Assembly Dynamics and Stoichiometry of the Apoptosis Signal-regulating Kinase (ASK) Signalosome in Response to Electrophile Stress

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Apoptosis signal-regulating kinase 1 (ASK1) is a key sensor kinase in the mitogen-activated protein kinase pathway that transduces cellular responses to oxidants and electrophiles. ASK1 is regulated by a large, dynamic multiprotein signalosome complex, potentially including over 90 reported ASK1-interacting proteins. We employed both shotgun and targeted mass spectrometry assays to catalogue the ASK1 protein-protein interactions in HEK-293 cells treated with the prototypical lipid electrophile 4-hydroxy-2-nonenal (HNE). Using both epitope-tagged overexpression and endogenous expression cell systems, we verified most of the previously reported ASK1 protein-protein interactions and identified 14 proteins that exhibited dynamic shifts in association with ASK1 in response to HNE stress. We used precise stable isotope dilution assays to quantitate protein stoichiometry in the ASK signalosome complex and identified ASK2 at a 1:1 stoichiometric ratio with ASK1 and 14–3–3 proteins (YWHAO, YWHAH, and YWHAE) collectively at a 0.5:1 ratio with ASK1 as the main components. Several other proteins, including ASK3, PARK7, PRDX1, and USP9X were detected with stoichiometries of 0.1:1 or less. These data support an ASK signalosome comprising a multimeric core complex of ASK1, ASK2, and 14–3–3 proteins, which dynamically engages other binding partners needed to mediate diverse stress-response signaling events. This study further demonstrates the value of combining global and targeted MS approaches to interrogate multiprotein complex composition and dynamics. Molecular & Cellular Proteomics 15: 10.1074/mcp.M115.057364, 1947–1961, 2016.

Apoptosis signal-regulating kinase 1 (ASK1, MAP3K5) is a mitogen activated protein kinase (MAPK) kinase (MAP3K) that acts as a critical sensor of cell stress (1). ASK1 is responsive to many stimuli, including oxidative stress (2), electrophile stress (3), endoplasmic reticulum stress (4), calcium stress (5), inflammatory signals (6), and many other endogenous and exogenous chemical signals (7–18). Recognition of these stresses by ASK1 results in activation of the p38 and JNK MAPK pathways (1) and subsequent apoptosis. A large multiprotein complex termed the ASK signalosome (19), has been reported to regulate ASK1 where proteins dynamically assemble around ASK1 as either an inactive or active signaling complex [recently reviewed in (20)].

The exact composition of this complex has not yet been determined, but over 90 proteins have been identified as ASK1-interacting proteins (http://thebiogrid.org/110381/table/homo_sapiens/map3k5.html). These interacting proteins have most commonly been identified using co-immunoprecipitation (co-IP) and Western blot methods in ASK1-overexpressing cells. Thus, although the ASK1 system is currently best understood as a series of binary interactions, there is evidence that ASK1 exists in a high molecular mass “pre-activation” complex of ~1500 kDa that undergoes protein compositional or stoichiometric remodeling to form an even higher mass “postactivation” complex upon treatment with H2O2 (21). The majority of the reported ASK1 protein-protein interactions have been investigated in the context of H2O2-mediated...
ASK Signalosome Dynamics

Activated activation of the system, in which H2O2 is thought to reversibly oxidize thioredoxin, thereby allowing the inhibitory effect of thioredoxin on ASK1 and thereby allowing a shift in ASK1-binding partners (2, 19). Among reported ASK1-interacting proteins is the homologous kinase ASK2 (MAP3K6) (22). Another homolog, ASK3 (MAP3K15), has also been identified (23); however, the roles of ASK2 and ASK3 in MAPK signaling remain largely unexplored.

We chose to investigate the ASK1 system in the context of exposure to the lipid electrophile 4-hydroxy-2-nonenal (HNE), which is a physiologically relevant stressor generated endogenously under conditions of oxidative stress (24, 25) that we and others (3) have shown to be capable of activating the ASK1 pathway. Unlike the reversible oxidation caused by H2O2, electrophile stress is characterized by covalent (nonreversible) addition of cellular nucleophiles, particularly protein Cys residues (24–27). Because of this mechanistic difference between H2O2-induced oxidative stress and HNE-induced electrophile stress, we reasoned that the mechanisms of ASK1 regulation may vary between these two stressors. Thus, studies of HNE-induced electrophile stress may reveal new insights into how the ASK system is able to respond to diverse stress signals.

To better define the molecular composition of the ASK system and its regulation in response to HNE, we used quantitative mass spectrometry to gain an “all components” view of ASK1-interacting proteins. We hypothesized that a subset of the previously reported ASK1-interacting proteins would constitute the ASK signalosome in unstressed cells and that the composition and stoichiometry of the complex would change in response to HNE stress. Here we report a systematic investigation of ASK signalosome components and the response of this signalosome to HNE treatment. We employed an affinity purification–mass spectrometry (AP-MS) approach with data-dependent shotgun liquid chromatography–tandem mass spectrometry (LC-MS/MS) and parallel reaction monitoring (PRM) methods to study four related cell models with both over-expression and endogenous expression of ASK1, ASK2, and ASK3 proteins.

EXPERIMENTAL PROCEDURES

DNA Constructs—Tandem-tagged constructs for ASK1, ASK2, and ASK3 were generated in pcDNA3.1 plasmids (V790–20, Life Technologies, Grand Island, NY) from Genscript (Piscataway, NJ). The ASK1 sequence previously described (1) was modified by substituting the HA tag with a tandem HA-FLAG tag. Partial clones of ASK2 containing the catalytic portion of each protein (plasmid # 23853) and ASK3 (plasmid # 23499) were obtained from Addgene (Cambridge, MA) and were originally created by William Hahn and David Root (28). The missing gene fragments were synthesized by Genscript and a tandem tag sequence was added to each gene (HA-V5 for ASK2 and HA-My c for ASK3). All three plasmids are available from Addgene (ASK1 - #69726, ASK2 - #69727, ASK3 - #69728).

Antibodies—

**For Co-immunoprecipitation**—Co-IPs of epitope-tagged proteins were performed with either EView red anti-HA beads (Sigma-Aldrich, St. Louis, MO, E6779), EView red anti-FLAG beads (Sigma-Aldrich, F2426), EView red anti-Myc beads (Sigma-Aldrich, E6654), or Anti-V5 agarose affinity gel (Sigma-Aldrich, A7345). The endogenous ASK1 IPs were performed using sc-5294 AC (Santa Cruz Biotechnology, Dallas, TX).

**For Immunocytochemistry (ICC)**—ICC staining was performed with the following primary antibodies at 1:500 dilutions: Beta Tubulin (ab7792), V5 (ab9116), and Myc (ab9132) purchased from Abcam (Cambridge, MA); ASK1 (sc-5294) purchased from Santa Cruz Biotechnology; HA (71–5500) purchased from Life Technologies (Carlsbad, CA); and FLAG (8146S) purchased from Cell Signaling Technology (Danvers, MA). Secondary staining for ICC was done with the following antibodies from Life Technologies at 1:500 dilutions: anti-rabbit 633 (A21071), anti-goat 546 (A11056), and anti-mouse 488 (A21202).

**For Western Blot**—Western blots were performed with the following primary antibodies: FLAG (8146S), ASK1-phospho-T845 (3765S), JNK (9252S), phospho-JNK (4668S), p38 (9212S), and phospho-p38 (9211S) purchased from Cell Signaling Technology; ASK1 (sc-5294) purchased from Santa Cruz Biotechnology; ASK2 (ab99426) and ASK3 (ab76806) purchased from Abcam; and ATAD3A (H00055210-D01P) and ATAD3B (H00083858-B01P) purchased from Novus Biologicals (Littleton, CO). Secondary antibodies were from Life Technologies: anti-mouse 680 (A21058), anti-rabbit 680 (A21109), and anti-goat 680 (A21084).

**Cell Lines and Cell Culture**—

**Stable Transfection**—HEK-293 cells (CRL-1573, ATCC) were grown in DMEM (Life Technology, 12430) supplemented with 10% fetal bovine serum (Atlas Biologicals, Fort Collins, CO) at 37 °C in a humidified atmosphere with 5% CO2. ASK1, 2, and 3 expressing cell lines were generated from HEK-293 cells by transfecting the corresponding expression plasmids into cells using Lipofectamine 2000 (Life Technologies, 11668019) following the manufacturer’s instructions. Twenty-four hours following transfection, cells were split 1:10 and after an additional 24 h were switched into selection medium consisting of the HEK-293 medium described above supplemented with 1.5 mg/ml Geneticin (Life Technologies, 10131–027) and maintained in this medium for 2 weeks to select for cells expressing the plasmid of interest. Stock cell cultures were maintained thereafter in HEK-293 media supplemented with 1 mg/ml Geneticin. Prior to harvesting, cells were plated in 15 cm dishes in HEK-293 media supplemented with 0.5 mg/ml Geneticin.

**HNE Stimulation**—All cell lines were treated prior to harvesting with ethanol (vehicle control, 0.08% v/v), 10 μM HNE, or 50 μM HNE. The three treatments were prepared in serum-free media and placed on the cells for 1 h at 37 °C. Cells were harvested with a cell scraper in the treatment medium and immediately centrifuged at 100 × g for 5 min at 4 °C. Following this, the cell pellets were washed twice with ice cold phosphate buffered saline (PBS) and stored at −80 °C until use.

For the ASK protein colocalization imaging studies, ASK1, ASK2, and ASK3 constructs were transiently cotransfected into HEK-293 cells using an Am MAXA NucleoFector 2b (Lonza, Allendale, NJ) and the cell line NucleoFector Kit V (Lonza, VCA-1003) using the manufacturer’s recommended HEK-293 electroporation protocol.

**RNA Interference**—HEK-293 cells were plated in 10 cm dishes and transfected at 30% confluence using Lipofectamine RNAiMax (Life Technologies, 13778–150) following the manufacturer’s suggested protocol. Forty-eight hours after transfection, cells were split into two new 10-cm dishes. Twenty-four hours later, cells were harvested, washed twice in PBS, and frozen at −80 °C until use. Anti-ASK1 Stealth siRNA duplexes with the following sequences: 5’-AAACAUU-
UCAGUAUGAUGGUUGUGG-3' and 5'-CCAAGGGCAUUUCACAAAUGUUU-3' (Life Technologies) specific for ASK1 (with no overlapping targeting on ASK2 or ASK3) were used at a concentration of 10 nm. Negative control siRNA (Life Technologies, 12935–300) designed for use with medium GC content Stealth siRNA was used at the same concentration.

**Immunocytochemistry**—Cells were plated in a 6-well plate at a density of 100,000 cells per well with five 12 mm glass coverslips in each well (Carolina Biological Supply, Burlington, NC, 633029). Three days later, the coverslips were washed once in PBS, fixed for 10 min at room temperature (RT) in 4% paraformaldehyde, blocked for 20 min at RT in 5% bovine serum albumin (BSA), washed twice with PBS and then mounted using Prolong Gold onto glass slides (VWR, 48312–003).

Total protein was added in a final volume of 2.5 ml to antibody beads washed twice with 1% BSA for 30 min at 37 °C in a humidified chamber, washed once with PBS, incubated with secondary antibody in 1% BSA for 1 h at RT, washed three times with PBS, incubated with primary antibody in 1% BSA for 30 min at 37 °C in a humidified chamber, washed twice with PBS, and then mounted onto a slide with Prolong Gold (Life Technologies, P-36931). For slides imaged with the Zeiss LSM510 META microscope, cells were permeabilized in 0.1% Triton X-100 for 5 min at RT, washed once with PBS, incubated with primary antibody in 1% BSA for 1 h at 37 °C in a humidified chamber, washed once with PBS, incubated with secondary antibody in 1% BSA for 30 min at 37 °C in a humidified chamber, washed twice with PBS, and then mounted onto a slide with Prolong Gold (Life Technologies, P-36931). For slides imaged with the Zeiss LSM510 META microscope, cells were permeabilized in 0.1% Triton X-100 for 5 min at RT, washed three times with PBS, incubated with primary antibody in 1% BSA overnight at 4 °C, washed six times with PBS, incubated with secondary antibody in 1% BSA for 1 h at RT, washed five times with PBS, and then mounted using Prolong Gold onto glass slides (VWR, 48312–003).

**Cell Viability Assays**—Cells were plated in a 96-well plate at a density of 10,000 cells/well and allowed to grow for 24 h. Medium was then removed and replaced with 100 μl/well of serum-free DMEM containing the appropriate concentration of HNE and the cells were incubated at 37 °C for 24 h. To measure cell viability, 10 μl/well of WST reagent (Sigma, 11644807001) was added and the cells were incubated for 1 h. Absorbance readings then were taken at 450 nm and 650 nm with a Spectramax M4 spectrophotometer (Molecular Devices, Sunnyvale, CA) according to the manufacturer’s protocol.

**Cominmunoprecipitation and Western Blotting**—Frozen cell pellets were lysed on ice in 500 μl of NETN buffer (50 mM HEPES pH 7.5, 150 mM NaCl, and 1% Igepal supplemented with 10 μM of HALT [Life technologies, 78444] protease and phosphatase inhibitor), per 15 cm plate for 30 min with occasional inversion. Lysates then were clarified by centrifugation at 10,000 × g for 10 min at 4 °C. Protein in the clarified lysates was measured with the BCA assay (Life Technology, 23225). Protein concentrations were adjusted to 2 mg/ml and 5 μg of total protein was added in a final volume of 2.5 ml to antibody beads pre-washed with NETN. For each IP, a 50 μl slurry of antibody-bead conjugate was washed twice with 1 ml of NETN prior to incubation with protein lysates. After addition of the protein lysate, the beads were rotated at 4 °C for 1 h to allow for capture of the target protein. Following incubation, the beads were pelleted by centrifugation at 100 × g and the supernatant was discarded. The beads were then washed twice with 1 ml of NETN buffer and the bound protein complexes were resuspended in 80 μl of lithium dodecyl sulfate (LDS) sample buffer diluted 1:1 with NETN and supplemented with 50 mM dithiothreitol (DTT). The samples were then frozen at −80 °C until use.

For Western blot analysis, cell lysis and IP were carried out as described above. Gels were loaded with either 50 μg of input protein or 5 μl of IP and run for 50 min at a constant 180 V. Proteins were then electrophoretically transferred to PVDF membranes (Life Technology, LC2002) using the BioRad (Hercules, California) wet transfer system (1703930) operated at a constant 300 mA current for 90 min at 4 °C. Membranes were blocked in a 1:1 mixture of Tris-buffered saline plus 0.05% Tween-20 (TBST) and blocking buffer (Rockland, Limerick, PA, MB-070) for 1 h at RT while rocking. Primary antibodies were diluted 1:2000 for tag antibodies, 1:1000 for protein antibodies, and 1:750 for phospho antibodies in the same buffer used for blocking, added to the membrane, and incubated at 4 °C overnight with rocking. After incubation, the membranes were washed three times with TBST, incubated with the appropriate secondary antibody diluted 1:10,000 in the same buffer used for the primary antibody, and allowed to rock for 30 min at RT. The membranes were then washed three times with TBST, visualized using a LiCor Odyssey (Lincoln, Nebraska), and analyzed using Odyssey v3.0 software.

**Size Exclusion Chromatography of ASK Complexes**—Size exclusion chromatography (SEC) was performed as described previously (21) with minor modifications (see supplemental Fig. S104). For the IP-PRM assays, 6 column runs were pooled and each target fraction (1–9) was immunopurified following the procedure described above with the exception of the buffer which consisted of the SEC buffer supplemented with 10 μI/mL of HALT.

**Preparation of Peptides for MS Analyses**—Bead-bound protein complexes were eluted in LDS buffer and heated at 95 °C for 10 min prior to being loaded on a 10% Bis-Tris gel (Life Technologies, NP0301). The gels were loaded with 35 μl of each sample elution in alternate lanes (with a blank lane between sample lanes) and then were run at a constant 180 V for 3 min, after which the run was paused. Each sample lane was then re-loaded with the remaining 35 μl of each sample and the gel runs were resumed for an additional 7 min to complete loading of proteins into the gel. Gels were then stained with Simply Blue safe stain (Life Technologies, LC6060) for 1 min in a microwave oven at maximum power and then allowed to destain in distilled water for 2–3 h. Each sample lane was cut as a single band and diced into ~1 mm cubes. The gel pieces were placed into individual Eppendorf tubes and washed twice with 200 μl of 100 mM ammonium bicarbonate (AmBic). Samples were then reduced with 5 μl DTT in AmBic for 30 min at 60 °C while shaking at 1000 rpm and then alkylated with 10 μl iodoacetamide in AmBic for 20 min in the dark at RT. Excess blue dye was then removed with three 200 μl washes in 50 μl AmBic/acetonitrile (1:1, v/v) and the gel pieces were dehydrated in 100% acetonitrile. The gel pieces were rehydrated in 200 μl of 25 mM AmBic with 300 ng of trypsin gold (Promega, Madison, WI) per sample and placed in a 37 °C incubator for 16 h. Peptides were extracted from the SDS gel with three 20 min incubations in 200 μl of 66% aqueous acetonitrile containing 1% formic acid and evaporated to dryness in vacuo. The peptides were resuspended in 30% aqueous acetonitrile containing 0.1% formic acid and stored at −80 °C until use. Labeled peptide standards for quantitative analyses (see below) were spiked in at this reconstitution step.

**Mass Spectrometry**—

**Shotgun LC-MS/MS**—Shotgun analyses of the single gel fraction samples were carried out on a Q Exactive Plus mass spectrometer (ThermoFisher Scientific, San Jose, CA) equipped with a Proxeon nLC1000 LC (ThermoFisher Scientific) and a Nanoflex source (ThermoFisher Scientific). Peptide mixtures were evaporated in vacuo and resuspended in 2% aqueous acetonitrile containing 0.1% formic acid and loaded onto an 11 cm long column with a 75 μm internal diameter (New Objective, Woburn, MA, PF360–75–10-N-S) packed with 3 μm particle size and 120 Å pore size ReproSil-Pur C18-AQ resin (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) and separated over a 100 min gradient with a mobile phase containing aqueous acetonitrile and 0.1% formic acid programmed from 2–5% acetonitrile over 5 min, then 5 to 35% acetonitrile over 80 min, then 35–90% acetonitrile over 5 min, followed by 10 min at 90% acetonitrile, all at a flow rate of 0.3 μl/min.
of 300 nL/min. A single MS1 scan from m/z 300–1800 at 70,000 resolution with an automatic gain control (AGC) value of 3e6 and max injection time of 64 msec was recorded as profile data. A top 12 method was used, whereby the 12 most intense precursors were automatically chosen for MS2 analysis and a dynamic exclusion window of 20 s was employed. For each MS2 scan, a resolution of 17,500, an AGC value of 2e6, a max injection time of 100 msec, a 2.0 m/z isolation window, and a normalized collision energy of 27 was used and centroid data were recorded.

Parallel Reaction Monitoring (PRM) Targeted MS—PRM assays were also performed on the ThermoFisher Scientific Q Exactive Plus instrument and LC system described above. For PRM analyses, peptides were separated over a 70 min gradient: from 2–5% acetonitrile over 5 min, then 5 to 35% acetonitrile over 50 min, then 35–90% acetonitrile over 5 min, followed by 10 min at 90% acetonitrile, all at a flow rate of 300 nL/min. The PRM method consisted of an MS1 scan at 17,500 resolution with an AGC value of 5e6, max injection time of 80 msec, a 0.5 m/z isolation window, a fixed first mass of 150 m/z, normalized collision energy of 27, and recorded as profile data. The targeted-MS2 methods were controlled with a timed inclusion list containing the target precursor m/z value, charge, and a 3 min retention time window that was determined from prior analyses of synthetic peptide standards as described (29). Lists of all peptides targeted for PRM analysis are given in Supplemental Tables S1–S3.

Targeted Protein Quantitation—Two targeted protein analysis methods were employed in this study, the labeled reference peptide (LRP) and stable isotope dilution (SID) methods. The first relied on normalization of each unlabeled target peptide to a heavy isotope labeled peptide standard called the labeled reference peptide (LRP) for relative quantitation (29). Three LRP standards (GYSFTTAER\(^\#\)/H11021, AAQDITAPGGAR\(^\#\)/H11022, and APLDNDIGVSEATR\(^\#\)/H11022) were purchased from New England Peptide. Each heavy peptide was synthesized with a terminal lysine or arginine that was uniformly labeled with C\(^{13}\) and quantified by the light SID peptide signal for the ASK bait protein. This final correction step minimizes measurement variation because of differing efficiencies of protein capture in each IP. For stoichiometric analysis, the peak area from the LRP peptide was further normalized to an IRP as described above. Specifically, the peak area for the light target peptide was divided by the peak area of the corresponding heavy peptide; this ratio was then further divided by the light SID peptide from the ASK bait protein. This final correction step minimizes measurement variation because of differing efficiencies of protein capture in each IP.

SID Data Analysis—Peptides targeted for SID measurements were first normalized to the appropriate heavy labeled standard and then further normalized to an IRP as described above. Specifically, the peak area for the light target peptide was divided by the peak area of the corresponding heavy peptide; this ratio was then further divided by the light SID peptide signal for the ASK bait protein. This final correction step minimizes measurement variation because of differing efficiencies of protein capture in each IP. For stoichiometric analysis, absolute molar amounts of each peptide present in the IP were calculated using the standard curve and these were expressed as a percent of total ASK bait protein present in order to estimate the stoichiometry of the complex.

Experimental Design and Statistical Rationale—

IP Method Comparison—For initial data-dependent LC-MS/MS studies to compare IP methods, three biological replicate samples were analyzed for each of the IP methods, together with 3 negative control replicates. Similarly, for data-dependent LC-MS/MS analyses of the composition of ASK signalosome complexes, three biological replicate samples were analyzed for each of the IP methods, together with 3 negative control replicates. These sample sizes and datasets enable confident estimates of measurement variation and protein complex composition based on spectral count data (44) and application of the SAINT algorithm and software (39, 40).

ASK Signalosome Dynamics in Response to Stress—To evaluate the dynamics of ASK signalosome changes in response to HNE electrophile stress, four biological replicate samples were performed for each cell line (five for ASK1-TAG). For these ASK signalosome dynamics studies, four biological replicate samples were analyzed for each of the IP methods, together with 3 negative control replicates. Similarly, for data-dependent LC-MS/MS analyses of the composition of ASK signalosome complexes, three biological replicate samples were analyzed for each of the IP methods, together with 3 negative control replicates. These sample sizes and datasets enable confident estimates of measurement variation and protein complex composition based on spectral count data (44) and application of the SAINT algorithm and software (39, 40).
dynamics experiments, 24 control replicates were prepared and each was run as two technical replicates interspersed between the IP samples to control for both nonspecific binding and any potential carryover. The 48 control PRM analyses were divided among the 4 cell lines based on run order and all technical replicates were treated as independent for statistical testing. For PRM studies of the effects of ASK1 knockdown, four biological replicates and six controls were prepared and run as described above. For studies of the composition of ASK signalosome fractions prepared by size exclusion chromatography, two biological replicates were performed for each sample along with one negative control replicate that was run as two technical PRM injections as described above. These sample sizes were chosen to be sufficiently large enough to document measurement variation and assess significance of differences with the tests enumerated below.

Statistical Tests—Concentration-dependent differences in protein-protein interactions with the bait protein were assessed using both the two-tailed Jonckheere-Terpstra test (45, 46) to identify trends in the concentration-response relationships and the Kruskal Wallis test (47) with Dunn’s posthoc testing (48) to identify significant differences in the means of each concentration point via the DescTools package in R. The Wilcoxon rank sum test (49) was used to determine if the peptides targeted were significantly enriched over the negative control IPs. These nonparametric test methods were chosen as the IP data in this study was found to be nonnormally distributed. All statistical tests were carried out in the R environment. Raw data files can be downloaded at: ftp://massive.ucsd.edu/MSV000079399 and processed Skyline files can be downloaded at https://panoramaweb.org/labkey/Liebler_ASK.url.

RESULTS

ASK-expressing HEK-293 Cell Lines—We first generated cell lines that express epitope-tagged versions of ASK1 and the related MAP3Ks, ASK2, and ASK3. ASK2 was chosen as it is a known interacting protein of ASK1 (22, 50–54). ASK3 (23, 55) is a highly similar MAP3K that we identified as a potential ASK1-interacting protein in a pilot shotgun study of ASK1 (data not shown). HEK-293 cells were chosen as they have been used for much of the published ASK1 research to date and are easily transfected. Because of the low endogenous expression of ASK1 and the lack of suitable antibodies for immunoprecipitating endogenous ASK2 and ASK3, we chose to clone a unique tandem affinity purification tag (TAG) in-frame with each of the ASK genes (Fig. 1A). These sample sizes were chosen to be sufficiently large enough to document measurement variation and assess significance of differences with the tests enumerated below.

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RESULTS

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ylation of ASK1 (pT838), as well as increased phospho-JNK and phospho-p38 staining, all of which are consistent with previous reports (1, 3).

Shotgun LC-MS/MS Analysis of ASK1-interacting Proteins—We optimized a tandem-IP method to minimize potential nonspecific interactions through the sequential IP steps (Fig. 1C). Although a tandem IP can reduce nonspecific interactions, it can also fail to detect true interactions that do not survive both capture steps. We performed an IP method comparison using the ASK1-TAG cells, as ASK1 has the largest number of reported interactors among the three ASK proteins. We tested three different purifications using the FLAG and/or HA tags: (1) anti-FLAG IP, followed by FLAG peptide release and recapture with anti-HA, (2) anti-FLAG IP, and (3) anti-HA IP. Each IP was performed in triplicate along with three negative control IPs consisting of untransfected HEK-293 cells. After shotgun LC-MS/MS analyses of the IPs, the data were searched and assembled together at a protein FDR of 0.88%. The complete list of protein identifications from this experiment are provided in supplemental Table S4. Across all three IP methods, we identified 28 out of 94 (30%) previously reported ASK1-interacting proteins.

We then applied the SAINT algorithm to determine which of the identified proteins in each IP experiment were best supported by the spectral count data as specific binding partners of the ASK proteins. SAINT uses the negative control data to generate a probability score for each protein present in a purification, which allows for a comparison of the robustness of each IP method employed for ASK1. The SAINT scores for all proteins identified in each IP are provided in supplemental Tables S5-S7. The HA IP generated the most interactions with a high SaintScore, whereas the FLAG IP generated the most identified interactions overall, and the tandem IP generated the fewest interactions (Fig. 2). However, application of a SAINT score threshold of ≥0.8 yielded only 32 proteins that passed the filter (Venn diagram in Fig. 2), of which only four were previously reported as ASK1-interactors (bold proteins in Venn diagram). Three of the reported interacting proteins were identified in all three IP methods; of these, ASK2 is a well-documented ASK1 interactor, whereas ATAD3A and NACC1 were only recently reported in another AP-MS study (54).

We decided to further examine the putative ATAD3A interaction, because it (along with ATAD3B) represented the highest number of spectral counts for ASK1 interacting proteins other than ASK2. We confirmed the interaction between ASK1 and both ATAD3A and ATAD3B in the ASK1-TAG cells (supplemental Fig. S3A) by co-IP Western blot analysis, but we were not able to confirm this interaction in the HEK-293 cells (supplemental Fig. S3B). We also could not detect the inter-
action between ASK1 and ATAD3A by co-IP and Western blotting in another cell line (RT4 cells) with a higher endogenous level of ASK1 expression (supplemental Fig. S3B). We therefore concluded that the apparent ASK1-ATAD3A/ATAD3B interaction was an artifact of the ASK1-TAG model.

Interaction Between ASK1, ASK2, and ASK3—Another protein of interest identified in all three IP methods was ASK3 (MAP3K15). We had previously identified this protein as a potential ASK1-interactor in a preliminary shotgun screen of ASK1-expressing cells and the presence of ASK3 in all three IP methods seemed to support this conclusion. To further investigate the association of the three ASK proteins (ASK1, ASK2, and ASK3), we transiently electroporated all three proteins into HEK-293 cells in order to perform colocalization studies. As can be seen in Fig. 3A, all three of the ASK proteins show similar subcellular distributions and colocalization (white color in the Merge panel of Fig. 3A).

We also performed a transient transfection of only ASK1 and used co-IP and Western blotting to detect ASK2 and ASK3 association with overexpressed ASK1 (Fig 3B). The available antibodies for ASK2 and ASK3 did not enable sufficiently sensitive detection for co-IP Western analysis at their endogenous expression levels in HEK-293 cells. Instead, we used a parallel reaction monitoring (PRM) targeted MS assay to demonstrate the association of all three ASK proteins in an endogenous ASK1 IP (Fig 3C). Furthermore, we performed siRNA knockdown of endogenous ASK1 in HEK-293 cells (supplemental Fig. S4A) and showed with PRM analysis that decreasing the amount of expressed ASK1 also decreased the amount of associated ASK2 and ASK3 in co-IP analyses (Fig. 3C). In addition, we monitored many of the previously reported ASK1-interacting proteins, but saw no other proteins that followed the same trend seen with ASK2 and ASK3 (see supplemental Fig. S4B and S4C and supplemental Tables S8, S9). This series of experiments confirmed the previously reported association of ASK2 with ASK1 and identified ASK3 as a new ASK1-interacting protein. Furthermore, the siRNA knockdown experiment suggested a proportional interaction among the three ASK proteins.

Targeted MS Analysis of ASK1-interacting Proteins—Our inability to detect previously reported ASK1-interacting proteins in data-dependent LC-MS/MS analyses of co-IPs suggested that they were present at relatively low levels compared with the overexpressed ASK1 and that more sensitive targeted methods would be required to detect these proteins. Thus, we re-analyzed the same co-IP samples described above with a PRM assay, which targeted 66 previously reported ASK1-interacting proteins listed in supplemental Table 3.
For these experiments we used the LRP method, which employs a single heavy isotope labeled peptide as a normalization standard for all target peptides in the analysis and provides a cost-effective means to quantitatively compare abundance of larger numbers of proteins between samples (29). This LRP-PRM assay was thus used to detect significant enrichment of the target proteins in ASK1-TAG co-IPs.

These analyses detected 61 of the 66 targeted ASK1-interacting proteins (92%) (supplemental Tables S10–S13). Significant enrichment was defined as either (1) detection in at least 2 of 3 biological replicates for proteins not detected in the control samples or (2) by a significant \((p < 0.05; \text{Wilcoxon rank test})\) and at least 3-fold enrichment over control samples (Fig. 4). Of the sixty-two enriched proteins, we detected 60 in the HA IP while only observing 29 in the FLAG IP and 6 in the tandem IP. These results thus confirmed the association of most previously reported ASK1 interacting proteins; albeit at levels that appeared to be much lower than ASK1.

**Targeted Assay Strategy for ASK1 Complex Dynamics and Stoichiometry Studies**—Because PRM analyses enabled detection of many previously reported ASK1-interacting proteins in our co-IP analyses, we pursued a targeted analysis strategy for studies of the dynamics of ASK signalosome components in response to HNE electrophile stress and to further characterize the stoichiometry of ASK1 interaction with other putative signalosome protein components. A targeted analysis strategy employing a combination of LRP-PRM and SID-PRM assays for these studies is represented in Fig. 5. We employed the LRP-PRM approach to target 94 proteins, including the 68 targeted in the co-IP analyses described above, as well as additional proteins hypothesized to have ASK1 interactions. These proteins and the targeted peptides and precursor \(m/z\) values are listed in supplemental Table S2. Because normalization to the LRP standard provides ratios for each peptide (peak area for peptide/peak area for LRP standard), these values can be used for relative quantitation between HNE exposures for the dynamics experiments.

For measurements of stoichiometry of ASK1-interacting proteins, we required absolute quantitation and therefore performed SID-PRM analyses for 26 peptide targets (supplemental Table S3) corresponding to a subset of 26 ASK1-interacting proteins identified as significant interactors in the co-IP
experiments above. Calibration curves for all 26 peptides are shown in supplemental Fig. S5 and the specific features of each assay (target peptides, transitions, LLOD, LLOQ, linearity) are presented in supplemental Table S14.

These two quantitative strategies were applied simultaneously in the same study of the dynamics of ASK signalosome proteins in response to HNE treatment. The combined LRP-PRM and SID-PRM analysis targeted 254 peptide precursors from the 94 proteins, three LRP standard peptide precursors, and 26 stable isotope labeled SID standard peptide precursors. For LRP-based quantitation, multiple peptides were targeted for most of the proteins. For SID-based quantitation, a single peptide per protein and its heavy isotopolog standard were measured. Quantitative comparisons of each protein between conditions were based on the data for a single peptide per protein generated either by LRP (normalized ratio) or by SID (molar quantity) measurements. For the 26 proteins for which SID measurements were possible, these were used in preference to LRP measurements. For proteins for which multiple peptides were targeted for LRP measurements, the one with the highest average peak area across all IPs was used for quantitative comparisons.

Dynamic Changes in ASK Signalosome Composition in Response to HNE—We performed WST cell death assays to determine the HNE concentrations to use for studies of ASK1 protein interaction dynamics. All three ASK-overexpressing cell lines and the nontransfected HEK-293 cell line yielded a similar EC_{50} value (~10 μM) for HNE (supplemental Fig. S6A). The effectiveness of this HNE concentration in triggering electrophile stress was confirmed by Western blot analysis of the activating Thr838 phosphorylation of ASK1 in the ASK1-TAG cell line, which showed a much higher level of activation at 20 μM than 5 μM (2-fold above and below the EC_{50} value, respectively) (supplemental Fig. S6B). These assays, along with evidence of maximal ASK1 MAPK pathway activation after 1 h of treatment (supplemental Fig. S2), led us to select a treatment time of 1 h with three concentrations: 0 μM HNE (ethanol vehicle control - below EC_{50}), 10 μM HNE (EC_{50}), and 50 μM HNE (above EC_{50}).

We treated the four cell lines with HNE in quadruplicate (quintuplicate for ASK1-TAG) at each concentration and confirmed the success and uniformity of the IPs by Western blotting (supplemental Fig. S7A) prior to running the PRM analyses of the corresponding samples. We first determined the amount of the ASK1, ASK2, and ASK3 proteins present in IPs from ASK1-TAG, ASK2-TAG, and ASK3-TAG cells, as well as from nontransfected control HEK-293 cells (supplemental Fig. S7B). ASK1 and ASK2 were both purified at higher amounts from their respective cell lines, whereas amounts of immunoprecipitated ASK3 from ASK3-TAG cells and ASK1 from nontransfected cells were over an order of magnitude lower.

To detect HNE-induced changes in the ASK protein complex composition for each cell line, we employed two different strategies. First, we compared the means of the LRP- and SID-normalized peak areas for each detected peptide at each concentration using the Kruskal-Wallis test with a Dunn’s posttest. Differences between the means are indicated by blue connecting bars in the graphs in Fig. 6A and 6B. We also performed a Jonckheere-Terpstra (JT) test to detect trends in response consistent with a concentration-dependent relationship. Fig. 6A and 6B depict measurements of NACC1, YWHAG, PSMC2, and HSP90AB1, all of which have significant \( p < 0.05 \) JT test values, indicating that these four peptides exhibit increased abundance with increasing HNE concentration.

As a further test of specificity, we performed Wilcoxon rank sum tests between each treatment condition and the negative control to assess whether or not the peptide was part of a true protein-protein interaction with the bait protein or if it was merely nonspecifically purified. A red mean bar was used to denote peptides that were enriched over the negative control at that concentration point with either a Wilcoxon \( p \) value \( \leq 0.05 \) or detection in at least half of the replicates at a given concentration point and no detection in any of the negative control replicates.

Fig. 6C summarizes the findings of the concentration-response relationships in the ASK complex in all four cell lines. We detected 79 peptides corresponding to 44 proteins in at least one of the cell lines. The ASK1-TAG cells had the largest number of significant changes in protein complex composition (red bars), whereas the ASK2 and ASK3 cell lines only had a few each. In total, 15 proteins exhibited concentration-dependent shifts in interaction with the three ASK proteins and 14 of these were observed with ASK1 alone. Furthermore, because of the low expression level of ASK3 in the ASK3-TAG cell line, the majority of the detected peptides were not significantly enriched over the negative control samples. Measurements in nontransfected HEK-293 cells expressing endogenous ASK1 had no significantly changing ASK-interacting proteins in response to HNE, probably because the low abundance of ASK1 made it difficult to quantify interactors. Data for the individual protein measurements summarized in Fig. 6C are presented in supplemental Figs. S8 and S9; the normalized protein measurements with statistical test results can be found in supplemental Tables S15–S19.

A well-known caveat with AP-MS studies is that artificial protein-protein associations may be observed in studies with overexpressed, epitope-tagged proteins. To address this possibility for the 14 proteins found to exhibit a dynamic association change with ASK1-TAG in response to HNE treatment, we measured levels of these proteins in the ASK1 IPs from the HEK-293 cells. Most (11 of 14) were detected in the endogenous ASK1 IPs at a higher level than in the negative control IPs (supplemental Fig. S10). Those 11 proteins thus are significantly associated with ASK1 at endogenous levels and the association is independent of an epitope tag. Moreover, if these associations had been driven simply by overex-
pression of ASK1-TAG protein, association differences upon HNE treatment would not be expected. Our failure to observe this HNE-dependent dynamic in untransfected cells may reflect the difficulty of measuring changes in association at the low abundance levels of endogenous ASK1.

Analysis of ASK Signalosome Populations Prepared by Size Exclusion Chromatography—Although several proteins displayed concentration-dependent changes in response to HNE treatment, there were fewer changes than might be anticipated from the ASK1-signalosome remodeling hypothesis (20, 21). We hypothesized that some of the dynamic shifts in the ASK complex composition could have been masked by a potentially heterogeneous mixture of pre- and postactivation ASK signalosome complexes. ASK1 has been reported to undergo a shift in complex size upon activation by oxidants and thereby change from a ~1500 kDa pre-activation complex to a much higher molecular weight postactivation complex (21).

To assess this possibility, we repeated the SEC fractionation of the ASK complex as described by Ichijo and colleagues (21) with minor modifications (supplemental Fig. S11A). Our measurements suggested a minimum complex...

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**Fig. 6. ASK complex dynamics in response to HNE treatment.** A and B. Four proteins that exhibited a positive concentration-response relationship to HNE in the ASK1-TAG cells (A) and the ASK2-TAG cells (B). C. Summary of all the peptides detected in the ASK complex dynamics study. Black signifies that the peptide was not detected in the IP; yellow indicates that the peptide was detected, but did not change significantly in response to HNE; and red indicates that the peptide was detected, was enriched above the negative control, and exhibited a significant HNE-dependent change for at least one treatment concentration.
size of \( \sim 669 \text{kDa} \), which is consistent with the analysis by Ichijo and colleagues [21]. However, we did not observe a shift in the distribution of ASK1 or phospho-ASK1 across SEC fractions (supplemental Fig. S11B and S11C). PRM analysis of ASK1, ASK2, and ASK3 in IPs from SEC-fractionated ASK1 complexes revealed a similar distribution of the ASK proteins as was seen for ASK1 in the Western blot analyses (supplemental Fig. S11D).

**ASK Protein Complex Stoichiometry**—SID-PRM measurements in the ASK signalosome dynamics study provided absolute quantitative estimates of ASK1-interacting proteins in the IPs. We used these measurements to estimate protein stoichiometry relative to bait protein as has been done for other systems [59–62]. The apparent stoichiometry varied somewhat depending on the cellular context. In the ASK1-TAG cell co-IPs (Fig. 7A), ASK2, ASK3, 14–3-3 proteins (YWHAQ, YWHAB, YWHAH, and YWHAE measured collectively by a common peptide), PARK7, PRDX1, and USP9X were present at a few percent of the level of ASK1. Analyses of the ASK2-TAG cell co-IPs (Fig. 7C) yielded data similar to the ASK1-TAG IPs, with ASK1, ASK3, 14–3-3 proteins, PARK7, PRDX1, and USP9X present at a few percent of the level of ASK2. Interestingly, measured levels of some of the interacting proteins appeared to increase with increasing HNE treatment concentration.

In co-IPs of endogenous ASK1 from untransfected cells (Fig. 7B), the stoichiometry of ASK2 relative to ASK1 was \( \sim 1:1 \) and that of 14–3-3 proteins to ASK1 was \( \sim 0.5:1 \). The other proteins measured at low levels in co-IPs of ASK1-TAG and ASK2-TAG were also present at less than 10% of endogenous ASK1 and PRDX1 was not detected as a partner of endogenously expressed ASK1.

ASK3-TAG co-IPs expressed a level of ASK3 lower than that of endogenous ASK1 in co-IPs from untransfected cells (supplemental Fig. S7B) and 14–3-3 proteins and PRDX1 were present in stoichiometries of \( \sim 0.5:1 \) and 0.7:1 relative to ASK3, respectively (Fig. 7D). Other detected interactors were present at levels less than 10% of ASK3.

**DISCUSSION**

ASK1 is thought to be regulated by a large multiprotein complex that changes composition dynamically in response to oxidative stress. The candidate membership list—ASK1 and its 90 reported interacting proteins—together would be
too large to comprise a signalosome complex of the reported 1500 kDa size (21). We therefore asked which members of this candidate list are constitutively bound to ASK1 and how does this repertoire of interacting molecules change upon activation of the ASK1 pathway by HNE electrophile stress. We first confirmed ASK1 interactions with most of the reported interactors with shotgun and targeted MS analyses. Using targeted PRM assays, we also identified 14 proteins that demonstrated dynamic shifts in their association with ASK1 under stress. Using precise SID-based measurements, we next provided the first ever quantitative examination of the ASK complex. We detected ASK2 as a 1:1 stoichiometric binding partner of ASK1 and several 14–3–3 proteins as ASK1 interactors with a collective stoichiometry of ~0.5:1. ASK3 and several other proteins exhibited stoichiometries of 0.1:1 or lower. These stoichiometries were consistent between ASK1-TAG and ASK2-TAG complexes and immunoprecipitates of endogenous ASK1. Moreover, the complex size calculated from our stoichiometry measurements is consistent with SEC analyses of ASK1 complexes. The data suggest a core ASK signalosome consisting of ASK1, ASK2, and 14–3–3 proteins, which interacts with a variety of other proteins to mediate ionization suppression and interferences because of the binding partners. However, the method is potentially subject to interference by unanticipated protein modification or cleavage, all of the SID measurement of the ASK signalosome in response to HNE stress. These two approaches enabled highly precise quantitative assessment of component stoichiometries similar to those in native ASK1 complexes from untransfected cells. Additionally, by overexpressing ASK1 and ASK2, we were able to detect dynamic protein-protein associations that could not be observed at endogenous ASK1 expression levels, likely because of the ~40-fold lower expression level of the ASK proteins in untransfected cells (supplemental Fig. S7). Thus, examining a multiprotein complex at multiple expression levels can provide insights that would otherwise be missed.

One of the disadvantages of overexpression systems is that they can generate false-positive associations. In our shotgun analysis of ASK1-TAG IPs, we identified the previously reported ASK1-interacting protein ATAD3A (54) as a putative ASK1 interactor by all three IP methods. We then attempted to validate this interaction by Western blotting and were able to do so in the overexpressing cell line, but not in two different nontransfected systems (supplemental Fig. S3). This suggested that the interaction between ATAD3A and ASK1 may be mediated by the presence of the affinity tags. The previous study by So et al. reporting an ASK1-ATAD3A interaction used a triple FLAG tag on ASK1 (54). We used a single FLAG epitope as part of our tandem tag on ASK1, so it is possible that the interaction between ASK1 and ATAD3A is mediated by the FLAG sequence. By utilizing the endogenously expressing cell line, we were able to identify this interaction as a likely artifact. Through quantitative MS analyses of ASK-TAG protein overexpression and native ASK protein expression in nontransfected cells, we were able to confirm the essential elements of the ASK signalosome hypothesis (20) and further extend our understanding of this system. Through our SEC
experiments, we confirmed previous observations of the distribution of the ASK1 protein across multiple high molecular weight complexes (21). In contrast to a report that H$_2$O$_2$ led to the formation of a higher molecular weight ASK signalosome complex (21), we did not observe the same shift with a similar degree of MAPK activation by HNE. We also detected most previously reported ASK1-interacting proteins at a significant enrichment over negative control IPs, thus confirming their associations with ASK1. Several of these proteins exhibited dynamic associations with the overexpressed ASK1 upon HNE treatment, which is consistent with previous observations of dynamic protein-protein associations with ASK1 in response to stress activation.

Although we did observe several dynamic protein-protein interactions with ASK1, most of these proteins associated with ASK1 in a low stoichiometric ratio. Our SID-PRM assay targeted 26 of the previously reported ASK1-interacting proteins and quantified 7 of them in ASK1 IPs from nontransfected cells. Of these 7 proteins, ASK2 was present at a nearly 1:1 ratio with ASK1 and several 14–3–3 family proteins were collectively present at an ~−0.5:1 ratio with ASK1. The remaining proteins were observed at a 0.1:1 or lower ratio with ASK1. Thus, our data points toward a core ASK1 complex that consists of ASK1, ASK2, and 14–3–3 family members that associates with other proteins to execute MAPK pathway signaling.

Interestingly, the expected size of a stoichiometric core ASK1 complex as we have described it here would be ~625 kDa (2 ASK1, 2 ASK2, and 1 14–3–3 family protein), which corresponds well with the smallest commonly observed ASK1 complex mass (~669 kDa) in both our SEC experiments (supplemental Fig. S11B and S11C) and similar SEC analyses reported by Noguchi et al. (21). This suggests a core ASK1 complex that may transiently associate with other proteins in a context-dependent fashion. Indeed, the MW range for ASK1 complexes in the SEC analyses extends from ~669 kDa to as much as 3000 kDa (21), which could indicate the simultaneous co-existence of many different ASK1 signalosome complexes.

The presence of multiple 14–3–3 family members in the ASK1 core complex suggests that they may serve a key role as adapter proteins for bringing the ASK complex into contact with many other identified ASK interacting proteins. Indeed, finding equal stoichiometry between ASK1 and ASK2 in the signalosome complex raises the possibility that previously reported ASK1-interacting proteins may interact with both ASK proteins. Regardless of ASK protein binding specificity, the diversity of ASK-interacting protein partners likely enables the ASK signaling pathway to respond to a wide variety of environmental stressors. ASK1 has been reported to interact with several other proteins to influence different signaling pathways (63–66) and recent studies explored the role of ASK1 in antiviral response and innate immunity (67–69). The core ASK complex thus may play a broader cellular role beyond activation of the MAPK pathway. The large number of identified interacting proteins certainly points toward multifunctional roles of the core ASK complex in the cell. Future investigations of the ASK1 core complex associations through targeted MS in different physiological contexts will be essential to test these hypotheses.

* This work was supported by National Institutes of Health Grant R01ES022936 (to D. C. L.). J. D. F. and C. M. B. were supported by the Training Program in Environmental Toxicology T32ES007028. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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