

Apoptosis Signal-regulating Kinase 1 (ASK1)-p38 Pathway-dependent Cytoplasmic Translocation of the Orphan Nuclear Receptor NR4A2 Is Required for Oxidative Stress-induced Necrosis*

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Background: The molecular mechanisms of p38 MAPK-mediated necrosis currently have not been well elucidated.

Results: During oxidative stress, NR4A2 is phosphorylated and translocated into the cytosol in an ASK1-p38-dependent manner, which ultimately leads to the promotion of necrosis.

Conclusion: ASK1-p38 MAPK pathway-dependent phosphorylation and subsequent cytoplasmic translocation of NR4A2 promote oxidative stress-induced necrosis.

Significance: We found a novel intracellular signaling pathway that regulates oxidative stress-induced and p38-mediated necrosis.

p38 mitogen-activated protein kinases (MAPKs) play important roles in various cellular stress responses, including cell death, which is roughly categorized into apoptosis and necrosis. Although p38 signaling has been extensively studied, the molecular mechanisms of p38-mediated cell death are unclear. ASK1 is a stress-responsive MAP3K that acts as an upstream kinase of p38 and is activated by various stresses, such as oxidative stress. Here, we show that NR4A2, a member of the NR4A nuclear receptor family, acts as a necrosis promoter downstream of ASK1-p38 pathway during oxidative stress. Although NR4A2 is well known as a nucleus-localized transcription factor, we found that it is translocated into the cytosol after phosphorylation by p38. Because the phosphorylation site mutants of NR4A2 cannot rescue the cell death-promoting activity, ASK1-p38 pathway-dependent phosphorylation and subsequent cytoplasmic translocation of NR4A2 may be required for oxidative stress-induced cell death. In addition, NR4A2-mediated cell death does not depend on caspases and receptor-interacting protein 1 (RIP1)-RIP3 complex, suggesting that NR4A2 promotes an RIP kinase-independent necrotic type of cell death. Our findings may enable a more precise understanding of molecular mechanisms that regulate oxidative stress-induced and p38-mediated necrosis.

Apoptosis signal-regulating kinase 1 (ASK1)² is a stress-responsive MAP3K that activates the c-Jun N-terminal kinase (JNK) and p38 MAPK pathways in response to various intra- and extracellular stresses (1). Among these stresses, oxidative stress is one of the most potent activators of ASK1 (2). We have previously demonstrated that ASK1 mediates oxidative stress-induced cell death by activating JNK and p38 (3–5).

Recently, we identified NR4A2, which is a member of the NR4A orphan nuclear receptor family, as a target molecule of p38 (6). The NR4A family consists of three members, NR4A1 (also called Nur77), NR4A2 (also called Nurr1), and NR4A3 (also called NOR-1). These proteins function as transcription factors that play important roles in various cellular functions, such as differentiation and cytokine production (7, 8).

In addition to their well known functions as transcription factors, NR4As possess cell death-promoting activities. In particular, the proapoptotic function of NR4A1 has been extensively studied in T cells (9, 10). Although initial studies argued that the proapoptotic activity of NR4A1 is dependent on its transcriptional activity (11), recent studies have indicated that the nuclear export of NR4A1 and NR4A3 also contributes to apoptosis (12–14). In addition, NR4A1 has been reported to be involved in necrosis, caspase-independent cell death, induced by the combined treatment of cells with LPS and pancaspase inhibitor, Z-VAD-fmk (15). Nevertheless, the cell death-promoting activity of NR4A2 in apoptosis or necrosis has not been studied.

In this study, we elucidated the roles of ASK1-p38 pathway-dependent phosphorylation of NR4A2 in oxidative stress-in-

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² The abbreviations used are: ASK1, apoptosis signal-regulating kinase 1; Z, benzoyloxycarbonyl; fmk, fluoromethyl ketone; NES, nuclear export signal; MEF, mouse embryo fibroblast; LDH, lactate dehydrogenase; RIP, receptor-interacting protein; CRM1, chromosome region maintenance 1.

ASK1-p38-NR4A2 Axis in H₂O₂-induced Necrosis

duced necrosis. Although we previously demonstrated that p38 directly phosphorylates NR4A2 and potentiates its transcriptional activity (6), here we identified that NR4A2 is translocated from the nucleus into the cytosol during oxidative stress after it is phosphorylated downstream of the ASK1-p38 pathway. The alanine substitution of the oxidative stress-induced phosphorylation sites of NR4A2 inhibits the cytoplasmic translocation and pro-necrosis activity of NR4A2 induced by oxidative stress, suggesting that p38-dependent phosphorylation of NR4A2 is a crucial regulation to confer the necrosis-promoting activity to NR4A2.

It has been reported that pretreatment with p38 inhibitor attenuates ischemia-reperfusion injury, which is a typical *in vivo* model of oxidative stress-induced necrosis (16), suggesting that p38 regulates not only apoptosis but also oxidative stress-induced necrosis. However, the substrates of p38 in the context of necrosis and, thus, the molecular mechanisms by which p38 promotes necrosis are largely unknown. Our findings further the understanding of the molecular mechanisms by which p38 exerts its necrosis-promoting activity during oxidative stress.

EXPERIMENTAL PROCEDURES

Expression Plasmids—FLAG-NR4A2 wild type and the cluster I, cluster II, cluster III, and cluster IV alanine mutants were described previously (6). FLAG-NR4A2 S126A, T129A, T132A, S140A, T168A, S181A, T185A, 3A, 4A, 5A, 6A, S126A/T129A, S126A/T132A, and T129A/T132A, and siRNA-resistant constructs of FLAG-NR4A2 WT, cluster II alanine, 3A, and nuclear export signal (NES) mutants were generated using site-directed mutagenesis (QuikChange kit, Agilent Technologies, Santa Clara, CA) and inserted into pcDNA3 vector with a FLAG tag. The experimental protocol was approved by the Animal Research Committee of the Graduate School of Pharmaceutical Sciences (University of Tokyo).

Cell Culture and Reagents—HeLa cells, mouse embryonic fibroblasts (MEFs), and A549 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 units/ml penicillin G in 5% CO₂ at 37 °C. The transfection of the expression plasmids was performed using PEI Max (Polysciences, Inc.) according to the manufacturer's instructions. For RNAi, the cells were transfected with the following siRNAs using Lipofectamine RNAi MAX (Invitrogen) according to the manufacturer's instructions: human ASK1 (siRNA 1, Stealth Select RNAi 10620312 200700 G09; siRNA 2, Stealth Select RNAi 10620312 149162 E09), mouse ASK1 (siRNA 1, Stealth Select RNAi 10620312 227058 C11; siRNA 2, Stealth Select RNAi 10620312 204579 E08), and NR4A2 (siRNA 1, Stealth Select RNAi 10620312 166630 G02; siRNA 2, Stealth Select RNAi 10620312 166630 G04). Stealth RNAi Negative CTL Medium GC Duplex siRNA 2 (Invitrogen) was used as a control. In this paper, the following inhibitors and reagents are used: SB202190 (Calbiochem), SB203580 (Calbiochem), PH797804 (Selleckchem), H₂O₂ (Wako), Z-VAD-fmk (Sigma), and necrostatin-1 (Sigma). The ASK1 inhibitor K811 is a nitrogen-containing heterocyclic derivative compound. It was synthesized at and obtained from Kyowa Hakko Kirin Co. Ltd. (Tokyo, Japan). The company has filed a patent application for K811 (International Publication

Number: WO 2012/011548 A1). Detailed information about K811, including its chemical structure, is described on the World Intellectual Property Organization Web site. K811 was dissolved in DMSO for *in vitro* analysis.

Antibodies—Antibody specific for phospho-ASK1 was generated previously (17). A rabbit polyclonal antibody specific for phospho-NR4A2 was raised against the phosphopeptide KPS(pS)PP(pT)PT(pT)PGFQ as described previously (17). The following antibodies were purchased from commercial sources: FLAG tag (1E6, Wako), NR4A2 (N6413, Sigma), ASK1 (EP553Y, Abcam), JNK1 (JNK-FL, Santa Cruz Biotechnology, Inc.), p38 (C-20-G, Santa Cruz Biotechnology), phospho-JNK (Thr¹⁸³/Tyr¹⁸⁵) (catalog no. 9251, Cell Signaling Technology), phospho-p38 (Thr¹⁸⁰/Tyr¹⁸²) (catalog no. 9211, Cell Signaling Technology), actin (A3853, Sigma), α -tubulin (sc-53029, Santa Cruz Biotechnology), and Lamin A/C (sc-7292, Santa Cruz Biotechnology).

Lactate Dehydrogenase (LDH) Assay—H₂O₂-induced necrotic cell death was monitored using the LDH-cytotoxic test (Wako) according to the manufacturer's protocols. Released LDH activity into the culture media was quantified as a percentage of the total LDH activity.

Measurement of Caspase-3 Activity—The activities of caspase-3 were measured using a CPP32/caspase-3 fluorometric protease assay kit (Medical and Biological Laboratories) according to the manufacturer's protocols.

Quantitative RT-PCR Analysis—Total RNA from HeLa cells was isolated using Isogen (Wako) and reverse-transcribed using the QuantiTect reverse transcription kit (Qiagen). Quantitative PCR was performed using Power SYBR Green PCR Master Mix (Roche Applied Science) on an ABI PRISM7000 sequence detection system (Applied Biosystems). The following oligonucleotides were used: RPS18, 5'-TTTGCGAG-TACTCAACACCAA-3' (forward) and 5'-GCATATCTTCG-GCCCACA-3' (reverse); NR4A1, 5'-CCACTGCCTCCTTCA-ACC-3' (forward) and 5'-GGCTTGGATACAGGGCAGC-3' (reverse); NR4A2, 5'-TGAAGAGAGACGCGGAGAAC-3' (forward) and 5'-AAAGCAATGGGGAGTCCAG-3' (reverse); NR4A3, 5'-ACACCCAGAGATCTTGATTATTCC-3' (forward) and 5'-GTAGAATTGTTGCACATGCTCAG-3' (reverse). To normalize the relative expression of each gene to that of the RPS18 control, a standard curve was prepared for each gene.

Immunoblotting Analysis—Cells were lysed in immunoprecipitation lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 10 mM EDTA, 1 mM PMSF, and 5 mg/ml leupeptin) supplemented with PhosSTOP (Roche Applied Science). Immunoblotting was performed as described previously (3).

λ -Phosphatase Treatment—Cell lysates were treated with 2 units/ml of λ -protein phosphatase (New England Biolabs) in a buffer containing 50 mM HEPES (pH 7.5), 5 mM DTT, 0.1 mM EDTA, 0.01% Brij35, and 2 mM MnCl₂ at 30 °C for 30 min. Reactions were stopped by adding SDS sample buffer.

Immunostaining Analysis and Cell Image Analyzer—After fixation in 4% formaldehyde for 10 min, the cells were permeabilized with 1% Triton X-100 in phosphate-buffered saline for 15 min and blocked with TBST solution containing 5% skim

milk for 30 min at room temperature. Anti-FLAG antibody (1E6, Wako) and anti-active caspase-3 antibody (ab13847, Abcam) were used as primary antibodies and visualized using Alexa Fluor 555 and 488 secondary antibodies, respectively (Molecular Probes). Hoechst 33342 solution (Dojindo) was used for nuclear staining. Images were acquired using confocal microscopy (TCS SP5, Leica). For imaging analysis using Array-Scan VTI (Thermo Scientific Cellomics), samples were immunostained as described above and analyzed. After cells were identified using the Hoechst signal of their nuclei, the localization of FLAG-NR4A2 was defined from 200 cells, using the Cellomics Cell Health Profiling V3 software.

RESULTS

The ASK1-p38 Pathway Is Required for H₂O₂-induced Caspase-independent Cell Death—H₂O₂ is one of the most potent stresses to activate ASK1 (2). We previously reported that ASK1 was required for H₂O₂-induced cell death in various types of cells (3–5). Because it was shown that H₂O₂ induces apoptosis and necrosis depending on its concentration (18), we began to examine which type of cell death ASK1 regulates in response to H₂O₂. First, we characterized the type of cell death that was induced by various concentrations of H₂O₂ in HeLa cells (Fig. 1, A–C). As shown in Fig. 1A, immunocytochemical analysis using an active caspase-3 antibody revealed that caspase-3 activity was detected when cells were treated with 1 mM, but not 3 mM, H₂O₂. To further analyze the dose-dependent H₂O₂-induced cell death features in HeLa cells, we utilized two different quantitative cell death assays. To detect apoptosis, caspase-3 activity was measured. As a necrosis marker, we measured the levels of LDH released into the culture media, which represents disruption of the plasma membrane. As shown in Fig. 1B, caspase-3 activity was significantly increased by treatment with 1 mM, but not 3 mM, H₂O₂. By contrast, LDH release was induced by 3 mM, but not 1 mM, H₂O₂ (Fig. 1C). Although we further examined caspase-3 activation every hour after 3 mM H₂O₂ treatment, caspase-3 activation was not observed at any time point (Fig. 1D). Moreover, treatment with the pancaspase inhibitor Z-VAD-fmk did not suppress LDH release induced by 3 mM H₂O₂ (Fig. 1E). These data indicate that, in HeLa cells, treatment with 3 mM H₂O₂ induces caspase-independent cell death.

Next, we investigated the type of cell death in which ASK1 and its downstream targets, p38 and JNK, were involved. As shown in Fig. 1, F and G, neither siRNA treatment with ASK1 nor pretreatment with p38 or JNK inhibitors suppressed the caspase-3 activation that was induced by 1 mM H₂O₂. Conversely, 3 mM H₂O₂-induced LDH release was significantly suppressed by ASK1 knockdown (Fig. 1H). In addition, pretreatment with p38 inhibitors, but not a JNK inhibitor, suppressed 3 mM H₂O₂-induced LDH release (Fig. 1I). We further examined the requirement of p38 for H₂O₂-induced HeLa cell death by using another p38 inhibitor, PH797804, whose high potency and selectivity has been established (19). As shown in Fig. 1J, 1 μM PH797804 inhibited H₂O₂-induced p38 activation without any obvious effects on the activity of other MAPKs. H₂O₂-induced LDH release was also inhibited by the pretreatment with 1 μM PH797804 to the same extent as 10 μM SB202190 (Fig.

1K). Although the suppressive effects of these p38 inhibitors on LDH release were less striking compared with that of ASK1 knockdown (Fig. 1H), this might be due to different experimental conditions to suppress the activity of proteins (one is the small compound-mediated enzymatic inhibition, and the other is the siRNA-based suppression of protein expression). It would also be possible that substrates of ASK1 other than p38 may be involved in H₂O₂-induced LDH release.

Next, we examined whether 3 mM H₂O₂-induced p38 activation is ASK1-dependent. As shown in Fig. 1L, two independent siRNAs that target different regions of the *ASK1* gene suppressed p38 activation, and their effects on JNK activation were different. The signal of phospho-JNK was even intensified by ASK1 siRNA 1 (Fig. 1L). These results suggest that ASK1 is required for H₂O₂-induced p38 activation, although the contribution of ASK1 to the 3 mM H₂O₂-induced JNK activation in HeLa cells is ambiguous. From these results, we concluded that the ASK1-p38 pathway is required for 3 mM H₂O₂-induced caspase-independent cell death, but not for 1 mM H₂O₂-induced apoptosis, in HeLa cells.

The ASK1-p38 Pathway Is Required for H₂O₂-induced RIP1-RIP3-independent Necrosis—Although it has been reported that necroptosis is not induced in HeLa cells due to low expression of RIP3 (20), we examined the effect of a RIP1 kinase inhibitor, necrostatin-1 (Nec-1), on 3 mM H₂O₂-induced HeLa cell death to confirm that this cell death is independent of RIP1-RIP3-dependent necrosis. As expected, treatment with Nec-1 did not inhibit 3 mM H₂O₂-induced LDH release (Fig. 2A). We also address this point by using other cells in which expression of endogenous RIP3 has been confirmed and, thus, reported to be sensitive to RIP kinase-induced necroptosis (20, 21). We tested the sensitivity of MEFs and a human lung adenocarcinoma cell line (A549) to H₂O₂-induced cell death. As a result, in both of these cells, H₂O₂-induced LDH release was observed even under the pretreatment with Nec-1 and Z-VAD-fmk (Fig. 2, B and C), suggesting the existence of RIP1-RIP3-independent necrosis pathways in MEFs and A549 cells.

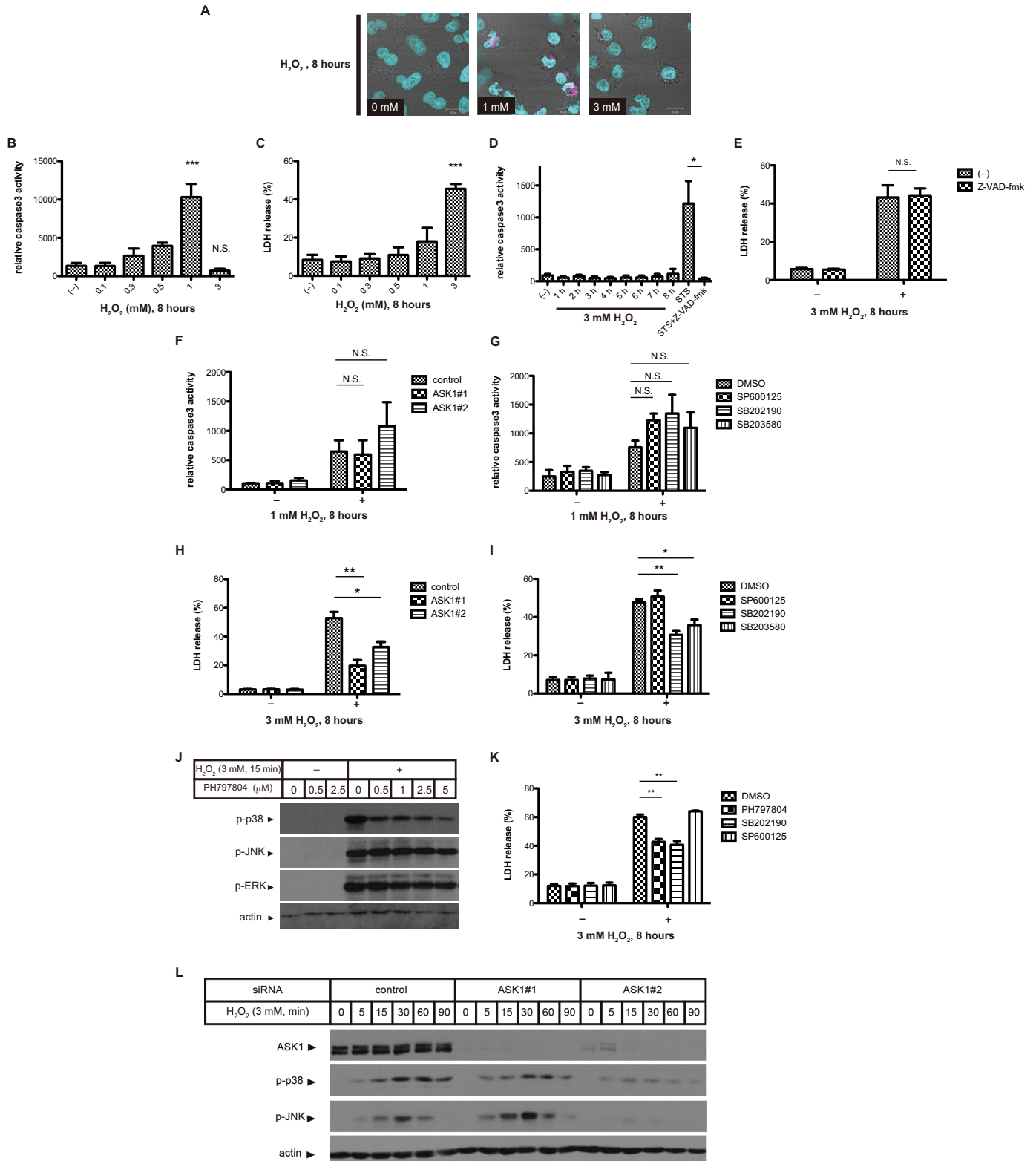
We next examined the potential involvement of ASK1-p38 pathway in H₂O₂-induced necrosis in MEFs and A549. Although p38 inhibitor did not attenuate H₂O₂-induced LDH release in A549 (Fig. 2E), the requirement of ASK1 and p38 for H₂O₂-induced LDH release was clearly observed in MEFs (Fig. 2, D, F, and G). These results suggest that the ASK1-p38 pathway is required for oxidative stress-induced necrosis independently of RIP kinases at least in HeLa cells and MEFs but not in A549. The mechanism behind the cell type-specific requirement of the ASK1-p38 pathway for H₂O₂-induced cell death should be elucidated.

NR4A2 Is Required for H₂O₂-induced RIP1-RIP3-independent Necrosis—Next, we attempted to identify the downstream targets of p38 in the context of H₂O₂-induced necrosis. We previously reported that ASK1-activated p38 directly phosphorylates NR4A2, which is a member of the NR4A nuclear receptor family (6). Several recent studies have suggested that other NR4A family members, such as NR4A1 and NR4A3, exert cell death-promoting activity in addition to their canonical roles in transcription (13). Therefore, we examined the potential involvement of NR4A2 in H₂O₂-induced necrosis. We knocked

ASK1-p38-NR4A2 Axis in H₂O₂-induced Necrosis

down NR4A2 using two independent siRNAs that target different regions of the *Nr4a2* gene. We confirmed the knockdown efficiency of these siRNAs using real-time PCR analysis. Treatment with NR4A2 siRNAs specifically suppressed the expression of NR4A2 without apparent effects on the expression of other NR4A family members in HeLa cells (Fig. 3A). We also

confirmed the knockdown efficiency of these siRNAs on NR4A2 protein by immunoblotting in both HeLa cells and MEFs (Fig. 3, B and C). Using these siRNAs, we examined the effect of NR4A2 knockdown on 3 mM H₂O₂-induced LDH release. As shown in Fig. 3D, we observed that 3 mM H₂O₂-induced LDH release was significantly attenuated by NR4A2



knockdown in HeLa cells, suggesting that NR4A2 is required for H₂O₂-induced necrosis. Consistent with the finding that ASK1-p38 pathway was also required for the H₂O₂-induced necrosis in MEFs (Fig. 2, *D*, *F*, and *G*), the knockdown of NR4A2 in MEFs also attenuated the H₂O₂-induced caspase- and RIP1-RIP3-independent cell death (Fig. 3*E*).

H₂O₂-induced Phosphorylation of NR4A2 Is Mediated by the ASK1-p38 Pathway—Because we observed a retarded migration of the NR4A2 band by immunoblotting after H₂O₂, we examined the possibility that this H₂O₂-induced band shift of NR4A2 can be ascribed to phosphorylation by using broadly specific λ-protein phosphatase. As expected, H₂O₂-induced retarded migration of the NR4A2 band was reversed by incubation with λ-protein phosphatase (*λPPase*; Fig. 4*A*), suggesting that NR4A2 is phosphorylated in response to H₂O₂. We also observed that the band shift of NR4A2 was similarly inhibited, at least in part, by pretreatment with ASK1 and p38 inhibitors (Fig. 4*B*). These results suggest that the H₂O₂-induced phosphorylation of NR4A2 is mediated by the ASK1-p38 pathway in HeLa cells.

We attempted to identify the H₂O₂-induced phosphorylation sites of NR4A2. There are 15 consensus phosphorylation sites (Ser-Pro or Thr-Pro) targeted by p38 in both human and mouse NR4A2. We previously divided these sites into four clusters that we designated cluster I (Ser¹¹, Ser¹⁶, and Thr³⁶), II (Ser¹²⁶, Thr¹²⁹, Thr¹³², Ser¹⁴⁰, Thr¹⁶⁸, Ser¹⁸¹, and Thr¹⁸⁵), III (Ser²⁵⁰, and Ser²⁵⁶), and IV (Ser³⁵¹, Ser³⁵⁶, and Ser³⁵⁹), as depicted in the diagram in Fig. 4*C* (6). To determine the phosphorylation site(s) by H₂O₂-activated p38, we utilized combinatorial alanine substitution mutants of each cluster of NR4A2. We have previously shown that the retarded migration of NR4A2 that is induced by co-expression of ASK1ΔN (a constitutively active form of ASK1 that lacks its N-terminal inhibitory domain) was suppressed in the cluster II mutant (6). We also found that the H₂O₂-induced, retarded migration of NR4A2 was suppressed in the cluster II mutant (Fig. 4*D*). Seven potential phosphorylation sites are located in cluster II, as shown in the sequence of NR4A2 in Fig. 4*E*. We therefore examined the effect of each alanine substitution on the H₂O₂-induced band shift of NR4A2. None of these single mutants showed clear suppression of the band shift by immunoblotting (Fig. 4*F*). To narrow down the phosphorylation sites, we tested the NR4A2 mutants by substituting each potential phosphorylation site in cluster II with Ala beginning at the N terminus of cluster II. We named these mutants as follows: 6A (Ser¹²⁶, Thr¹²⁹, Thr¹³², Ser¹⁴⁰, Thr¹⁶⁸, and Ser¹⁸¹ to Ala), 5A (Ser¹²⁶, Thr¹²⁹, Thr¹³², Ser¹⁴⁰, and Thr¹⁶⁸ to Ala), 4A (Ser¹²⁶, Thr¹²⁹, Thr¹³², and Ser¹⁴⁰

to Ala), and 3A (Ser¹²⁶, Thr¹²⁹, and Thr¹³² to Ala). The H₂O₂-induced retarded migration of NR4A2 was suppressed in the 3A mutant to the similar extent with the cluster II mutant (Fig. 4*G*). Moreover, double alanine mutation (Ser¹²⁶/Thr¹²⁹, Thr¹²⁹/Thr¹³², and Ser¹²⁶/Thr¹³²) did not show clear effects on the band shift of NR4A2 (Fig. 4*H*), suggesting that Ser¹²⁶, Thr¹²⁹, and Thr¹³² all have the potential to be phosphorylated by H₂O₂-induced p38, and the phosphorylation of one of the three phosphorylation sites is sufficient for the retarded migration of NR4A2.

In order to confirm the p38-dependent phosphorylation sites of NR4A2, we generated an anti-phospho-NR4A2 antibody (p-NR4A2 S126/T129/T132) raised against a phosphopeptide, KPSPSPPTPTTPGFGQ (where pS and pT represent phosphoserine and phosphothreonine, respectively). As shown in Fig. 4*I*, under the H₂O₂-treated condition, the band of wild-type NR4A2 but not of 3A mutant was clearly detected by immunoblotting using this antibody, indicating that H₂O₂-induced phosphorylation of Ser¹²⁶, Thr¹²⁹, and Thr¹³² of NR4A2 can be successfully monitored by this antibody. Moreover, we observed that the H₂O₂-induced enhanced signal of the phospho-NR4A2 antibody was similarly attenuated by the pretreatment with ASK1 and p38 inhibitors but not by the pretreatment with JNK inhibitor (Fig. 4, *J* and *K*), suggesting that H₂O₂-induced phosphorylation of NR4A2 at Ser¹²⁶, Thr¹²⁹, and Thr¹³² is mediated by the ASK1-p38 pathway.

H₂O₂-induced Cytoplasmic Translocation of NR4A2 Is Regulated by the ASK1-p38 Pathway—We investigated whether the role of NR4A2 in H₂O₂-induced cell death is dependent on its function as a transcriptional factor by using actinomycin D, a general inhibitor of mRNA transcription. H₂O₂-induced necrosis in HeLa cells is not affected by the pretreatment with actinomycin D (Fig. 5*A*). Therefore, we concluded that transcriptional activity of NR4A2 is not required for H₂O₂-induced necrosis in HeLa cells.

It has been previously reported that, in some cases, the nuclear export of NR4A1 and NR4A3 is involved in their cell death-promoting activity (13). Moreover, a recent report has suggested that NR4A2 is exported from the nucleus in response to oxidative stress (22). Because transcriptional activity was not required for H₂O₂-induced necrosis in HeLa cells, as shown in Fig. 5*A*, we speculated that cytoplasmic translocation of NR4A2 might be involved in H₂O₂-induced necrosis independently of its transcriptional activity. We found that NR4A2 was translocated to the cytosol after H₂O₂ treatment (Fig. 5, *B* and *C*). Moreover, we also found that the ASK1-p38 pathway is involved in this H₂O₂-induced cytoplasmic translocation of

FIGURE 1. The ASK1-p38 pathway is required for 3 mM H₂O₂-induced caspase-independent cell death. *A–C*, after HeLa cells were treated with the indicated concentration of H₂O₂ for 8 h, cells were subjected to immunocytochemistry analysis using an anti-active caspase-3 antibody (*A*), and caspase-3 activity (*B*) or LDH release was measured (*C*). *Error bars*, S.E. (***, *p* < 0.001, one-way analysis of variance and Bonferroni's multiple comparison test). *D* and *E*, after incubation with 3 mM H₂O₂ with or without 10 μM Z-VAD-fmk for the indicated time periods, caspase-3 (*D*) and LDH assays (*E*) were performed. *Error bars*, S.E. (**, *p* < 0.01, Student's *t* test). *F* and *H*, 48 h after transfection of the ASK1 siRNAs, HeLa cells were treated with the indicated concentration of H₂O₂. 8 h later, the caspase-3 (*F*) and LDH assays (*H*) were performed. *Error bars*, S.E. (*, *p* < 0.05; **, *p* < 0.01, one-way analysis of variance and Dunnett's multiple comparison test compared with the control). *G* and *I*, HeLa cells pretreated with 10 μM SP600125, 10 μM SB202190, and 10 μM SB203580, respectively, were incubated with the indicated concentration of H₂O₂. 8 h later, caspase-3 (*G*) and LDH assays (*I*) were performed. *Error bars*, S.E. (*, *p* < 0.05; **, *p* < 0.01, one-way analysis of variance and Dunnett's multiple comparison test compared with the control). *J*, HeLa cells pretreated with the indicated concentration of PH797804 were incubated with the indicated concentration of H₂O₂ for the time indicated and were subjected to immunoblotting. *K*, HeLa cells pretreated with 1 μM PH797804, 10 μM SB202190, and 10 μM SP600125 were incubated with the indicated concentration of H₂O₂. 8 h later, LDH assays were performed. *Error bars*, S.E. (**, *p* < 0.01, one-way analysis of variance and Dunnett's multiple comparison test compared with the control). *L*, 48 h after transfection of the ASK1 siRNAs, HeLa cells were treated with 3 mM H₂O₂ for the indicated time periods and subjected to immunoblotting. *N.S.*, not significant.

ASK1-p38-NR4A2 Axis in H₂O₂-induced Necrosis

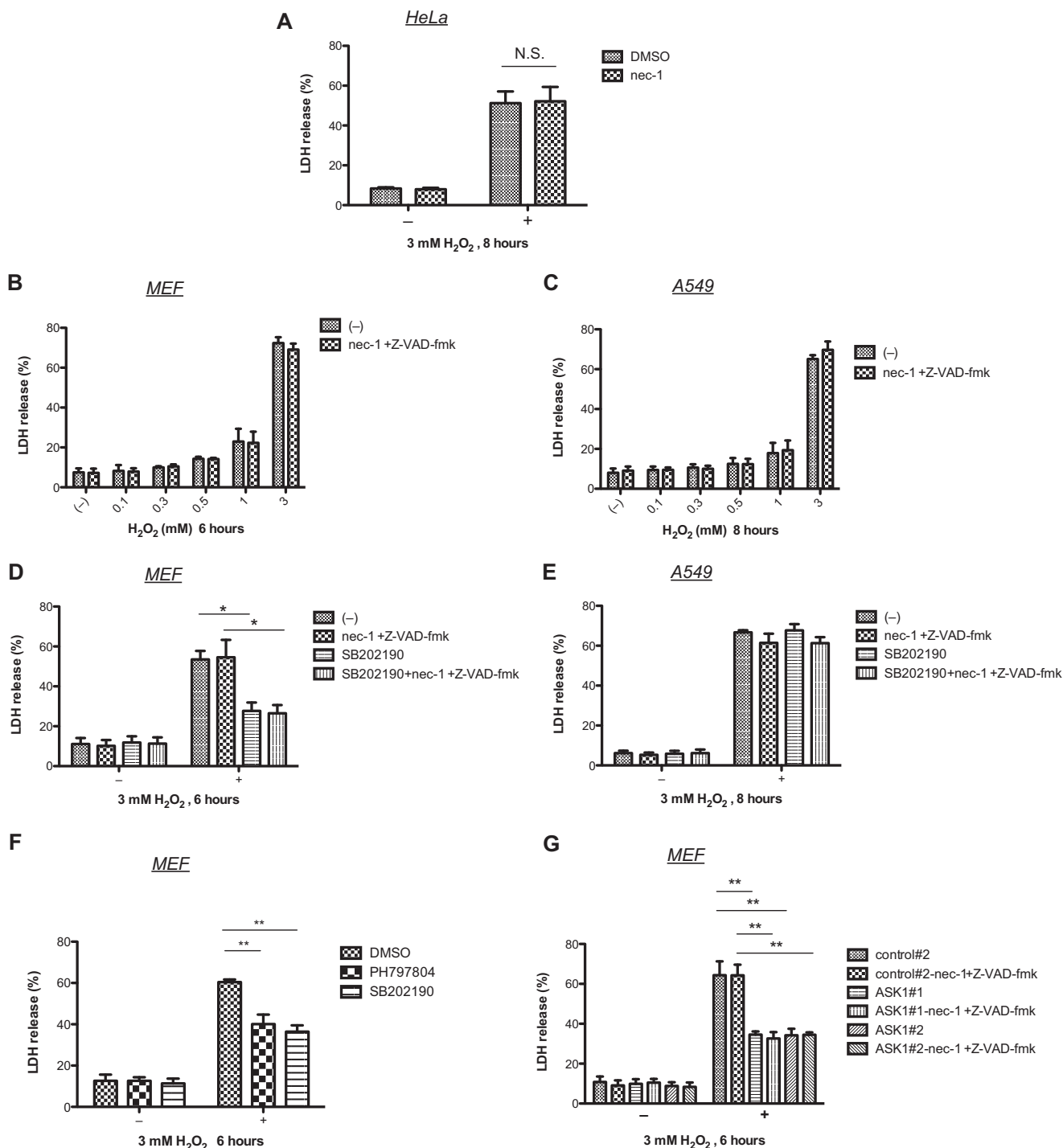


FIGURE 2. The ASK1-p38 pathway is required for H₂O₂-induced caspase- and RIP1-RIP3-independent necrosis. A, HeLa cells were treated with 3 mM H₂O₂ for 8 h with or without 10 μM Nec-1 and subjected to the LDH assay. Error bars, S.E. B, after MEFs were treated with the indicated concentration of H₂O₂ with or without 10 μM Z-VAD-fmk and 10 μM Nec-1 for 6 h, cells were subjected to an LDH assay. Error bars, S.E. C, after A549 cells were treated with the indicated concentration of H₂O₂ with or without 10 μM Z-VAD-fmk and 10 μM Nec-1 for 8 h, cells were subjected to an LDH assay. Error bars, S.E. D, MEFs pretreated with the indicated inhibitor compounds were incubated with 3 mM H₂O₂. The concentration of SB202190 is 10 μM. 6 h later, LDH assays were performed. Error bars, S.E. (*, *p* < 0.05, one-way analysis of variance and Bonferroni's multiple comparison test). E, A549 cells pretreated with the indicated inhibitor compounds were incubated with 3 mM H₂O₂. 8 h later, LDH assays were performed. Error bars, S.E. F, HeLa cells pretreated with 1 μM PH797804 and 10 μM SB202190 were incubated with the indicated concentration of H₂O₂. 8 h later, LDH assays were performed. Error bars, S.E. (**, *p* < 0.01, one-way analysis of variance and Dunnett's multiple comparison test compared with the control). G, 48 h after transfection of the ASK1 siRNAs, HeLa cells were treated with the indicated concentration of H₂O₂. 8 h later, LDH assays were performed. Error bars, S.E. (**, *p* < 0.01, one-way analysis of variance and Bonferroni's multiple comparison test). N.S., not significant.

NR4A2. Although some NR4A2 is still translocated to cytosol in response to H₂O₂, even by the treatment with ASK1 siRNA or p38 inhibitor, NR4A2 remaining in the nucleus was clearly

increased under these conditions (Fig. 5, B and C). To verify the H₂O₂-induced cytoplasmic translocation of NR4A2 and its dependence on the ASK1-p38 pathway more clearly, we evalu-

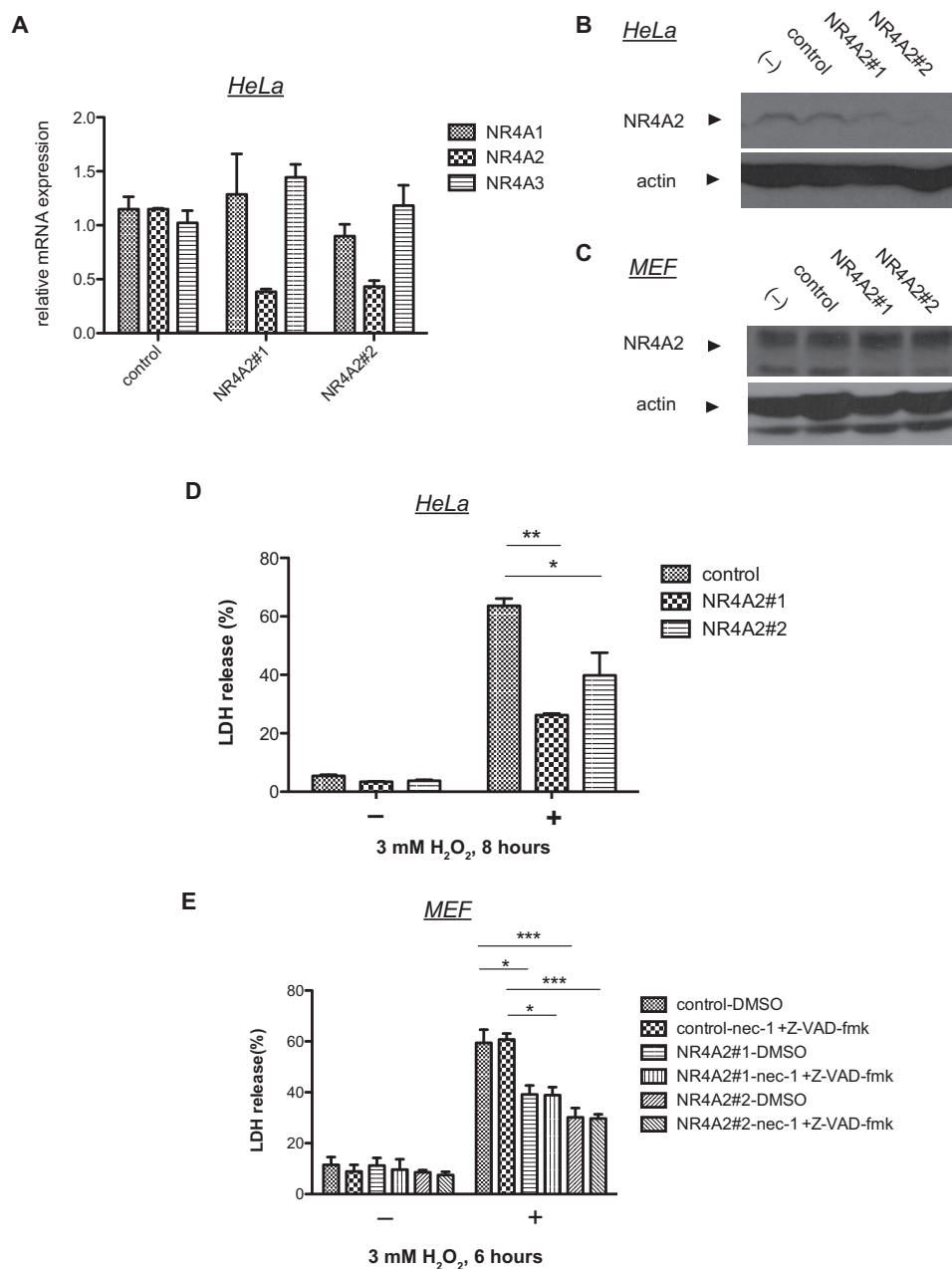


FIGURE 3. **NR4A2 is required for H₂O₂-induced caspase- and RIP1-RIP3-independent necrosis.** *A*, the knockdown efficiency of siRNAs in HeLa cells was evaluated using quantitative RT-PCR. *Error bars*, S.E. *B*, 48 h after transfection of the NR4A2 siRNAs, HeLa cells were subjected to immunoblotting. *C*, 48 h after transfection of the NR4A2 siRNAs, MEFs were subjected to immunoblotting. *D*, NR4A2 is required for caspase- and RIP1-RIP3-independent necrosis in MEF cells. 48 h after transfection of the NR4A2 siRNAs, MEFs were treated with 3 mM H₂O₂. 6 h later, LDH assays were performed. *Error bars*, S.E. (*, $p < 0.05$; ***, $p < 0.001$, one-way analysis of variance and Bonferroni's multiple comparison test). *E*, 48 h after transfection with the NR4A2 siRNAs, the cells were treated with 3 mM H₂O₂ for 8 h and subjected to the LDH assay. *Error bars*, S.E. (*, $p < 0.05$; **, $p < 0.01$, one-way analysis of variance and Dunnett's multiple comparison test compared with the control).

ated cytoplasmic and nucleic NR4A2 quantitatively by using a cell image analyzer (Array Scan VTI, Thermo Scientific). As shown in Fig. 5*D*, in the algorithm that we set up, the cell image analyzer correctly recognizes nucleic and cytoplasmic FLAG-NR4A2 (green and red, respectively). By using this algorithm, we measured the cytoplasmic and total intensity of FLAG-NR4A2 from 200 cells in each well and calculated the ratio of these parameters. As a result, the ratio of the cytoplasmic intensity of NR4A2 to the total intensity of NR4A2 was increased after H₂O₂ treatment (Fig. 5, *E* and *F*). In addition, consistent

with immunocytochemistry data (Fig. 5, *B* and *C*), ASK1 knockdown and pretreatment with p38 inhibitor significantly reduced the ratio of these parameters (Fig. 5, *E* and *F*).

We also confirmed the H₂O₂-induced cytoplasmic translocation of NR4A2 and its dependence on ASK1-p38 pathway biochemically. As shown in Fig. 5*G*, cytoplasmic fraction of NR4A2 was increased after H₂O₂ treatment and reduced by the pretreatment with p38 and ASK1 inhibitor.

From these results, we concluded that the ASK1-p38 pathway is required for efficient cytoplasmic translocation of

ASK1-p38-NR4A2 Axis in H₂O₂-induced Necrosis

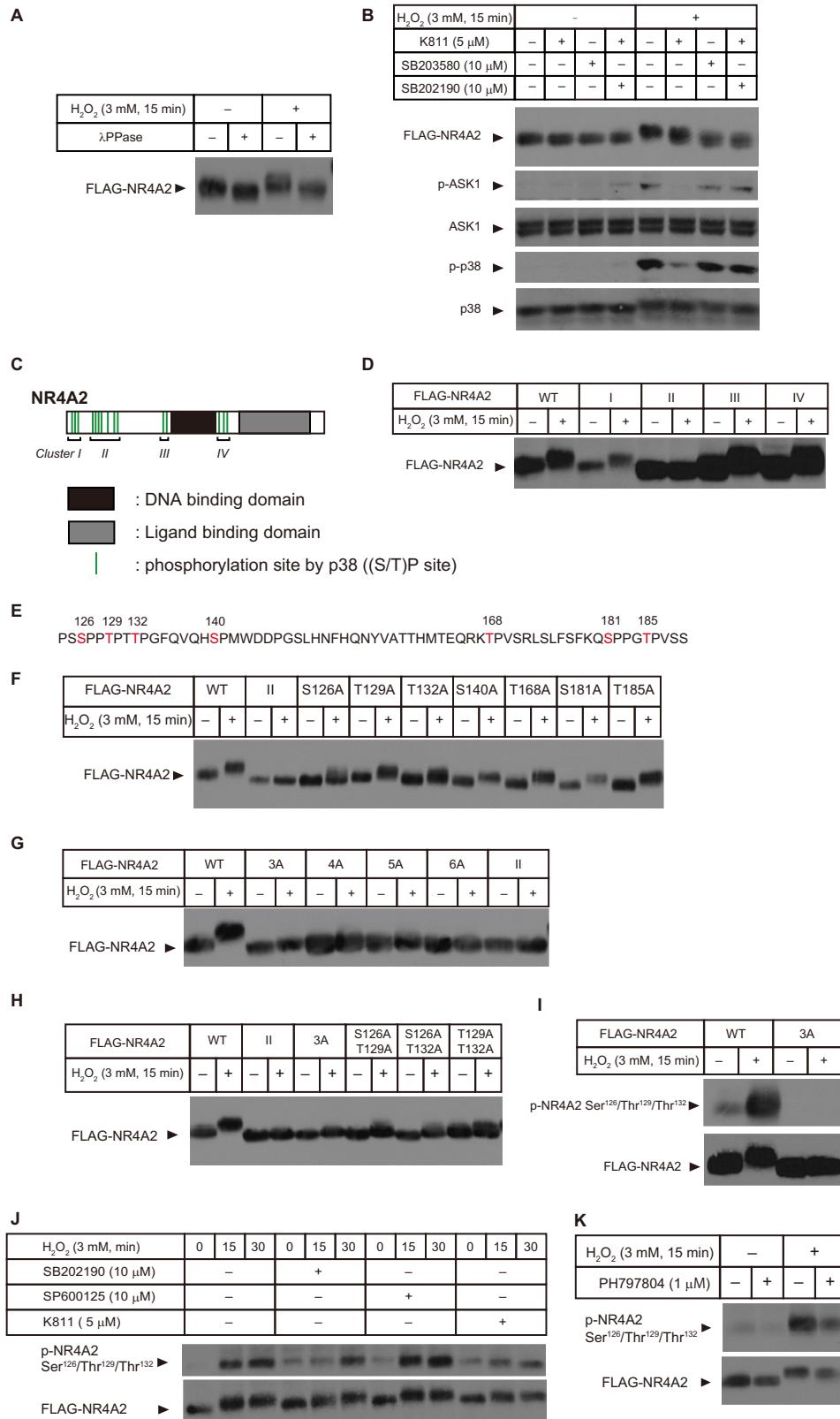
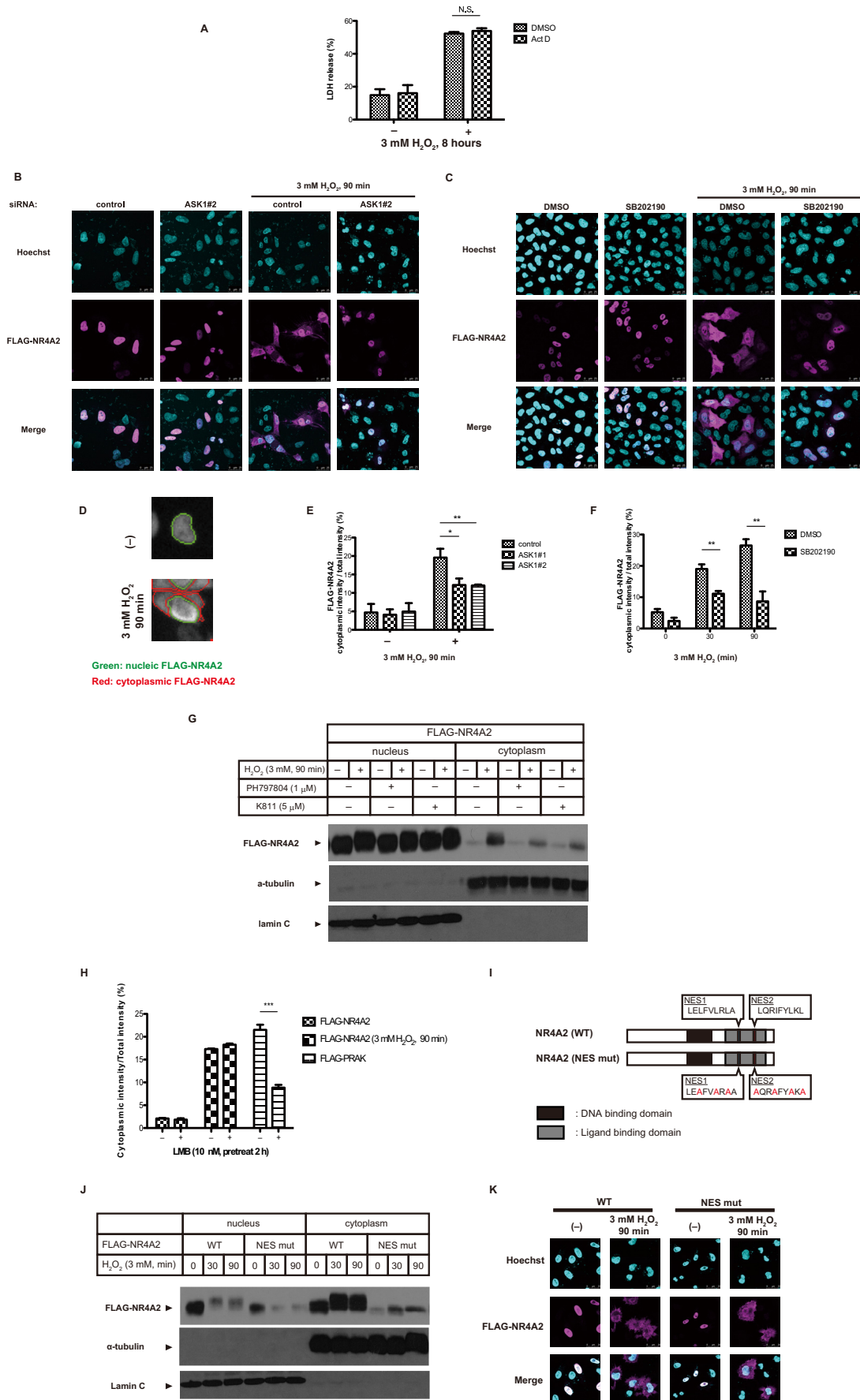


FIGURE 4. H₂O₂-induced phosphorylation of NR4A2 is mediated by the ASK1-p38 pathway. *A*, 24 h after transfection with FLAG-NR4A2, HeLa cells were treated with 3 mM H₂O₂. 15 min later, the cell lysates were incubated with or without λ-protein phosphatase (λPPase) and subjected to immunoblotting. *B*, 24 h after transfection with FLAG-NR4A2, HeLa cells were treated with the indicated inhibitor compounds, and 30 min later, the cells were incubated with 3 mM H₂O₂ for 15 min and subjected to immunoblotting. *C*, consensus phosphorylation sites for p38 ((S/T)P site) in NR4A2. *D*, *F*, *G*, *H*, and *I*, 24 h after transfection with the indicated expression plasmids, HeLa cells were treated with 3 mM H₂O₂ for 15 min and subjected to immunoblotting. *E*, consensus phosphorylation sites for p38 ((S/T)P site) in cluster II. *J* and *K*, 24 h after transfection with FLAG-NR4A2, HeLa cells were treated with the indicated inhibitor compounds, and 30 min later, the cells were incubated with 3 mM H₂O₂ for the indicated time periods and subjected to immunoblotting.



ASK1-p38-NR4A2 Axis in H₂O₂-induced Necrosis

NR4A2 in response to H₂O₂. As for the partial suppressive effects of ASK1 siRNA or p38 inhibitor on cytoplasmic translocation of NR4A2, it is conceivable that the efficiency of siRNA or inhibitors may not be sufficient or that certain regulators other than the ASK1-p38 pathway may also contribute to NR4A2 nuclear export.

Because it has been reported that NR4A2 has two NESs and is exported from nucleus to cytosol in a chromosome region maintenance 1 (CRM1/exportin 1)-dependent manner (22), we examined whether H₂O₂-induced cytoplasmic translocation of NR4A2 is CRM1-dependent by using leptomycin B, a CRM1 inhibitor (Fig. 5H), or by mutating the NES of NR4A2 (Fig. 5I). The pretreatment with leptomycin B reduced the cytoplasmic translocation of p38-regulated/activated kinase (PRAK/MAPKAPK5), which has been known to be exported from nucleus to cytoplasm in a CRM1-dependent manner (Fig. 5H). However, H₂O₂-induced cytoplasmic translocation of NR4A2 was not suppressed (Fig. 5H). In addition, NES-disrupted NR4A2 mutant was also translocated from nucleus to cytoplasm after H₂O₂ treatment similarly to NR4A2 WT in both cellular fractionation and immunocytochemistry analyses (Fig. 5, J and K, respectively). These results suggest that H₂O₂-induced cytoplasmic translocation of NR4A2 occurs in a CRM1-independent and unknown nuclear export mechanism.

Alanine Substitution of Ser¹²⁶, Thr¹²⁹, and Thr¹³² Reduces H₂O₂-induced Cytoplasmic Translocation of NR4A2—As shown in Fig. 4, J and K, we identified Ser¹²⁶, Thr¹²⁹, and Thr¹³² of NR4A2 as H₂O₂-induced ASK1-p38 pathway-dependent phosphorylation sites, which are successfully monitored by the p-NR4A2 S126/T129/T132 antibody using immunoblotting. Consistent with these results, we observed that the signal of the p-NR4A2 S126/T129/T132 antibody was increased by H₂O₂ treatment also in immunocytochemistry analysis (Fig. 6A, top panels). The specificity of this antibody to phospho-NR4A2 in immunocytochemistry analysis was confirmed, because the signal of this antibody was suppressed by alanine substitution at Ser¹²⁶, Thr¹²⁹, and Thr¹³² (Fig. 6A, bottom panels). Interestingly, the signal of this antibody was enhanced not only in the nucleus but also in the cytosol in response to H₂O₂ treatment (Fig. 6A, top panels), suggesting that NR4A2 is phosphorylated by the ASK1-p38 pathway under oxidative stress conditions and is subsequently translocated from the nucleus to the cytosol.

In order to further confirm the requirement of ASK1-p38 pathway-dependent phosphorylation of NR4A2 for H₂O₂-in-

duced cytoplasmic translocation of NR4A2, we examined whether cytoplasmic translocation of NR4A2 could be suppressed by the alanine substitution of ASK1-p38 pathway-dependent phosphorylation sites. As we mentioned above, the ASK1-p38 pathway was required for efficient cytoplasmic translocation of NR4A2 in response to H₂O₂ (Fig. 5, B, C, E, F, and G). Consistent with these data, H₂O₂-induced cytoplasmic translocation of NR4A2 was suppressed by alanine substitution of ASK1-p38 pathway-dependent phosphorylation sites, although a small population of these mutants still translocated into the cytosol (Fig. 6B). To confirm the suppression of cytoplasmic translocation of NR4A2 by alanine substitution more clearly, we measured the cytoplasmic and nucleic NR4A2 quantitatively using a cell image analyzer in the same way that is shown in Fig. 5, D–F. The H₂O₂-induced cytoplasmic translocation of NR4A2 was significantly suppressed by the cluster II mutant but not by the cluster I, III, or IV mutant (Fig. 6C). In addition, the 3A mutant was also refractory to H₂O₂-induced cytoplasmic translocation (Fig. 6D). These findings suggest that ASK1-p38 pathway-dependent phosphorylation of NR4A2 at Ser¹²⁶, Thr¹²⁹, and Thr¹³² is required for the efficient cytoplasmic translocation of NR4A2 in response to oxidative stress.

Phosphorylation of NR4A2 at Ser¹²⁶, Thr¹²⁹, and Thr¹³² Is Required for H₂O₂-induced Necrosis—Finally, we examined whether ASK1-p38 pathway-dependent phosphorylation of NR4A2 is required for the H₂O₂-induced necrosis. We performed rescue experiments to examine whether the inhibitory effect of NR4A2 knockdown on H₂O₂-induced necrosis could be reversed by expressing a siRNA-resistant construct of NR4A2 and its alanine mutants (cluster II mutant and 3A mutant). These constructs were successfully expressed, even when cells were treated with NR4A2 siRNAs (Fig. 6E). H₂O₂-induced LDH release was significantly rescued by the expression of wild-type NR4A2 but not by the cluster II mutant (Fig. 6F). Furthermore, the expression of the 3A mutant did not rescue H₂O₂-induced LDH release (Fig. 6G). These findings suggest that ASK1-p38 pathway-dependent phosphorylation of NR4A2 at Ser¹²⁶, Thr¹²⁹, and Thr¹³² is also required for H₂O₂-induced necrosis as well as H₂O₂-induced efficient cytoplasmic translocation.

DISCUSSION

In this study, we observed that NR4A2 is translocated from the nucleus to the cytoplasm in an ASK1-p38 pathway-depen-

FIGURE 5. The ASK1-p38 pathway mediates H₂O₂-induced cytoplasmic translocation of NR4A2. A, HeLa cells were treated with 3 mM H₂O₂ for 8 h with or without 100 nM actinomycin D and subjected to the LDH assay. Error bars, S.E. B and E, 24 h after transfection with the ASK1 siRNA, FLAG-NR4A2 was transiently transfected into HeLa cells. Twenty-four hours later, the cells were treated with 3 mM H₂O₂ for 90 min and subjected to immunocytochemistry analysis with anti-FLAG antibody. The images were monitored using confocal microscopy (B), and the cytoplasmic intensity per total intensity of FLAG-NR4A2 was quantitated using a cell image analyzer (E). Error bars, S.E. (*, $p < 0.05$; **, $p < 0.01$, one-way analysis of variance and Dunnett's multiple comparison test compared with the control) (E). C and F, 48 h after transfection with FLAG-NR4A2, HeLa cells that were pretreated with 10 μ M SB202190 for 30 min were incubated with 3 mM H₂O₂. After the indicated periods, the cells were subjected to immunocytochemistry analysis with the anti-FLAG antibody. The images were monitored using confocal microscopy (C), and H₂O₂-induced translocation of each NR4A2 was quantified using a cell image analyzer (F). Error bars, S.E. (**, $p < 0.01$, Student's *t* test). D, the intensity of nuclear and cytoplasmic NR4A2, which is enclosed by green and red lines, respectively, was detected from 200 cells using a cell image analyzer. G, 48 h after transfection with FLAG-NR4A2, HeLa cells that were pretreated with 1 μ M PH797804 and 5 μ M K811 for 30 min were incubated with 3 mM H₂O₂. After the indicated periods, the cells were lysed and separated into nucleic and cytoplasmic fractions and then subjected to immunoblotting. H, 48 h after transfection with FLAG-NR4A2, HeLa cells that were pretreated for the indicated times with the indicated concentrations of leptomycin B (LMB) were incubated with 3 mM H₂O₂. After the indicated periods, the cells were subjected to immunocytochemistry analysis with the anti-FLAG antibody. I, there are two NESs in NR4A2. J, 48 h after transfection with FLAG-NR4A2, HeLa cells were incubated with 3 mM H₂O₂. After the indicated periods, the cells were lysed and separated into nucleic and cytoplasmic fractions and then were subjected to immunoblotting. K, 48 h after transfection with FLAG-NR4A2, HeLa cells were incubated with 3 mM H₂O₂. After the indicated periods, the cells were subjected to immunocytochemistry analysis. N.S., not significant.

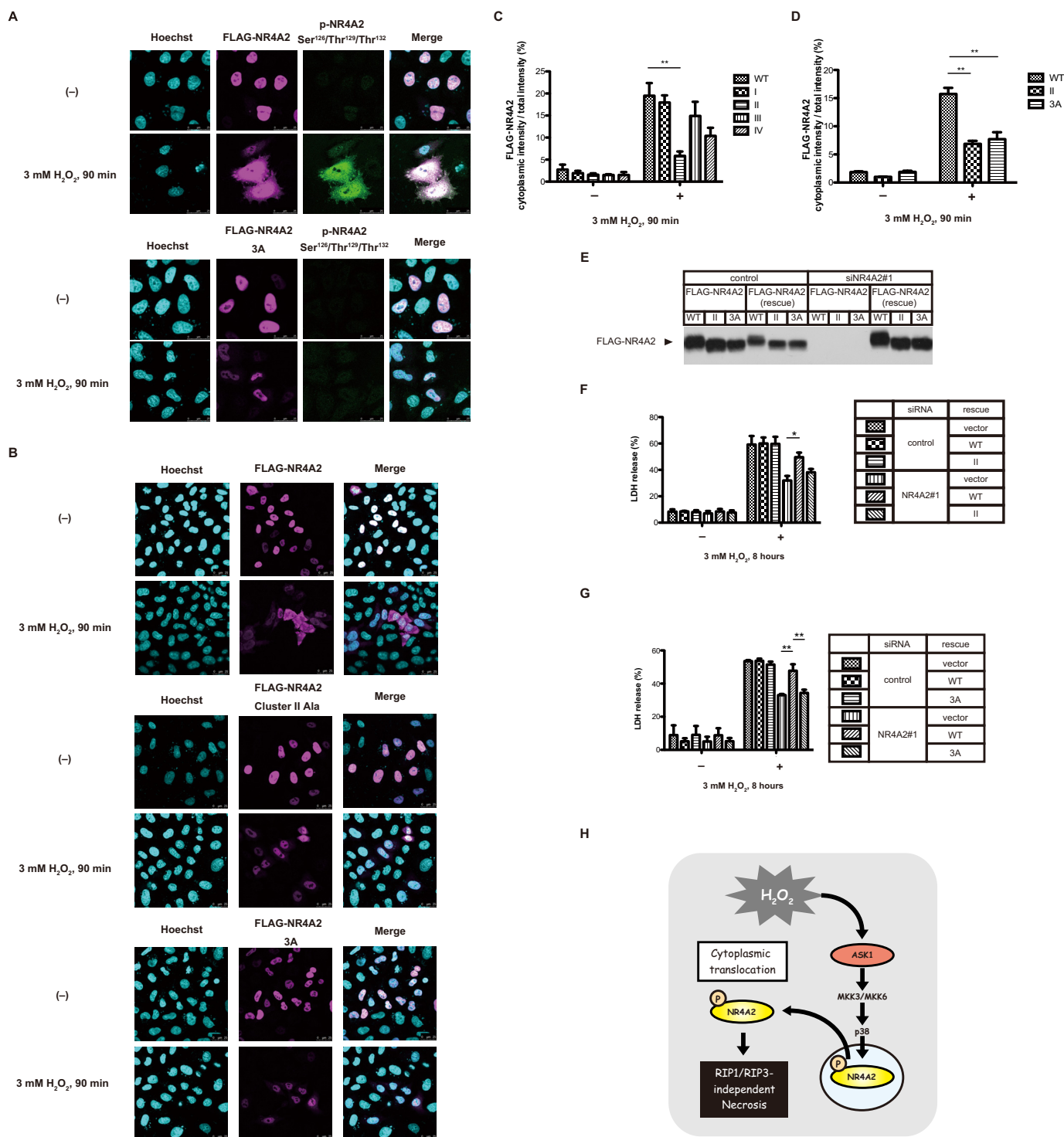


FIGURE 6. Alanine substitution of Ser¹²⁶, Thr¹²⁹, and Thr¹³² reduces H₂O₂-induced cytoplasmic translocation of NR4A2 and necrosis. *A*, 48 h after transfection with the indicated expression plasmids, HeLa cells were treated with 3 mM H₂O₂ for 90 min. After the indicated periods, the cells were subjected to immunocytochemistry analysis with the anti-FLAG antibody and phospho-NR4A2 antibody. The images were monitored using confocal microscopy. *B–D*, 48 h after transfection with the indicated expression plasmids, HeLa cells were treated with 3 mM H₂O₂ for 90 min. After the indicated periods, the cells were subjected to immunocytochemistry analysis with the anti-FLAG antibody. The images were monitored using confocal microscopy (*B*). H₂O₂-induced translocation of each NR4A2 was quantified using a cell image analyzer (**, $p < 0.01$, one-way analysis of variance and Dunnett's multiple comparison test compared with the control) (*C* and *D*). *E*, siRNA-resistant constructs of NR4A2 were successfully expressed. 24 h after transfection of the NR4A2 siRNA, the indicated NR4A2-expressing plasmids were transiently transfected, and 24 h later, HeLa cells were lysed and subjected to immunoblotting. *F* and *G*, 24 h after transfection of the NR4A2 siRNA and the indicated siRNA-resistant NR4A2-expressing constructs, the cells were treated with 3 mM H₂O₂ for 8 h and subjected to the LDH assay. Error bars, S.E. (**, $p < 0.01$, one-way analysis of variance and Bonferroni's multiple comparison test). *H*, schematic model of this study.

dent manner in response to oxidative stress. Because the alanine substitution of ASK1-p38 pathway-dependent NR4A2 phosphorylation sites reduces the cytoplasmic translocation of NR4A2, the ASK1-p38 pathway-dependent phosphorylation of NR4A2 appears to be required for the efficient translocation of NR4A2 into the cytosol (Fig. 6, B–D). Moreover, the alanine substitution of these phosphorylation sites did not rescue the necrosis-promoting activity of NR4A2 under oxidative stress, suggesting that the ASK1-p38 pathway-dependent phosphorylation of NR4A2 is also required for the induction of necrosis (Fig. 6, F and G). Taking these results together, we proposed the model of ASK1-p38-NR4A2 axis-regulated H₂O₂-induced necrosis as shown in Fig. 6H. Oxidative stress-induced phosphorylation of NR4A2 induces the efficient cytoplasmic translocation of NR4A2 in an ASK1-p38 pathway-dependent manner, and this localization change of NR4A2 may play important roles in necrosis induction. Because this cell death was not inhibited by RIP kinase inhibitor, ASK1-p38-NR4A2 axis-regulated H₂O₂-induced necrosis is RIP1-RIP3-independent. Although the involvement of p38 in necrosis has been implicated (16), the molecular mechanisms by which p38 promotes necrosis have been largely unknown. Our findings may provide insights into the molecular mechanisms of p38-mediated necrosis.

NR4A2 is a member of the NR4A orphan nuclear receptor family (23), whose well known function is transcriptional regulation. Because NR4A family members lack intrinsic ligand binding and classical trans-activation domains that are common to other nuclear receptor families, NR4A family members appear to activate gene expression in a constitutive, ligand-independent manner (24). Therefore, it has been considered that post-translational modifications, such as phosphorylation, may be important for the transcriptional activity of NR4As. We previously reported that p38 directly phosphorylates unidentified sites in cluster II of NR4A2 and activates its transcriptional activity (6). In this study, we identified Ser¹²⁶, Thr¹²⁹, and Thr¹³² as phosphorylation sites that are required for its efficient cytoplasmic translocation (Fig. 6, B and D) and subsequent necrosis induction (Fig. 6G). Interestingly, these sites also resided in cluster II (Fig. 4E). Therefore, it is conceivable that the transcriptional activity of NR4A2 is up-regulated by p38-dependent phosphorylation under oxidative stress conditions. However, pretreatment with a transcription inhibitor, such as actinomycin D, did not inhibit H₂O₂-induced necrosis (Fig. 5A). Although the reason for p38-dependent phosphorylation within the same region in NR4A2 having different roles remains to be elucidated, the above mentioned result suggests that NR4A2 induces necrosis at least in a transcription-independent manner.

Although our experiments using the NR4A2 alanine mutants suggest that phosphorylation of Ser¹²⁶, Thr¹²⁹, and Thr¹³² is required for efficient cytoplasmic translocation of NR4A2 (Fig. 6, B–D), the overexpression of NR4A2 aspartic acid mutant (NR4A2 3D) showed neither cytoplasmic localization nor necrosis-promoting activity under steady-state conditions (data not shown). However, we cannot exclude the possibility that the NR4A2 3D mutant does not act as a phosphomimetic mutant in this case.

For a long time, necrosis has been regarded as an accidental and uncontrollable cell death that does not involve intracellular signaling, unlike apoptosis. However, it has now been recognized that necrosis can also occur in a regulated manner (25). Increasing evidence has suggested that H₂O₂-induced necrosis is also a regulated process. For example, cyclophilin D and its interaction partner p53 mediate H₂O₂-induced necrosis by regulating mitochondrial permeability transition pore opening (26–28). In another report, poly(ADP-ribose) polymerase 1 overactivation and apoptosis-inducing factor nuclear translocation were found to be important processes for the induction of necrosis under oxidative stress (29). Because the suppression of NR4A2 did not completely attenuate the H₂O₂-induced necrosis, it is possible that not only NR4A2 but also the other regulators mentioned above might be involved downstream of ASK-p38 pathways. Moreover, to understand the mechanisms by which cytoplasmic NR4A2 is exported from the nucleus in response to H₂O₂-induced necrosis, it will also be important to investigate the relationship between cytoplasmic NR4A2 and the above mentioned necrosis regulators.

Recently, cytoplasmic localization of NR4A2 has been observed in some pathologic conditions, such as in a brain ischemic reperfusion injury model (30) and in bladder cancer tissue (31). Although the involvement of the necrosis-promoting activity of cytoplasmic NR4A2 in these models is not known, we hope that our findings will lead to the discovery of new therapeutic targets for these disease conditions.

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