Molecular Biology of the Cell
Vol. 18, 3903–3913, October 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

Philippe J. Nadeau,* Steve J. Charette,* Michel B. Toledano,† and Jacques Landry*

*Centre de recherche en cancérologie de l'Université Laval, L'Hôtel-Dieu de Québec, Québec G1R 2J6, Canada; and †Laboratoire Stress Oxydants et Cancer, Service de Biologie Moléculaire Systémique, Département de Biologie Joliot-Curie, Commissariat à l'Énergie Atomique-Saclay, 91191 Gif-sur-Yvette Cedex, France

Submitted May 24, 2007; Revised July 9, 2007; Accepted July 16, 2007
Monitoring Editor: Carole Parent

Apoptosis signal-regulated kinase-1 (Ask1) lies upstream of a major redox-sensitive pathway leading to the activation of Jun NH_2-terminal kinase (JNK) and the induction of apoptosis. We found that cell exposure to H_2O_2 caused the rapid oxidation of Ask1, leading to its multimerization through the formation of interchain disulfide bonds. Oxidized Ask1 was fully reduced within minutes after induction by H_2O_2. During this reduction, the thiol-disulfide oxidoreductase thioredoxin-1 (Trx1) became covalently associated with Ask1. Overexpression of Trx1 accelerated the reduction of Ask1, and a redox-inactive mutant of Trx1 (C35S) remained trapped with Ask1, blocking its reduction. Preventing the oxidation of Ask1 by either overexpressing Trx1 or using an Ask1 mutant in which the sensitive cysteines were mutated (Ask1-ΔCys) impaired the activation of JNK and the induction of apoptosis while having little effect on Ask1 activation. These results indicate that Ask1 oxidation is required at a step subsequent to activation for signaling downstream of Ask1 after H_2O_2 treatment.

INTRODUCTION

H_2O_2 is emerging as an important intracellular signaling molecule affecting a variety of cellular responses. In mammals, H_2O_2 concentration transiently increases in response to different membrane-receptor agonists such as peptide growth factors, hormones, cytokines, and neurotransmitters. This increase of H_2O_2 concentration is important for downstream signaling from the corresponding membrane receptors (Deyulia *et al*., 2005; Rhee *et al*., 2005; Stone and Yang, 2006). Some of these H_2O_2 regulatory effects are mediated by protein-thiol oxidation, as shown for the oxidative inhibition of protein tyrosine phosphatase upon receptor-tyrosine kinases engagement. H_2O_2 can oxidize cysteine residues to the sulfenic (protein-SOH), sulfinic (protein-SO_2H), and sulfonic acid (protein-SO_3H) forms, also leading to intra- or intermolecular disulfide bonds (protein-SS-protein) and to protein glutathionylation (protein-SSG). Similarly to protein post-translational modification by serine, threonine, and tyrosine phosphorylation, oxidation of cysteine residues can trigger changes in protein conformation and activity (Filomeni *et al*., 2005). In bacteria, OxyR is an archetypical H_2O_2 sensor at the top of a redox-sensitive pathway, being activated by H_2O_2 through formation of an intramolecular disulfide bond and controlling the transcription of antioxidant defense genes (Storz and Tartaglia, 1992; Zheng *et al*., 1998). In yeast, H_2O_2 sensing involves the thiol-based peroxidase Orp1/Gpx3, which, upon oxidation by H_2O_2, mediates the formation of an intramolecular disulfide bond in the Yap1 transcription factor, causing its activation (Lee *et al*., 1999; Delaunay *et al*., 2002).

The mitogen-activated protein kinase kinase kinase apoptosis signal-regulated kinase-1 (Ask1) and its downstream stress-activated kinases p38 and Jun NH_2-terminal kinase (JNK) constitute an important mammalian signaling pathway that can promote cell survival, apoptosis, proliferation, or differentiation, depending on the cell type and/or cellular context (Takeda *et al*., 2000; Sayama *et al*., 2001; Matsuzawa and Ichijo, 2005; Sumbayev and Yasinska, 2005). Ask1 can be activated by H_2O_2; thus, it is part of a redox-signaling pathway (Gotoh and Cooper, 1998; Tobiume *et al*., 2001; Kadowaki *et al*., 2005; Noguchi *et al*., 2005). The activation of Ask1 by H_2O_2 is regulated by the disulfide reductase thioredoxin-1 (Trx1). According to the current model, reduced Trx1 tightly binds the N-terminal domain of Ask1, inhibiting Ask1 multimerization and kinase activity. On H_2O_2 treatment, Trx1 is oxidized and dissociates from Ask1, thereby allowing Ask1 activation (Saitoh *et al*., 1998; Liu *et al*., 2000; Liu and Min, 2002). This differential binding modulated by the state of oxidation and that results in inhibition of a signaling protein constituted a new and unexpected function for thioredoxin. Thioredoxin is a universal disulfide reductase that operates by catalyzing thiol–disulfide exchange reactions. Among its classical functions as a thiol-transferase, thioredoxin contributes to DNA synthesis by reducing the catalytic disulfide of ribonucleotide reductase,

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E07–05–0491) on July 25, 2007.

Address correspondence to: Jacques Landry (jacques.landry@med.ulaval.ca).

Abbreviations used: Ask1, apoptosis signal-regulated kinase-1; JNK, Jun NH_2-terminal kinase; siRNA, small interfering RNA; Trx, thioredoxin.

the enzyme reducing ribonucleotides to deoxyribonucleotides (Thelander, 1974). Thioredoxin also contributes to H_2O_2 scavenging by recycling oxidized peroxiredoxins, an important thiol-based peroxidase family enzyme (Rhee *et al*., 2005). Thioredoxin as a thiol reductase has also regulatory functions, capable of reducing, either directly or indirectly, several redox-sensitive transcriptional factors (Matthews *et al*., 1992; Hirota *et al*., 1997, 1999; Ueno *et al*., 1999).

We have here revisited the mechanisms of Ask1 regulation by H_2O_2. We found that exposure of cells to H_2O_2 induced the rapid oxidation of Ask1 to disulfide-linked high-molecular-multimeric species. We show that oxidation-induced Ask1 multimerization is essential for full JNK activation and apoptosis induction by H_2O_2 and that Trx1 negatively regulates Ask1 signaling by directly reducing the oxidized form of Ask1.

MATERIALS AND METHODS

Plasmids and Reagents

pcDNA3-Ask1-HA and pcDNA3-Ask1K709M-HA contains hemagglutinin (HA)-tagged human wild-type Ask1 and kinase-inactive Ask1 mutant, respectively (Ichijo *et al*., 1997). They were provided by Dr. Hidenori Ichijo (Graduate School of Pharmaceutical Science, Tokyo, Japan). Ask1 deletants were produced by polymerase chain reaction (PCR) amplification of pcDNA3-Ask1-HA by using primers delimiting the region to be deleted. The various cysteine-to-alanine Ask1 mutants and the threonine-838-to-alanine (pcDNA3-Ask1T838A-HA) mutant were constructed using PCR following the QuikChange site-directed mutagenesis protocol (Stratagene, La Jolla, CA). All newly developed constructs were verified by automated DNA sequencing (Plateforme de séquençage et de génotypage du génome, Centre hospitalier universitaire Laval, Québec, Canada). pRK-FLAG-Trx1 and pRK-FLAG-Trx1C35S encode a human Flag-tagged wild-type Trx1 and redox-inactive Trx1 mutant, respectively (Liu and Min, 2002). They were provided by Dr. Wang Min (Yale University School of Medicine, New Haven, CT). pcDNA3-MYC-Ask1 and pCMV5-MYC-Trx1 expressing Myc-tagged human Ask1 and Trx1, respectively (Liu *et al*., 2000), were provided by Dr. John Kyriakis (Molecular Cardiology Research Institute, Boston, MA). Iodoacetamide, *N*-ethylmaleimide, H_2O_2, 2-mercaptoethanol, thapsigargin, 4′,6-diamidino-2-phenylindole (Dapi), gelatin, and poly-L-lysine were all purchased from Sigma Diagnostics Canada (Mississauga, ON, Canada).

Cell Culture and Transfection

HeLa, human embryonic kidney (HEK)293, and 293T (derived from HEK293) cells were maintained in a humidified 5% CO_2 atmosphere in DMEM (catalog no. 12100-046; Invitrogen Canada, Burlington, ON, Canada) containing 2.2 g/l $NaHCO_3$ and 0.4 g/l NaCl, and supplemented with 10% fetal bovine serum (Sigma Diagnostics Canada). The dishes were coated either with 0.1% gelatin (HEK293 cells), 1 mg/ml poly-L-lysine (293T cells), or not coated (HeLa cells). The cells were transfected 24 h after plating with 0.01 to 4.0 μg DNA plasmid by calcium phosphate precipitation as described previously (Landry *et al*., 1989). Small interfering RNA (siRNA) targeting human Trx1 was based on nucleotides 256-276 (Jeong *et al*., 2004) and obtained from Dharmacon RNA Technologies (Lafayette, CO). The nontargeting siRNA, siCONTROL #1 (Dharmacon RNA Technologies), was used as a negative control. siRNA were transfected in six-well plates at 50 nM for 48 h using Lipofectamine 2000 (Invitrogen Canada) according to the manufacturer's protocol developed for HEK293 cells, except that Lipofectamine 2000 was used at a concentration of 2.5 μl per 3 ml of total medium.

Antibodies

Anti-p38α has been described previously (Guay *et al*., 1997). The Ask1 phospho-specific antibody was raised in rabbit against the human Ask1 peptide $CTET_{838}(PO_3H_2)FTGT_{842}(PO_3H_2)LQY$ coupled to the keyhole limpet hemocyanin. The anti-Trx1 antibody and the phospho-specific antibodies against p38, MKK4, and MKK7 were from Cell Signaling Technology (Danvers, MA). The anti-phospho-JNK was from Santa Cruz Biotechnology (Santa Cruz, CA) or Cell Signaling Technology. The mouse monoclonal antibodies anti-Ask1 and anti-JNK were from Santa Cruz Biotechnology; antibodies against the HA (HA.11) and the Flag (FLAG M2) tags and against α-tubulin were from Sigma Diagnostics Canada. Anti-Myc (9E10) was from American Type Culture Collection (Manassas, VA).

Cells Extracts, Immunoprecipitation, and Western Blot Analysis

Cells were washed with ice-cold PBS and harvested in SDS-sample buffer lacking 2-mercaptoethanol and supplemented with 100 mM iodoacetamide. In some experiments, the cells (2.0×10^5) were precipitated in 20% trichloroacetic acid (TCA) before SDS solubilization as described previously (Lee *et al*., 2002). Extracts were homogenized, boiled, and 5% 2-mercaptoethanol was added (reducing gels) or not (nonreducing gels). For immunoprecipitation, the cells were washed with ice-cold PBS, extracted in ice-cold lysis buffer containing 20 mM Tris-Cl, pH 7.4, 100 mM KCl, 1.25 mM $MgCl_2$, 0.5% Igepal, 3.3% glycerol, Complete protease inhibitors (Roche Diagnostics, Indianapolis, IN), and 100 mM iodoacetamide; agitated on ice for 15 min; and centrifuged at $15{,}000 \times g$ for 10 min at 4°C. Supernatants were incubated with anti-HA Affinity Matrix (Roche Diagnostics) for 18 h at 4°C, washed three times with 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% NP-40 and 3.3% glycerol, and resuspended in SDS-sample buffer containing or not 5% 2-mercaptoethanol. For Western blot analysis, the samples were separated on SDS-polyacrylamide gels and transferred onto Biotrace nitrocellulose membranes (Pall Life Sciences, East Hills, NY). To detect phosphorylated Ask1 the membrane was incubated with anti-phospho-Ask1 at 4°C for 18 h in TBST buffer (10 mM Tris-Cl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20) supplemented with 3.5% albumin, 0.5% ovalbumine, 1 μg/ml phosphothreonine (Sigma Diagnostics Canada) and 2 μg/ml blocking peptide (CTETFTGTLQY) of human Ask1. For anti-Flag, the incubation was in TBST supplemented with 5% free fatty acid powder milk. All other primary antibodies were incubated in TBST containing 5% albumin. Primary antibodies were detected using anti-rabbit or anti-mouse immunoglobulin G (IgG) coupled with horseradish peroxidase and revealed with the West Pico or West Dura Supersignal Chemiluminescent kit (Pierce Chemical, Rockford, IL) according to the manufacturer's instructions. Chemiluminescence was visualized and digitalized using Fluor-S MultiImager (Bio-Rad, Hercules, CA) or with imaging films (Eastman Kodak, Rochester, NY).

Immunofluorescence Microscopy

HeLa cells were fixed in 3.7% formaldehyde, permeabilized for 15 min in 0.1% saponin, and blocked with bovine serum albumin before addition of the HA.11 antibody. Antigen–antibody complexes were revealed with Alexa Fluor 488-labled anti-mouse IgG (Invitrogen Canada). The cell nuclei were stained with 1.6 μg/ml 4,6-diamidino-2-phenylindole (DAPI). Observations were made with a Nikon Eclipse E800 upright microscope equipped with a 40× 0.75 numerical aperture objective lens.

RESULTS

In Vivo Oxidation of Ask1 by H_2O_2

Ask1 plays an important role in the cell response to oxidative stress, being essential for the activation of the stress-activated protein kinase JNK (Gotoh and Cooper, 1998; Tobiume *et al*., 2001; Machino *et al*., 2003; Saadatzadeh *et al*., 2004; Kadowaki *et al*., 2005). As expected, exposure of HEK293 cells to H_2O_2 induced the rapid phosphorylation of JNK (Figure 1E), which was increased severalfold by pretransfecting the cells with a HA-tagged version of Ask1 (Ask1-HA) (Figure 1A, lanes 3 and 4). The activation of human Ask1 is accompanied by the phosphorylation of Thr838 (Thr845 in mouse) (Tobiume *et al*., 2002); accordingly, a T838A phosphorylation mutant was unable to boost the phosphorylation of JNK (Figure 1A, lanes 5 and 6).

According to the current model (Saitoh *et al*., 1998), Ask1 is kept inactive in resting cells by association with Trx1, and it becomes activated and phosphorylated upon release of this interaction, which is triggered by Trx1 oxidation in response to H_2O_2 treatments. We investigated whether the H_2O_2-induced activation of Ask1 could be directly regulated by oxidation. Cell extracts from Ask1-HA–expressing 293T cells exposed to 100 or 400 μM H_2O_2 were migrated on nonreducing SDS-polyacrylamide gel electrophoresis (PAGE) and analyzed by Western blot using anti-HA antibodies (Figure 1B). In untreated cells, Ask1-HA migrated as a single band of the expected molecular weight (~155 kDa). On exposure to 100 μM H_2O_2, two to three prominent anti-HA–reactive bands of electrophoretic mobility lower than Ask1 (>200 kDa) showed up as early as 0.5 min after H_2O_2 exposure, and they disappeared after 15 min (Figures 1B, nonreducing, and 2B). These bands disappeared when migrated on reducing SDS-PAGE, establishing that they correspond to oxidized Ask1 in the presumable form of disulfide-

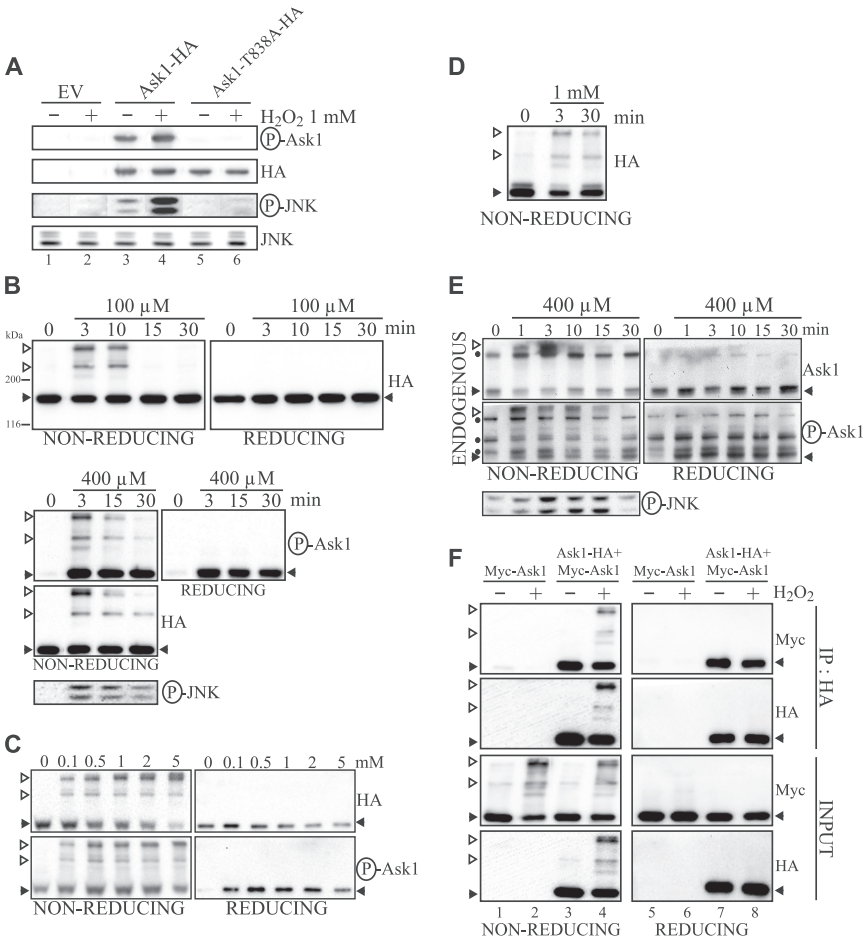

Figure 1. H_2O_2 induces disulfide bond-mediated Ask1 multimerization. (A) 293T cells were transfected with the empty vector pcDNA3 (EV), pcDNA3-Ask1-HA, or pcDNA3-Ask1T838A-HA. Twenty-four hours later, the cells were left untreated (−) or exposed (+) to 1 mM H_2O_2 for 3 min. Extracts were fractionated on reducing SDS-gels and analyzed by Western blot with specific antibodies to detect phosphorylated and total Ask1-HA and JNK, as indicated. Lane numbers are referred to in the text. (B–E) 293T cells transfected with pcDNA3-Ask1-HA for 24 h (B–D) or untransfected HEK293 cells (E) were treated with 100 μM (B) or 400 μM (B and E) or 1 mM (D) H_2O_2 for various times between 0 and 30 min as indicated (B, D, and E) or for 3 min at the indicated concentrations (C). The cells were scraped directly in SDS-sample buffer (B, C, and E) or first precipitated in TCA before SDS-solubilization (D). The extracts were fractionated on reducing or nonreducing gels as indicated and immunoblotted with appropriate antibodies to detect total (HA) or phosphorylated (P-Ask1) transfected Ask1 (B–D), total (Ask1) or phosphorylated (P-Ask1) endogenous Ask1 (E), or phosphorylated JNK (P-JNK). When indicated, the numbers on the left refer to the position of molecular weight standards migrated under nonreducing conditions. The expected position of monomeric Ask1 is indicated by closed arrowheads. Higher molecular weight Ask1 complexes are identified by open arrowheads. Bands that reacted nonspecifically with the antibodies (as determined by knocking down Ask1 with siRNA) are indicated by dots. (F) 293T cells were transfected with pcDNA3-MYC-Ask1 alone or with pcDNA3-Ask1-HA. Twenty-four hours later, the cells were left untreated (−) or exposed (+) to 1 mM H_2O_2 for 3 min. Total cell extracts (INPUT) or anti-HA immunoprecipitated extracts (IP:HA) were migrated on nonreducing (left) or reducing (right) gels and were analyzed by Western blot either with anti-HA (HA) or anti-MYC (Myc). The expected position of monomeric MYC-Ask1 and Ask1-HA are indicated by closed arrowheads. Lane numbers are referred to in the text.

linked homo- or heteromultimeric complexes (Figure 1, B and C, reducing). Similar results were obtained after exposure to 400 μM H_2O_2. The oxidized species tended to be more persistent; nevertheless, they disappeared by 15–30 min. In fact, the oxidation of Ask1 by H_2O_2 was strictly dose dependent, with the highest dose of H_2O_2 (5 mM) shifting Ask1-HA almost totally to its oxidized forms within 3 min (Figure 1C). The absence of detectable high-molecular-weight species at time 0 and at times later than 15 or 30 min strongly argued against the possibility that Ask1 might have been artifactually oxidized during cell fractionation. Moreover, the same results were obtained when extracts were prepared under acidic conditions that minimize thiol exchange (Figure 1D). Endogenous Ask1 was also oxidized in response to H_2O_2 treatments (Figure 1E). Endogenous Ask1 became oxidized within 1 min of exposure to 400 μM H_2O_2, and it remained in high-molecular-weight complexes for up to 15 min (Figure 1E, open arrowhead). These complexes also disappeared when migrated on reducing SDS-PAGE (Figure 1E, reducing).

The oxidized species all migrated more slowly than the 200-kDa molecular weight marker (see in most figures throughout the text). The biggest species was barely entering the gel; therefore, it possibly corresponded to a molecular weight of >500 kDa. This suggested that oxidation likely caused intermolecular bonds between different molecules of Ask1 or between Ask1 and another protein of a similar large size, causing homologous or heterologous multimerization. To determine whether another large protein was present in the cross-linked species, Ask1-HA was immunoprecipitated, migrated on a nonreducing gel, and proteins from the major oxidized bands were extracted, digested with trypsin, and subjected to mass spectrometry analyses. In spite of a 32–50% peptide recovery obtained for Ask1 in the three different bands analyzed, no other protein large enough so as to explain the shift in molecular weight was identified in any of these bands (data not shown). We concluded that the high-molecular-weight species were mainly constituted of disulfide-linked multimeric (up to tetramer) complexes of Ask1. To support this conclusion, coimmunoprecipitation experiments were performed in 293T cells after cotransfecting Ask1-HA and MYC-Ask1 (Figure 1F). Ask1 is found in cells as oligomer (Tobiume *et al.*, 2002); consequently, the immunoprecipitation of Ask1-HA also pulled down MYC-Ask1. These molecules were not covalently linked in control cells, and they migrated as monomers both in reducing and nonreducing gels (lanes 3 and 7). However, after treatment with H_2O_2, migration of the Ask1-HA immunoprecipitates under nonreducing conditions revealed the presence of MYC-Ask1 with the oxidized species (lane 4). This interaction was covalent since the same extract separated under reducing conditions only showed monomeric forms of Ask1-HA and MYC-Ask1 (lane 8). Hence, Ask1 forms covalently linked homo-oligomers after H_2O_2 treatments. Whether smaller

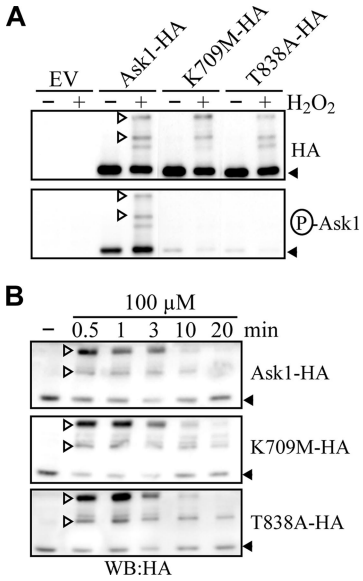

Figure 2. Role of phosphorylation in the oxidation of Ask1. 293T cells were transfected with the empty vector pcDNA3 (EV), pcDNA3-Ask1-HA, pcDNA3-Ask1K709M-HA, or pcDNA3-Ask1T838A-HA. Twenty-four hours later, the cells were left untreated (−) or treated (+) with 1 mM H_2O_2 for 3 min (A) or 100 μM H_2O_2 for different times (B). Cell extracts were migrated on nonreducing gels and immunoblotted with specific antibodies as indicated to detect total HA-tagged Ask1 or phosphorylated Ask1. The expected position of monomeric Ask1 is indicated by closed arrowheads. Higher molecular weight Ask1 complexes are identified by open arrowheads.

proteins are also covalently linked to the Ask1 complex cannot be ruled out. We looked at how Ask1 oxidation relates to phosphorylation using a phospho-specific Ask1 antibody (Figure 1, B, C, and E). Exposure to H_2O_2 induced an onset of Ask1 phosphorylation that occurred as fast as oxidation, being already maximum after 1–3 min. However, phosphorylation differs from oxidation during prolonged exposures. After 30 min, phosphorylation was still maximum (see the reducing gels in Figure 1, B and E), whereas the oxidized species were totally reduced. Intriguingly, JNK phosphorylation kinetics followed more closely Ask1 oxidation than phosphorylation, being transient and highly down-regulated after 30 min (Figure 1, B and E). Similar results were obtained whether transfected (Figure 1B) or endogenous (Figure 1E) proteins were monitored. Phosphorylated Ask1 (both endogenous and transfected Ask1-HA) was also detected with the high-molecular-weight oxidized species (Figure 1, B, C, and E). However, Ask1 phosphorylation was not a prerequisite for its oxidation (Figure 2, A and B). Both the T838A phosphorylation mutant and the K709M kinase dead mutant did not phosphorylate, but they oxidized at levels and kinetics similar to wild-type Ask1.

Oxidized Ask1 Is Reduced by Trx1

The aforementioned data showed that Ask1 oxidation is transient, reverting to its reduced form after ~15 min (Figures 1 and 2), which indicated that the oxidized disulfide-linked multimers are potently reduced back to monomers (or noncovalently linked oligomers). Because Trx1 is a disulfide reductase that mediates thiol–disulfide exchange reactions, we investigated whether the ascribed capacity of Trx1 to regulate Ask1 (Saitoh *et al*., 1998; Liu and Min, 2002; Noguchi *et al*., 2005) might be a consequence of Trx1-mediated Ask1 reduction. First, we evaluated the effect of overexpressing Myc-Trx1 on the Ask1 redox state. The disulfide-bond mediated oligomerization of Ask1 in response to H_2O_2 was substantially decreased in cells overexpressing Trx1 (Figure 3A). In addition, in the cells overexpressing Trx1, a new HA-antibody reactive band migrating just above monomeric Ask1-HA was seen in nontreated cells, the intensity of which increased upon H_2O_2 exposure and then decreased to the level of untreated cells after 20 min (Figure 3A, arrow). To test whether this band was a covalently bound Trx1–Ask1 complex, we reacted the same membrane with an anti-Myc antibody. We observed two bands migrating at 14 (T1) and 28 (T2) kDa, likely corresponding to monomeric and disulfide-linked dimeric Myc-Trx1; a few bands between 30 and 45 kDa; and a high-molecular-weight band (Figure 3A, right, arrow) migrating at the same level as the putative Trx1–Ask1 complex identified with the anti-HA antibody. Similarly to the HA-antibody–reactive band, this Myc-reactive band was present in untreated cells, increased in intensity after H_2O_2 treatment, and decreased at 20 min. The anti-Myc antibody also detected additional faint bands and a smear in the highest position of the gels consistent with an association of Trx1 with disulfide-bound Ask1 species.

To confirm that these bands corresponded to disulfide-linked Trx1–Ask1 complexes, we performed an anti-HA immunoprecipitation of lysates from cells that coexpressed Ask1-HA and Flag-Trx1, followed by anti-HA or anti-Flag Western blotting. The anti-HA antibody (Figure 3B, top left) revealed reduced Ask1 (black arrowhead) and a unique oxidized Ask1 band (empty arrowhead) that matched in size the largest oxidized Ask1 band seen in whole lysates (Figure 3A). This oxidized Ask1 band showed up at 1-min exposure to H_2O_2 and disappeared after 3 min. The anti-Flag antibody (Figure 3B, bottom) revealed in the same immunoprecipitates a band (arrow) at the size of the suspected Trx1–Ask1 complex seen in whole extracts and several species extending up to the largest Ask1 band. Like oxidized Ask1-HA, these Flag-reactive bands occurred 1 min after exposure to H_2O_2, and they started disappearing after 3 min. Because they were revealed by the anti-Flag antibody within the anti-HA immunoprecipitate, these bands should contain disulfide-linked Flag–Trx1 and Ask1–HA complexes possibly with different stoichiometric compositions. Note that within the anti-HA immunoprecipitate, bands corresponding to monomeric or dimeric Trx1 alone were not seen, indicating that Trx1 mostly associate with Ask1 through disulfide linkage. Moreover, no association was observed between Trx1 and a Ask1 mutant (ΔCys mutant; see below) with reduced sensitivity to oxidation (data not shown).

Substitution of the so-called resolving C-terminal cysteine of the CXPC Trx–dithiol redox center of *Escherichia coli* thioredoxin stabilizes its interactions with substrates (Holmgren, 1995; Powis and Montfort, 2001). Thus, we assayed the effect of mutating the resolving Cys35 Trx1 cysteine (Flag-Trx1C35S) to further confirm the Trx1–Ask1 redox linkage. Anti-HA immunoprecipitates of lysates from cells that coexpressed Ask1-HA and Flag-Trx1C35S were probed by Western blot after nonreducing SDS-PAGE (Figure 3B, right). The anti-HA antibody also revealed oxidation of Ask1 to a high-molecular-weight multimers (top), although in this case, all Ask1 was shifted to its oxidized form, and it did not return to its reduced state for up to 60 min. The Flag antibody (bottom) revealed a series of bands similar to those seen with wild-type Trx1, except that the more slowly migrating bands were much more intense and persisted longer (up to 60 min). In these conditions where Ask1 was not

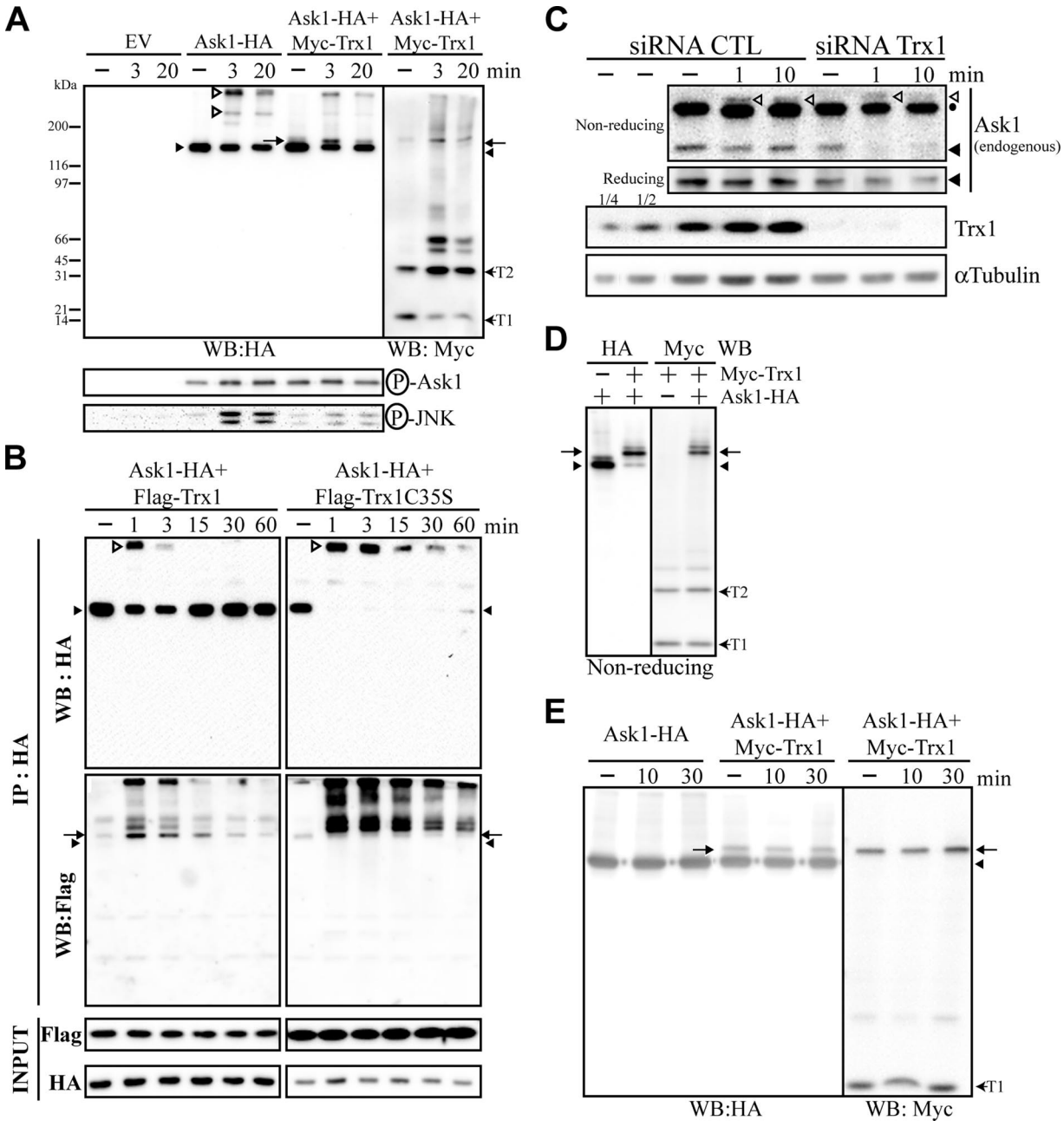

Figure 3. Regulation of Ask1 by Trx1. (A, B, D, and E) 293T cells were transfected with the empty vector pcDNA3 (EV), pcDNA3-Ask1-HA alone, or pcDNA3-Ask1-HA together with either pCMV5-Myc-Trx1, pRK-FLAG-Trx1, or pRK-FLAG-Trx1C35S. Twenty-four hours later, the cells were left untreated (−) or exposed to 1 mM H_2O_2 (A and B) or to 4 μM thapsigargin (E) for the time indicated. Total cell extracts (A, E, and INPUT in B), TCA-precipitated cell extracts (D), or anti-HA immunoprecipitated extracts (IP:HA in B) were analyzed by Western blot with either anti-HA (WB:HA), anti-Myc (WB:Myc), anti-Flag (WB:Flag), phospho-specific–Ask1 (P-Ask1), or JNK (P-JNK) antibodies as indicated. All samples were migrated in nonreducing gels except for the phospho-specific Ask1 and JNK blots in A and the INPUT samples in B. The expected position of monomeric Ask1 is indicated by closed arrowheads. Higher molecular weight Ask1 complexes are identified by open arrowheads. T1 and T2 indicate the expected positions of monomeric (14 kDa) and dimeric (28 kDa) Trx1. The arrows indicate the position of a Trx1–Ask1 complex migrating just above monomeric Ask1. (C) Effect on endogenous Ask1: 293T cells were transefected with siCONTROL (siRNA CTL) or with an siRNA against Trx1 (siRNA Trx1). Forty-eight hours later, the cells were left untreated (−) or exposed to 1 mM H_2O_2 for the times indicated. Total cell extracts were migrated under nonreducing or reducing conditions and probed with anti-Ask1 or under reducing conditions and probed with anti-Trx1 and anti-α-tubulin. The first (labeled "1/4") and second tracks (labeled "1/2") of the anti-Trx1 blot were loaded with 4 and 2 times less material as the third tracks and serve as a scale to evaluate the efficiency of the Trx1 knockdown (<25%). Monomeric Ask1 (closed arrowheads) and higher molecular weight Ask1 complexes (open arrowheads) migrated as indicated. The dot indicates a strong (relative to endogenous Ask1) nonspecific band recognized by the anti-Ask1 antibody in the nonreducing gels. When indicated, the numbers on the left refer to the position of molecular weight standards migrated under nonreducing conditions.

reduced, a decrease in the total level of Ask1 (oligomers + monomers) was seen after 60 min (top right). This reduction did not seem to be due to the degradation of Ask1 during the treatment, because the total amount of Ask1 seen in the reducing gels did not decrease (INPUT lane). It is likely that the loss of Ask1 was due to further multimerization and trapping of the oxidized species in the SDS-PAGE stacking gel. Similar results were obtained with endogenous Ask1 when Trx1 was knocked down using specific siRNA (Figure 3C). Depletion of endogenous Trx1 to <25% of its normal level enhanced the oxidation of Ask1 in response to H_2O_2 treatments, leading to the loss of monomeric Ask1 in the nonreducing gels (black arrowhead) but to little change in total Ask1 in the reducing gel.

The association between Ask1-HA and Myc-Trx1 was also observed when extracts were prepared by acid precipitation, and in fact, they even seemed more stable than when solubilized directly (Figure 3D). This suggested that the association was not induced by oxidative conditions generated during extraction and that it was dependent on disulfide reactions occurring in the cells between Trx1 and Ask1. Furthermore, the oxidation of Ask1 and its reduction by Trx1 was specific to the H_2O_2 activation signal. No enhanced association was observed between Ask1 and Trx1 when Ask1 was activated by thapsigargin, an endoplasmic reticulum (ER) stress-inducing chemical that activates Ask1 (Nishitoh *et al*., 2002; Figure 5E), but that does not lead to Ask1 oxidation. The basal level of interaction between Trx1 and Ask1 was kept unchanged after thapsigargin treatment (Figure 3E, arrow). These data strongly suggest that oxidized Ask1 generated during H_2O_2 treatments is rapidly reduced by Trx1.

Ask1 Cysteine Mutants Insensitive to Oxidation

The oxidation of Ask1 to large multimeric species presumably involves the formation of disulfide bonds. We sought to identify which of the 23 cysteine residues of Ask1 contributed these redox linkages. As a first step toward this goal, we analyzed the redox behavior of deletion mutants, each lacking one of the three major domains of Ask1: C-terminal, N-terminal, and kinase domains (ΔC-Ask1, ΔN-Ask1, and ΔK-Ask1, respectively) (Figure 4A). On H_2O_2 treatments, all deletion mutants oxidized with kinetics similar to the wild-type protein, indicating that each still contained redox-active cysteine residues (Figure 4B).

Thus, we individually analyzed the redox behavior of each of the three Ask1 domains (C-Ask1, K-Ask1, and N-Ask1) (Figure 4, C–E). All three single-domain proteins oxidized upon exposure to H_2O_2. The C domain formed dimers upon oxidation, and these dimers were still produced after any single substitutions of its three cysteine residues. However, the double mutants C1006-1352A and C1006-1361A, and the triple mutant C1006-1352-1361A did not or only barely oxidized. The Ask1 K domain also formed dimers upon oxidation (Figure 4D). Individual replacement of each of its five cysteine residues identified Cys835 as important for oxidation. The N domain formed several multimeric complexes of different sizes upon oxidation (Figure 4E). We analyzed eight of its 15 cysteine residues. A C22-30A and a C250A N-domain mutant still potently oxidized, whereas the C22-30-250A mutant was relatively resistant to oxidation (Figure 4E). A C391-621-622-638-643A mutant studied in the context of the whole protein also oxidized normally (data not shown).

We next combined these domain substitution mutants in the context of the full-length protein (Figure 5A). None of the oxidation-resistant single domain mutants were significantly impaired for oxidation (Figure 5B). However, the triple domain mutant ΔCys (C22-30-250A-835-1006-1052-1361A) was barely oxidized (Figure 5C, lanes 5 and 6; D, lanes 3 and 4; and E, lanes 3 and 6). We conclude that Ask1 contains several cysteines that can serve as redox-active residues to mediate H_2O_2-induced protein oxidation, precluding identification of the disulfide bonds of native Ask1. ΔCys was used for functional experiments.

Ask1 Oxidation Is required for JNK Activation and Apoptosis Induction by H_2O_2

Ask1 oxidation by H_2O_2 was not affected by mutations impeding its phosphorylation (Figure 2), indicating that phosphorylation is not a prerequisite for oxidation. Next, we next evaluated whether, reciprocally, Ask1 oxidation was required for its phosphorylation upon H_2O_2 treatment.

JNK is activated in response to H_2O_2 in control-untransfected cells (Figure 1E); however, its level of phosphorylation is increased severalfold in cell transfected with wild-type Ask1 (Figures 1A, 3A, and 5, B, C, and E). As reported previously (Saitoh *et al*., 1998), we found that Trx1 overexpression severely inhibits the activation of JNK downstream of Ask1 in response to H_2O_2 (Figure 3A, bottom). Trx1 overexpression may inhibit H_2O_2-induced Ask1 activation by promoting its reduction, but it could also act through inhibitory association as proposed previously (Saitoh *et al*., 1998). We thus examined the ability of oxidation-resistant Ask1 mutants to activate JNK in response to H_2O_2. Transfection of the Ask1 mutants NΔCys, KΔCys, and CΔCys, which oxidized as well as Ask1, resulted in an enhancement of JNK activation after mild 100 μM H_2O_2 treatments as strong as found after transfecting wild-type Ask1 (Figure 5B). In contrast, the ΔCys mutant, which only barely oxidized, did not activate JNK even after a treatment with 1 mM H_2O_2 (Figure 5C, lanes 5 and 6, and D, lanes 3 and 4). Ask1 activates JNK indirectly through the activation of MKK4 and MKK7 (Chang and Karin, 2001; Chen *et al*., 2001). ΔCys was also very inefficient in inducing the phosphorylation of MKK4 and MKK7 (Figure 5D, lanes 3 and 4).

ΔCys contains seven amino acid substitutions that could alter its activity by a redox-independent manner. Thus, we tested whether this mutant was still activated in response to thapsigargin. Thapsigargin triggered the phosphorylation of JNK as strongly as H_2O_2 in cells transfected with wild-type Ask1, but it did not lead to Ask1 oxidation (Figure 5E, lanes 7 and 8). Thapsigargin also fully activated JNK downstream of the ΔCys mutant (lane 9), demonstrating that the defective activation of this mutant by H_2O_2 was due to its impaired oxidation and not to a redox-independent defect caused by effects of the mutations on the structure of the protein.

We also looked at whether inhibiting Ask1 oxidation during H_2O_2 treatment affects Ask1 phosphorylation. Lowering Ask1 oxidation by overexpressing Trx1 did not cause a reduction in the level of H_2O_2-induced Ask1 phosphorylation, suggesting that oxidation might have a minimal effect on the induction of phosphorylation (Figure 3A). However, for unclear reasons, it also increased the basal level of Ask1 phosphorylation. As expected, all the mutants that were not impaired for oxidation, i.e., NΔCys, KΔCys, and CΔCys, were phosphorylated at the same level as wild-type Ask1 in both control and H_2O_2-treated cells (Figure 5B).The ΔCys mutant was generally slightly less phosphorylated in response to H_2O_2, however, the basal level also was reduced compared with wild-type Ask1 in such a way that induction was the same (Figure 5C, lanes 5 and 6; D, lanes 3 and 4; and E, lanes 3

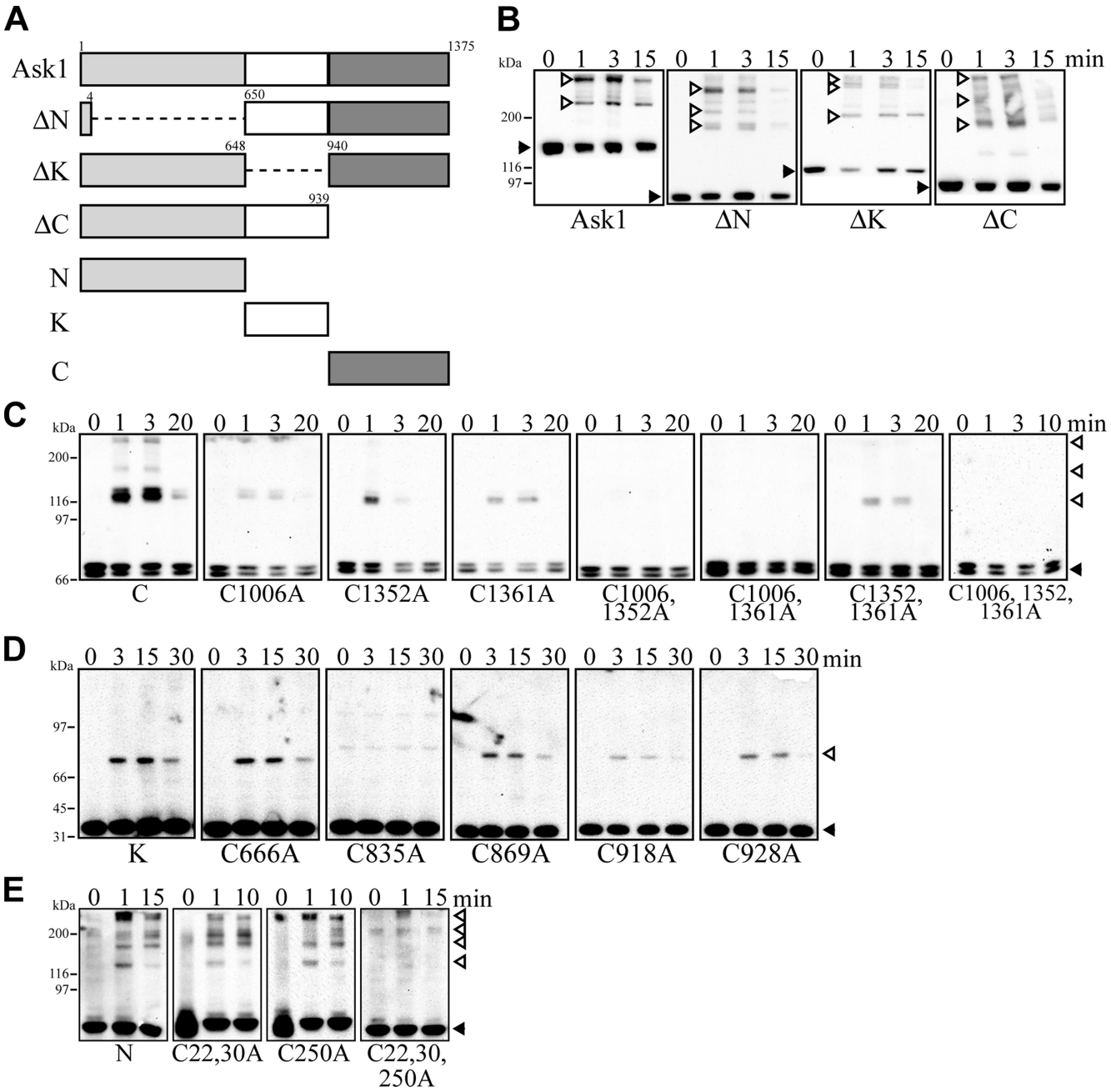

Figure 4. Identification of oxidation-sensitive cysteines. (A) Schematic representation of the N (N-terminal), K (kinase), and C (C-terminal) domain of Ask1 and of the Ask1 mutant and deletant constructs used in this figure. The numbers indicate amino acids at the borders. All constructs have an HA-tag at the C-terminal end. (B–E) 293T cells were transfected with the various Ask1 constructs as indicated at the bottom of each panel. The cells were transfected with full-length Ask1 (Ask1), Ask1 deleted of domain N, K, or C (B), with domain C-only (C), K-only (D), or N-only (E) proteins. The labels "Cx,yA" indicate that the cysteine residues at position x and y of the corresponding Ask1 domain-only protein have been replaced by alanine residues. Twenty-four hours after transfection, the cells were treated with 100 (C and E) or 400 μM (B and D) H_2O_2 for the time (minutes) indicated at the top of the panels. Extracts were migrated on nonreducing gels and immunoblotted with anti-HA. The expected positions of the monomeric Ask1 constructs are indicated by closed arrowheads. Higher molecular weight Ask1 complexes are identified by open arrowheads. The numbers on the left refer to the position of molecular weight standards migrated under nonreducing conditions.

and 6). In fact, both control and activated levels of ΔCys were also lower than that of control Ask1 in cells treated with thapsigargin (Figure 5E, lanes 2 and 3 and 8 and 9). We concluded that, most likely, the ΔCys mutant was slightly less active, but it was still as inducible as the wild-type cells. Overall, the results showed that the formation of disulfide bonds between Ask1 molecules is not required for Ask1 phosphorylation but that it is essential for JNK activation in response to H_2O_2.

Finally, the role of oxidation in the biological activation of Ask1 by H_2O_2 was tested by looking at apoptosis induction. As expected from the known apoptotic activity of Ask1

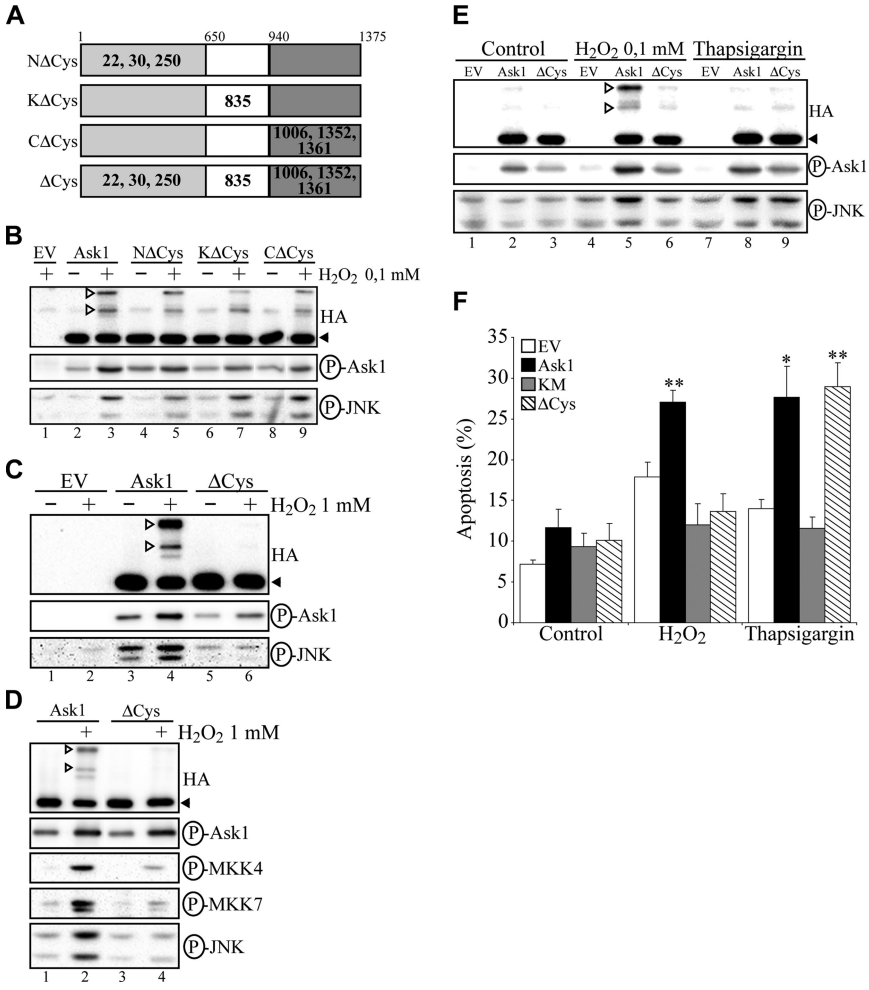

Figure 5. Role of oxidation in H_2O_2-induced Ask1 signaling pathways. (A) Schematic representation of the various cysteine substitution mutants of the N (N-terminal), K (kinase), and C (C-terminal) domain of Ask1 used in this figure. The numbers indicated within each domain correspond to the cysteine residues that have been replaced by alanine residues. All constructs have an HA-tag at the C-terminal end. (B–E) 293T cells were transfected with the various Ask1 constructs as indicated at the top of each panel and defined in A, or transfected with the empty vector pcDNA3 (EV). Twenty-four hours after transfection, the cells were left untreated (−) or treated (+) with 0.1 mM (B and E) or 1 mM H_2O_2 (C and D) for 3 min, or with 10 μM thapsigargin for 10 min (E). Extracts were migrated on nonreducing gels and immunoblotted with anti-HA or on reducing gels and probed with phospho-specific Ask1 (P-Ask1), JNK (P-JNK), MKK4 (P-MKK4), or MKK7 (P-MKK7) antibodies. The expected position of the monomeric Ask1 constructs is indicated by closed arrowheads. Higher molecular weight Ask1 complexes are identified by open arrowheads. Lane numbers are referred to in the text. (F) HeLa cells were transfected with the pcDNA3 empty vector (EV), pcDNA3-Ask1-HA (Ask1), pcDNA3-Ask1K709M (KM), or pcDNA3-Ask1ΔCys-HA (ΔCys). Forty-eight hours later, the cells were left untreated (Control) or exposed to 500 μM H_2O_2 or to 10 μM thapsigargin for 3 h. The percentage of HA-positive cells showing condensed or fragmented nuclei was determined by immunofluorescence microscopy. In the EV group, all cells were considered in the counts. The data are means + SEM from four (Control and H_2O_2) or three (Thapsigargin) distinct experiments. $^{**}p < 0.01$; $^{*}p < 0.05$ (one-way analysis of variance), comparing apoptosis in cells transfected with Ask1-HA or ΔCys-HA with EV transfected cells within the same treatment (H_2O_2 or thapsigargin).

(Ichijo *et al.*, 1997; Gotoh and Cooper, 1998; Nishitoh *et al.*, 2002), overexpression of wild-type Ask1, but not of a kinase dead mutant of Ask1, caused a marked sensitization to both H_2O_2-induced and thapsigargin-induced apoptosis. However, the ΔCys mutant had no stimulating activity on H_2O_2-induced apoptosis, whereas it did sensitize to thapsigargin-induced apoptosis to the same level as wild-type Ask1 (Figure 5F).

We conclude that the formation of disulfide-containing multimers of ASK1 is essential for the activation of JNK and induction of apoptosis in response to H_2O_2.

DISCUSSION

The results of this study show that disulfide-bond–mediated Ask1 oligomerization is essential for signaling downstream of Ask1 after H_2O_2 treatments. The results agree with previous models suggesting that Trx1 is a negative regulator of Ask1 (Saitoh *et al.*, 1998), but they propose a new mechanism of regulation in which Trx1 reduces oxidized Ask1, thereby hampering or turning off the signal.

Ask1 Is Oxidized during Oxidative Stress

Modification of cysteine is a posttranslational modification emerging as a new mechanism for the regulation of protein (Filomeni *et al.*, 2005). In bacteria and yeast, oxidation leading to the formation of intramolecular disulfide bonds and activation of the transcriptional factors OxyR and Yap1 play an important role in the response of these organisms to oxidative stress (Zheng *et al.*, 1998; Delaunay *et al.*, 2002). Likewise, an important component of mammalian cell responses to oxidative stress involves the oxidation of active cysteines in many types of proteins, thereby regulating several activities. Some transcription factors, such as activator protein-1 and nuclear factor-κB (NF-κB) bear oxidation-sensitive cysteines and they are regulated through oxidation during oxidative stress (Abate *et al.*, 1990; Toledano and Leonard, 1991; Matthews *et al.*, 1992). The formation of intramolecular disulfides in the catalytic core of many phosphatases, including protein tyrosine phosphatase and lipid phosphatases such as phosphatase with sequence homology to tensin (PTEN), results in their reversible inhibition (Lee *et al.*, 2002; Rhee *et al.*, 2005; Salmeen and Barford, 2005; Stone and Yang, 2006). Oxidative stress-induced glutathionylation inhibits the kinase activity of the mitogen-activated protein kinase kinase kinase 1 (Cross and Templeton, 2004). In contrast, glutathionylation of Ras mediates redox signaling in response to angiotensin (Adachi *et al.*, 2004). Oxidative stress induces an intermolecular covalent interaction between the two SUMO-conjugating enzymes Uba2 and Ubc9, and it negatively regulates proteins sumoylation (Bossis and Melchior, 2006). Oxidation of Ask1 seems to be unique in several aspects. First, the oxidation of Ask1 leads to multimerization mediated by intermolecular disulfide bonds. Such

intermolecular interactions have the potential of stabilizing the noncovalently associated oligomers of Ask1, perhaps changing its structure. This could have favored transactivation, because many kinases are activated through oligomerization-dependent events leading to phosphorylation (Gotoh and Cooper, 1998; Kyriakis and Avruch, 2001; Tobiume *et al*., 2002). However, for Ask1, downstream signaling, not its phosphorylation, seems to require oxidation. Second, several cysteines in Ask1 seem to be sensitive to oxidation, making Ask1 an extremely sensitive target of oxidation. From our mutational analysis, we concluded that five or six cysteines at position 22 and/or 30, 250, 835, 1052 or 1361, and 1006 need to be replaced to obtain a protein that is relatively insensible to disulfide-bond–mediated multimerization. Whether oxidation at all these sites really occur in the intact protein remains to be determined. The mechanism by which Ask1 is oxidized also remains to be determined. Oxidation may occur indirectly as with the yeast Yap1 protein where a thiol peroxidase, Orp1/Gpx3, is first oxidized before oxidizing Yap1 (Delaunay *et al*., 2002). H_2O_2-induced disulfide bond formation can also be initiated by the direct oxidation of a cysteine to Cys-SOH followed by the attack of a nearby Cys-thiol. This is the likely mechanism of action for many phosphotyrosine phosphatase and also PTEN, in which an active Cys is located in an environment of basic amino acid residues that makes it sensitive to direct oxidation (Rhee *et al*., 2005; Salmeen and Barford, 2005).

Trx1 Is a Reductant of Ask1

Thioredoxin is thought to regulate the redox state of numerous cellular proteins, although a direct in vivo biochemical demonstration of this function lacks in mammals. Thioredoxin reduces disulfide bonds by a thiol–disulfide exchange reaction involving the nucleophilic attack of one of the two cysteines of the substrate disulfide bond by the N-terminal cysteine of the thioredoxin CXPC dithiol redox center (Cys32 in Trx1), leading to a disulfide linkage between thioredoxin and its substrate (Powis and Montfort, 2001). A second thiol–disulfide exchange between the C-terminal cysteine of the thioredoxin–dithiol redox center (Cys35) and the remaining oxidized cysteine of the substrate results in the complete reduction of the substrate and the formation of an intramolecular disulfide bond between Cys32 and Cys35 of thioredoxin. A specific role of thioredoxin in substrate reduction has been suggested on the basis of its disulfide linkage with candidate substrate in vitro, as shown for NF-κB, Ref-1, and PTEN (Hirota *et al*., 1997, 1999; Lee *et al*., 2002), and in vivo by the cellular effect of its overexpression, although in the later case, the observed effects could be totally indirect. In the present study, the demonstrated in vivo direct linkage between Trx1 and Ask1 and the stabilization of this linkage with a mutant of Trx1 lacking Cys35 provide very strong evidence of the functional interaction between these proteins and the specific reductase function of Trx1 toward Ask1.

Previous studies, including our own, led to the suggestion that Trx1 associates with Ask1 in unstressed cells and dissociates upon oxidative stress (Saitoh *et al*., 1998; Liu *et al*., 2000; Dorion *et al*., 2002; Liu and Min, 2002; Song and Lee, 2003; Noguchi *et al*., 2005). We confirmed here that a significant portion of Trx1 associates with Ask1 in unstressed cells. However, this association seemed exclusively covalent, mediated by disulfide linkages. We also showed that this association increases immediately after treatment with H_2O_2 and that it decreases after 5–10 min in parallel with the recycling of reduced Ask1. The redox nature of the interaction between Trx1 and Ask1 has not been investigated in the aforementioned studies, and the increased interaction might have been missed by focusing at later times after exposure to H_2O_2. Nevertheless, it was shown that a double Trx1 mutant in which both Cys32 and Cys35 were changed for a Ser residue was not immunoprecipitated with Ask1 (Liu and Min, 2002), which is consistent with their disulfide-mediated association that we report here. The observation of a covalent association between Trx1 with Ask1 in unstressed cells is surprising considering the transient nature of the interaction between Trx1 and its substrates. It is possible that this basal covalent association reflects a low level of Ask1 oxidation by endogenous reactive oxygen species (ROS) and its continuous reduction by Trx1. It was proposed that Ask1 Cys250 was important in the Ask1–Trx1 interaction in unstressed cells (Zhang *et al*., 2004), which could suggest that this residue contributes to the basal Ask1–Trx1 mixed disulfide we have identified in unstressed cells. We confirmed that the ΔCys mutant (which includes a mutation at Cys250) did not form a covalent complex with Trx1 (data not shown).

An Alternative Model for the Redox Regulation of Ask1

The finding that the formation of disulfide bonds between Ask1 molecules is required for downstream activation of JNK and apoptosis during H_2O_2 treatment, and that Trx1 negatively regulates this oxidation, is consistent with most published data concerning the role of Trx1 in regulating Ask1 activity, but requires a reevaluation of the current model (Figure 6). In resting cells, homo-oligomers of Ask1 are formed through C-terminal coiled-coil domain interactions (Tobiume *et al*., 2002). These preformed homo-oligomers assemble with other component to form a high-molecular-weight complex (1500–2000 kDa) called the Ask1 signalosome (Noguchi *et al*., 2005). On H_2O_2 stimulation, a molecular rearrangement of the signalosome into a higher-molecular-weight complex (>3000 kDa) allows Ask1 phosphorylation/activation, a process which was considered to be triggered by the oxidation of Trx1, leading to its dissociation from Ask1 and allowing the association of Ask1 with other proteins such as tumor necrosis factor-associated factor (TRAF)-2/6 that are essential for Ask1 activation (Liu *et al*., 2000; Noguchi *et al*., 2005) (Figure 6, Trx1 oxidation model). We confirmed that Ask1 exist in resting cells as noncovalently associated Ask1 homo-oligomers, but we found that these homo-oligomers became covalently cross-linked after treatments. ΔCys, a mutant with diminished sensitivity to oxidation by H_2O_2, has a normal capacity to be phosphorylated and activated (see the results with tharsigargin) but has an impaired ability to activate JNK and to mediate apoptosis in response to H_2O_2. By triggering Ask1 intermolecular disulfide bond formation, H_2O_2 might promote a more stable or different Ask1 multimeric structure, which, although not required for autophosphorylation and activation, might favor a different type of interaction between Ask1 molecules and between Ask1 and essential cofactors, such as TRAF-2/6 and protein kinase D (Gotoh and Cooper, 1998; Noguchi *et al*., 2005; Zhang *et al*., 2005), thereby inducing competence for downstream signaling. The overexpression of Trx1, which speeds up the reduction of Ask1, or the substitution of the redox-active cysteine residues might prevent the formation of such competent complexes (Figure 6, Ask1 oxidation model).

Ask1 oxidation may also be required in the case of other stimuli. For example, ROS production seems essential for Ask1 activation induced by tumor necrosis factor (TNF) or lipopolysaccharide, because, in these cases also, Ask1 activation is blocked by antioxidants (Liu *et al*., 2000; Mat-

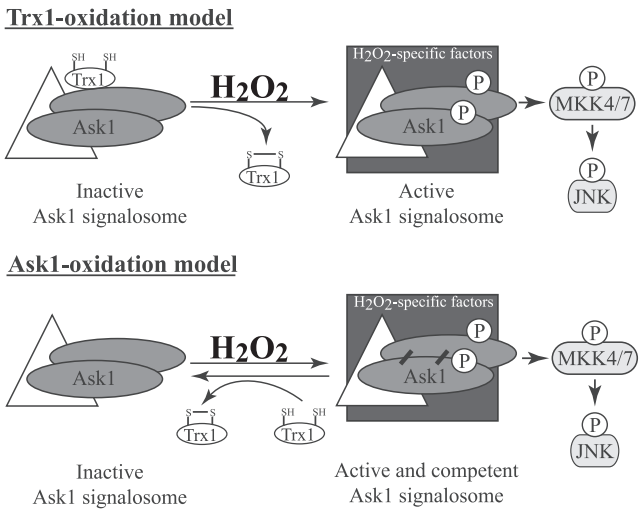

Figure 6. New model for the activation of the Ask1-signaling pathway by H_2O_2. Trx1-oxidation model (Old model): Trx1 oxidation model. In resting cells, multimers of Ask1 are found associated with other factors (triangle) in a complex called the signalosome. In this complex, the association of Trx1 with Ask1 maintains the signalosome in an inactive form. Treatment with H_2O_2 leads to the oxidation of Trx1 and its release from the signalosome, allowing the binding of additional factors, including TRAF6, which leads to the phosphorylation/activation of Ask1. Ask1-oxidation model (Proposed model): Ask1 oxidation model. In addition to phosphorylation, treatment with H_2O_2 induces the formation of disulfide bonds between Ask1 molecules (thick lines within the Ask1 molecules). Both phosphorylation and oxidation, which occur independently of each other, are required for competent signaling to MKK4/7, JNK, and apoptosis (not shown). Whereas phosphorylation is required and sufficient for Ask1 activation, oxidation is essential for the subsequent phosphorylation of MKK4/7 and JNK, likely by eliciting the recruitment of specific factors mediating MKK4/7 phosphorylation. In this model, Trx1 associates transiently with Ask1. It regulates negatively the pathway by reducing Ask1 multimers.

suzawa *et al*., 2005). However, oxidation is not required in all cases of stimulation of the Ask1 pathway. We showed here that JNK was phosphorylated and apoptosis was induced in the absence of Ask1 oxidation (the ΔCys mutant) in response to the ER stress agent thapsigargin, indicating that different stimulus-specific mechanisms of substrate recruitment exist after Ask1 stimulation. It is possible that the MKK4/7-JNK recruiting function provided by mixed disulfide bond formation upon activation by H_2O_2 can occur alternatively through the use of stimulus-specific adaptors after other types of activating treatments, e.g., death-associated protein-6, inositol-requiring enzyme 1, or ASK1-interacting protein-1 upon activation by Fas, ER stress, or TNF, respectively (Chang *et al*., 1998; Nishitoh *et al*., 2002; Zhang *et al*., 2004). Different activators could also activate different pools of Ask1 already associated with different specific recruiting scaffolds or regulators.

ACKNOWLEDGMENTS

We thank Dr. Hidenori Ichijo, John Kyriakis, and Wang Min for providing plasmids. This work was supported by the Canadian Institutes of Health Research and the Canada Research Chair in Stress Signal Transduction (to J.L.).

REFERENCES

Abate, C., Patel, L., Rauscher, F. J., 3rd, and Curran, T. (1990). Redox regulation of fos and jun DNA-binding activity in vitro. Science *249*, 1157–1161.

Adachi, T., Pimentel, D. R., Heibeck, T., Hou, X., Lee, Y. J., Jiang, B., Ido, Y., and Cohen, R. A. (2004). S-Glutathiolation of Ras mediates redox-sensitive signaling by angiotensin II in vascular smooth muscle cells. J. Biol. Chem. *279*, 29857–29862.

Bossis, G., and Melchior, F. (2006). Regulation of SUMOylation by reversible oxidation of SUMO conjugating enzymes. Mol. Cell *21*, 349–357.

Chang, H. Y., Nishitoh, H., Yang, X., Ichijo, H., and Baltimore, D. (1998). Activation of apoptosis signal-regulating kinase 1 (ASK1) by the adapter protein Daxx. Science *281*, 1860–1863.

Chang, L., and Karin, M. (2001). Mammalian MAP kinase signalling cascades. Nature *410*, 37–40.

Chen, Z., Gibson, T. B., Robinson, F., Silvestro, L., Pearson, G., Xu, B., Wright, A., Vanderbilt, C., and Cobb, M. H. (2001). MAP kinases. Chem. Rev. *101*, 2449–2476.

Cross, J. V., and Templeton, D. J. (2004). Oxidative stress inhibits MEKK1 by site-specific glutathionylation in the ATP binding domain. Biochem. J. *381*, 675–683.

Delaunay, A., Pflieger, D., Barrault, M. B., Vinh, J., and Toledano, M. B. (2002). A thiol peroxidase is an H2O2 receptor and redox-transducer in gene activation. Cell *111*, 471–481.

Deyulia, G. J., Jr., Carcamo, J. M., Borquez-Ojeda, O., Shelton, C. C., and Golde, D. W. (2005). Hydrogen peroxide generated extracellularly by receptor-ligand interaction facilitates cell signaling. Proc. Natl. Acad. Sci. USA *102*, 5044–5049.

Dorion, S., Lambert, H., and Landry, J. (2002). Activation of the p38 signaling pathway by heat shock involves the dissociation of glutathione S-transferase Mu from Ask1. J. Biol. Chem. *277*, 30792–30797.

Filomeni, G., Rotilio, G., and Ciriolo, M. R. (2005). Disulfide relays and phosphorylative cascades: partners in redox-mediated signaling pathways. Cell Death Differ. *12*, 1555–1563.

Gotoh, Y., and Cooper, J. A. (1998). Reactive oxygen species- and dimerization-induced activation of apoptosis signal-regulating kinase 1 in tumor necrosis factor-alpha signal transduction. J. Biol. Chem. *273*, 17477–17482.

Guay, J., Lambert, H., Gingras-Breton, G., Lavoie, J. N., Huot, J., and Landry, J. (1997). Regulation of actin filament dynamics by p38 map kinase-mediated phosphorylation of heat shock protein 27. J. Cell Sci. *110*, 357–368.

Hirota, K., Matsui, M., Iwata, S., Nishiyama, A., Mori, K., and Yodoi, J. (1997). AP-1 transcriptional activity is regulated by a direct association between thioredoxin and Ref-1. Proc. Natl. Acad. Sci. USA *94*, 3633–3638.

Hirota, K., Murata, M., Sachi, Y., Nakamura, H., Takeuchi, J., Mori, K., and Yodoi, J. (1999). Distinct roles of thioredoxin in the cytoplasm and in the nucleus. A two-step mechanism of redox regulation of transcription factor NF-kappaB. J. Biol. Chem. *274*, 27891–27897.

Holmgren, A. (1995). Thioredoxin structure and mechanism: conformational changes on oxidation of the active-site sulfhydryls to a disulfide. Structure *3*, 239–243.

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.

Jeong, W., Chang, T. S., Boja, E. S., Fales, H. M., and Rhee, S. G. (2004). Roles of TRP14, a thioredoxin-related protein in tumor necrosis factor-α signaling pathways. J. Biol. Chem. *279*, 3151–3159.

Kadowaki, H. *et al*. (2005). Amyloid beta induces neuronal cell death through ROS-mediated ASK1 activation. Cell Death Differ. *12*, 19–24.

Kyriakis, J. M., and Avruch, J. (2001). Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol. Rev. *81*, 807–869.

Landry, J., Chrétien, P., Lambert, H., Hickey, E., and Weber, L. A. (1989). Heat shock resistance conferred by expression of the human HSP27 gene in rodent cells. J. Cell Biol. *109*, 7–15.

Lee, J., Godon, C., Lagniel, G., Spector, D., Garin, J., Labarre, J., and Toledano, M. B. (1999). Yap1 and Skn7 control two specialized oxidative stress response regulons in yeast. J. Biol. Chem. *274*, 16040–16046.

Lee, S. R., Yang, K. S., Kwon, J., Lee, C., Jeong, W., and Rhee, S. G. (2002). Reversible inactivation of the tumor suppressor PTEN by H2O2. J. Biol. Chem. *277*, 20336–20342.

Liu, H., Nishitoh, H., Ichijo, H., and Kyriakis, J. M. (2000). Activation of apoptosis signal-regulating kinase 1 (ASK1) by tumor necrosis factor receptor-associated factor 2 requires prior dissociation of the ASK1 inhibitor thioredoxin. Mol. Cell. Biol. *20*, 2198–2208.

Liu, Y., and Min, W. (2002). Thioredoxin promotes ASK1 ubiquitination and degradation to inhibit ASK1-mediated apoptosis in a redox activity-independent manner. Circ. Res. *90*, 1259–1266.

Machino, T., Hashimoto, S., Maruoka, S., Gon, Y., Hayashi, S., Mizumura, K., Nishitoh, H., Ichijo, H., and Horie, T. (2003). Apoptosis signal-regulating kinase 1-mediated signaling pathway regulates hydrogen peroxide-induced apoptosis in human pulmonary vascular endothelial cells. Crit. Care Med. *31*, 2776–2781.

Matsuzawa, A., and Ichijo, H. (2005). Stress-responsive protein kinases in redox-regulated apoptosis signaling. Antioxid. Redox Signal. *7*, 472–481.

Matsuzawa, A., Saegusa, K., Noguchi, T., Sadamitsu, C., Nishitoh, H., Nagai, S., Koyasu, S., Matsumoto, K., Takeda, K., and Ichijo, H. (2005). ROS-dependent activation of the TRAF6-ASK1–p38 pathway is selectively required for TLR4-mediated innate immunity. Nat. Immunol. *6*, 587–592.

Matthews, J. R., Wakasugi, N., Virelizier, J. L., Yodoi, J., and Hay, R. T. (1992). Thioredoxin regulates the DNA binding activity of NF-kappa B by reduction of a disulphide bond involving cysteine 62. Nucleic Acids Res. *20*, 3821–3830.

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.

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.

Powis, G., and Montfort, W. R. (2001). Properties and biological activities of thioredoxins. Annu. Rev. Biophys. Biomol. Struct. *30*, 421–455.

Rhee, S. G., Kang, S. W., Jeong, W., Chang, T. S., Yang, K. S., and Woo, H. A. (2005). Intracellular messenger function of hydrogen peroxide and its regulation by peroxiredoxins. Curr. Opin. Cell Biol. *17*, 183–189.

Saadatzadeh, M. R., Bijangi-Vishehsaraei, K., Hong, P., Bergmann, H., and Haneline, L. S. (2004). Oxidant hypersensitivity of Fanconi anemia type C-deficient cells is dependent on a redox-regulated apoptotic pathway. J. Biol. Chem. *279*, 16805–16812.

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.

Salmeen, A., and Barford, D. (2005). Functions and mechanisms of redox regulation of cysteine-based phosphatases. Antioxid. Redox Signal. *7*, 560–577.

Sayama, K., Hanakawa, Y., Shirakata, Y., Yamasaki, K., Sawada, Y., Sun, L., Yamanishi, K., Ichijo, H., and Hashimoto, K. (2001). Apoptosis signal-regulating kinase 1 (ASK1) is an intracellular inducer of keratinocyte differentiation. J. Biol. Chem. *276*, 999–1004.

Song, J. J., and Lee, Y. J. (2003). Differential role of glutaredoxin and thioredoxin in metabolic oxidative stress-induced activation of apoptosis signal-regulating kinase 1. Biochem. J. *373*, 845–853.

Stone, J. R., and Yang, S. (2006). Hydrogen peroxide: a signaling messenger. Antioxid. Redox Signal. *8*, 243–270.

Storz, G., and Tartaglia, L. A. (1992). OxyR: a regulator of antioxidant genes. J. Nutr. *122*, 627–630.

Sumbayev, V. V., and Yasinska, I. M. (2005). Regulation of MAP kinase-dependent apoptotic pathway: implication of reactive oxygen and nitrogen species. Arch. Biochem. Biophys. *436*, 406–412.

Takeda, K., Hatai, T., Hamazaki, T. S., Nishitoh, H., Saitoh, M., and Ichijo, H. (2000). Apoptosis signal-regulating kinase 1 (ASK1) induces neuronal differentiation and survival of PC12 cells. J. Biol. Chem. *275*, 9805–9813.

Thelander, L. (1974). Reaction mechanism of ribonucleoside diphosphate reductase from *Escherichia coli*. Oxidation-reduction–active disulfides in the B1 subunit. J. Biol. Chem. *249*, 4858–4862.

Tobiume, K., Matsuzawa, A., Takahashi, T., Nishitoh, H., Morita, K., Takeda, K., Monowa, O., Noda, T., and Ichijo, H. (2001). ASK1 is required for sustained activations of JNK/p38 MAP kinases and apoptosis. EMBO Rep. *2*, 222–228.

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.

Toledano, M. B., and Leonard, W. J. (1991). Modulation of transcription factor NF-kappa B binding activity by oxidation-reduction in vitro. Proc. Natl. Acad. Sci. USA *88*, 4328–4332.

Ueno, M., Masutani, H., Arai, R. J., Yamauchi, A., Hirota, K., Sakai, T., Inamoto, T., Yamaoka, Y., Yodoi, J., and Nikaido, T. (1999). Thioredoxin-dependent redox regulation of p53-mediated p21 activation. J. Biol. Chem. *274*, 35809–35815.

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.

Zhang, W., Zheng, S., Storz, P., and Min, W. (2005). Protein kinase D specifically mediates apoptosis signal-regulating kinase 1-JNK signaling induced by H2O2 but not tumor necrosis factor. J. Biol. Chem. *280*, 19036–19044.

Zheng, M., Aslund, F., and Storz, G. (1998). Activation of the OxyR transcription factor by reversible disulfide bond formation. Science *279*, 1718–1721.