao, M. V. (2001) Akt phosphorylates and negatively regulates apoptosis signal-regulating kinase 1. *Mol. Cell. Biol. 21*, 893–901.
29. Yang, M., Hu, S., Wu, B., Miao, Y., Pan, H., and Zhu, S. (2007) Ghrelin inhibits apoptosis signal-regulating kinase 1 activity via upregulating heat-shock protein 70. *Biochem. Biophys. Res. Commun. 359*, 373–378.
30. Zhang, R., Luo, D., Miao, R., Bai, L., Ge, Q., Sessa, W. C., and Min, W. (2005) Hsp90-Akt phosphorylates ASK1 and inhibits ASK1-mediated apoptosis. *Oncogene 24*, 3954–3963.
31. Hwang, J. R., Zhang, C., and Patterson, C. (2005) C-Terminus of heat shock protein 70-interacting protein facilitates degradation of apoptosis signal-regulating kinase 1 and inhibits apoptosis signal-regulating kinase 1-dependent apoptosis. *Cell Stress Chaperones 10*, 147–156.
32. Zhang, Z., Hao, J., Zhao, Z., Ben, P., Fang, F., Shi, L., Gao, Y., Liu, J., Wen, C., Luo, L., and Yin, Z. (2009) β-Arrestins facilitate ubiquitin-dependent degradation of apoptosis signal-regulating kinase 1 (ASK1) and attenuate H_2O_2-induced apoptosis. *Cell. Signalling 21*, 1195–1206.