Cytosolic Peroxiredoxin Attenuates the Activation of JNK and p38 but Potentiates That of ERK in HeLa Cells Stimulated with Tumor Necrosis Factor-α*

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Tumor necrosis factor-α (TNF-α) induces the activation of all three types of mitogen-activated protein kinase (MAPK): c-Jun NH₂-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK). This cytokine also induces the production of several types of reactive oxygen species, including H₂O₂. With the use both of HeLa cells expressing wild-type or dominant negative forms of the cytosolic peroxidase peroxiredoxin II and of mouse embryonic fibroblasts deficient in this protein, we evaluated the roles of H₂O₂ in the activation of MAPKs by TNF-α. In vitro kinase assays as well as immunoblot analysis with antibodies specific for activated MAPKs indicated that H₂O₂ produced in response to TNF-α potentiates the activation of JNK and p38 induced by this cytokine but inhibits that of ERK. Our results also suggest that cytosolic peroxiredoxins are important regulators of TNF signaling pathways.

The responses of cells to extracellular stimuli are mediated by a complex system of intracellular signaling. Such signaling thus controls many aspects of cell function, including proliferation, differentiation, and death. Protein kinases and phosphatases are common components of signaling pathways and regulate the function of proteins by phosphorylation and dephosphorylation, respectively. Oxidants and antioxidants have also been increasingly recognized as important signaling molecules that modify protein function through oxidation and reduction of redox-sensitive amino acid residues such as cysteine and methionine.

Tumor necrosis factor-α (TNF-α)[1] is an extracellular stimu-

lus whose intracellular signaling relies extensively on phosphorylation and redox systems (1–4). This cytokine induces both proapoptotic and prosurvival effects that are achieved through the activation of various signaling pathways, including those mediated by caspases, nuclear factor-κB, and mitogen-activated protein kinases (MAPKs). The MAPK pathway is a common signal transduction system and typically comprises a MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK), and MAPK. MAPKs function in intracellular signaling by phosphorylating a wide range of effector proteins, most notably transcription factors. On the basis of their structures, MAPKs have been classified into three multimember subfamilies: c-Jun NH₂-terminal kinases (JNKs), p38 MAPKs, and extracellular signal-regulated kinases (ERKs). Although the outcomes of MAPK activation depend on the nature of the extracellular signal and on cell type, ERKs tend to influence cell proliferation and survival, whereas JNKs and p38 MAPKs primarily function in responses to stressors such as apoptotic stimuli (4–6).

Like many other extracellular stimuli, TNF-α induces the generation of reactive oxygen species (ROS), including the superoxide anion (O₂⁻) and hydrogen peroxide (7, 8). Although the mechanism of ROS generation has not been well characterized, studies with various inhibitors suggest that NADPH oxidase (9, 10), 5-lipoxygenase (11), or the electron transport chain of mitochondria (12, 13) might be the source of H₂O₂ produced in cells in response to TNF-α. The ROS thus produced participate in various TNF-α signaling pathways. A role for ROS in TNF-α-induced activation of JNK and p38 has been demonstrated by various observations. Thus, antioxidants such as N-acetylcysteine (NAC) and thioredoxin (Trx) block TNF-α-induced activation of JNK and p38 in various cell types (8, 10, 14, 15), and specific inhibitors of 5-lipoxygenase as well as inhibition of NADPH oxidase by diphenyliodonium or by dominant negative forms of Rac suppress TNF-α-induced JNK activation (10, 16). In contrast, the role of ROS in TNF-α-induced ERK activation has been less well characterized. NAC was shown to inhibit TNF-α-induced activation of ERK as well as of JNK and p38 in macrophages (17). In addition, ROS produced in cardiac fibro-

Opposite Effects of H_2O_2 on JNK or p38 versus ERK

blasts in response to angiotensin II (18) or in smooth muscle cells in response to platelet-derived growth factor (PDGF) (19) promote the activation of all three types of MAPK through redox-sensitive mechanisms.

The ROS initially produced by mitochondria or by NADPH oxidase is O_2^-, which is then converted to H_2O_2 enzymatically or nonenzymatically. It seems that O_2^- and H_2O_2 act differently in signaling pathways as suggested by that T cell receptor-induced ERK activation was enhanced specifically by H_2O_2 but not by O_2^-, whereas T cell receptor-induced cell death was mediated by O_2^- but not by H_2O_2 (20). In addition, various stimuli, including TNF-α induce the production of nitric oxide (21). Given that antioxidants (such as NAC and Trx) and inhibitors of ROS production (such as diphenyliodonium and dominant negative Rac) affect the intracellular concentrations of several oxidants, including O_2^-, H_2O_2, and various NO derivatives, studies with these reagents are not able to identify the oxidant species that participate in receptor signaling. Recent study also reveals that NAC inhibits TNF-α-induced NFκB activation not by scavenging ROS but by lowering the affinity of the receptor to TNF-α (22). In contrast, O_2^- and H_2O_2 are selectively removed by superoxide dismutase and catalase, respectively, and studies with cells overexpressing these enzymes suggest that both O_2^- and H_2O_2 participate in a variety of receptor signaling pathways. For example, overexpression of Mn^{2+}-dependent superoxide dismutase resulted in inhibition of TNF-α-induced activation of caspases, nuclear factor κB, and JNK (23), indicating the O_2^- acts in various TNF-α signaling pathways. Although the effects of catalase overexpression on TNF-α-induced activation of MAPK pathways have not been described, the effects of catalase on MAPK pathways activated by other stimuli appear to be dependent on stimulant and cell type (19, 24–27).

We have now investigated the effects of H_2O_2 on the activation of each type of MAPK induced by TNF-α in HeLa cells. Instead of catalase, we overexpressed peroxiredoxin (Prx) in these cells to eliminate H_2O_2 selectively. A dominant negative form of Prx was also constructed to study the effects of diminished H_2O_2 elimination. Members of the Prx family of peroxidases catalyze the reduction of H_2O_2 with the use of reducing equivalents provided by Trx (28–30). Six mammalian isoforms of Prx, which are distributed differentially within cells, have been identified: Prx I, II, and VI are localized to the cytosol; Prx III is restricted to mitochondria; Prx IV is secreted; and Prx V is located in mitochondria and peroxisomes. Prx enzymes are abundant in most cell types (31).

All Prx proteins exist as obligate homodimers with two identical active sites, and they all contain a conserved Cys residue, corresponding to Cys[51] in mammalian Prx I and II, in the NH$_2$-terminal portion of the molecule (28–30). Most Prx proteins, including four (Prx I–IV) of the six mammalian isoforms, contain an additional conserved Cys residue in the COOH-terminal region that corresponds to Cys[172] of mammalian Prx I and II. The Prx enzymes that contain two conserved Cys residues are known as 2-Cys Prxs to distinguish them from the small number of 1-Cys Prxs, which contain only the conserved Cys in the NH$_2$-terminal domain. In 2-Cys Prx enzymes, the NH$_2$-terminal conserved Cys is oxidized by H_2O_2 to cysteine-sulfenic acid (Cys[51]-SOH), which then reacts with Cys[172]-SH of the other subunit of the homodimer to produce an intermolecular disulfide (32). Reduction of the disulfide intermediate is mediated by Trx.

In addition to examining the effects of overexpression of wild-type or dominant negative forms of Prx in HeLa cells, we have compared the TNF-α-induced activation of MAPKs in mouse embryonic fibroblasts (MEFs) derived from wild-type

and Prx II knockout animals. Our observations indicate that TNF-α-induced activation of JNK and p38 is positively regulated by H_2O_2, whereas ERK activation in response to this cytokine is inhibited as a result of H_2O_2 production.

EXPERIMENTAL PROCEDURES

Antibodies, Plasmids, and Reagents—Mouse monoclonal antibodies to the FLAG epitope (M2) were obtained from Sigma; rabbit polyclonal antibodies to JNK1 (C-17), to p38 (C-20), to ERK2 (C-14), and to ASK-1 (H-300) were from Santa Cruz Biotechnology; and rabbit polyclonal antibodies to phospho-JNK, phospho-ERK, and phospho-p38 were from New England Biolabs. Rabbit polyclonal antibodies to human Prx I, II, or III have been described previously (33). Recombinant human TNF-α was from Life Technologies, and 2′,3′-dihydro-2′,7′-dichlorofluorescein diacetate (DCFH-DA) and 5(and 6)-chloromethyl-2′,7′-dichlorofluorescein diacetate (CM-H$_2$DCFDA) were from Molecular Probes. An *Escherichia coli* expression plasmid encoding a glutathione *S*-transferase (GST) fusion protein of c-Jun-(1–79) was provided by N. Holbrook (Yale University Medical School); mammalian expression vectors encoding hemagglutinin epitope (HA)-tagged forms of JNK3 and ERK2 were by J. M. Kyriakis (Harvard University Medical School) and S. Gutkind (National Institutes of Health), respectively; pGEX-4T-1/SEK1 (K129R) was from L. Zon (Howard Hughes Medical Institute, Harvard Medical School).

A mammalian expression vector for wild-type Prx II, pCRIIWT, has been described (34). A vector encoding a dominant negative form (C52S,C172S) of Prx II, pCRIIDN, was constructed by standard PCR-mediated site-directed mutagenesis with pCRIIWT as the template. To add a FLAG tag to the NH$_2$ terminus of Prx II, we prepared a 53-nucleotide primer (5′-AAGCATATGGGACTACAAGGACGATGACGATA-AGGGTGCCTCCGGTAACGCGCG-3′, FLAG coding sequence underlined) and performed PCR with pCRIIWT or pCRIIDN as the template. The resulting PCR products were subcloned into pCR3.1-Uni to generate pCRIIWT-FLAGN and pCRIIDN-FLAGN, respectively.

Construction of a Retrovirus Encoding Prx II—A full-length cDNA for human Prx II was cloned between the XhoI and BamHI sites of a bicistronic retroviral vector (pLXIN, Clontech). The resulting construct was introduced by transfection into RetroPack PT67 cells (Clontech), which were then cultured in the presence of G418 (500 μg/ml) to establish stable retrovirus-producing cell lines. The isolated virus clones were evaluated for the extent of Prx II expression induced after infection of Prx II$^{-/-}$ MEFs. The titer of the chosen clone was $\sim 2 \times 10^6$ plaque-forming units/ml.

Cell Culture—The generation of Prx II$^{-/-}$ mice has been described (35). MEFs were prepared at embryonic day 10 from embryos obtained by mating Prx II$^{+/-}$ animals. The cells were genotyped by Southern blot and PCR analysis of genomic DNA; the PCR primers were 5′-GCTTG-GGTGGAGAGGCTATTCG-3′ and 5′-GTAAAGCACGAGGAAGCGGT-CAGCC-3′ for the *neo* cassette and 5′-GATGATCTCCGTGGGGCAAA-CAAAAGTGAAG-3′ and 5′-ATGGCCTCCGGCAACGCGCAAATCG-3′ for the wild-type allele (35). MEFs, NIH 3T3 cells, and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with either 10% calf serum (NIH 3T3 cells) or 10% fetal bovine serum (MEFs and HeLa cells) as well as with penicillin (100 units/ml) and streptomycin (100 units/ml). NIH3T3 and HeLa cells were continuously passaged for 2 months after thawing. For generation of stably transfected cell lines, HeLa cells (1×10^6 in 60-mm dishes) were transfected with 6 μg of either pCR3.1, pCRIIWT, or pCRIIDN with the use of SuperFect (Qiagen) and were selected by culture in the presence of G418 (1 mg/ml). The selected clones were maintained in medium containing G418 (0.2 mg/ml).

In Vitro Kinase Assays—HeLa cells (1×10^6) that had been maintained in DMEM containing 0.5% fetal bovine serum for 24 h were stimulated with TNF-α (15 ng/ml), washed once with ice-cold phosphate-buffered saline, and then lysed for 20 min at 4 °C in 1 ml of a lysis buffer containing 25 mM HEPES-NaOH (pH 7.0), 1 mM EDTA, 2 mM EGTA, 1 mM dithiothreitol, 25 mM β-glycerophosphate, 1 mM Na$_3$VO$_4$, 1 mM NaF, microcystine-LR (20 ng/ml), 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, aprotinin (5 μg/ml), and leupeptin (5 μg/ml). For the ASK1 assay, the same lysis buffer used above except that dithiothreitol was omitted. The cells were scraped and further disrupted by vigorous vortex mixing for 1 min. The lysates were centrifuged at 15,000 × g for 20 min, and the resulting supernatants were incubated first for 1 h on ice with 1 μg of antibodies to JNK1, to ERK2, or HA, or 4 μg of antibodies to ASK1 and then, after the addition of 20 μl of protein A-Sepharose CL-4B (Amersham Biosciences), overnight at 4 °C. The beads were isolated, washed twice with lysis buffer and twice

with kinase reaction buffer (20 mM MOPS-NaOH (pH 7.2), 25 mM β-glycerophosphate, 10 mM MgCl₂, 1 mM dithiothreitol, 1 mM Na₃VO₄, 0.1% Triton X-100), and then incubated for 20 min at 37 °C in 40 μl of kinase reaction buffer containing 37.5 μM unlabeled ATP, 0.6 μCi of [γ-^{32}P]ATP, and 6 μg of GST-c-Jun-(1–79) or myelin basic protein (MBP) (Sigma), or 1 μg of GST-SEK1 (KR) as substrate. The reaction was terminated by the addition of 25 μl of 3× SDS sample buffer, and samples were then heated at 95 °C for 5 min and subjected to SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to a nitrocellulose membrane, and the radioactivity incorporated into GST-c-Jun-(1–79), MBP, or GST-SEK1 (KR) was quantified with an image analyzer (PhosphorImager, Amersham Biosciences). The membrane was subsequently subjected to immunoblot analysis with antibodies to JNK1, to ERK2, to HA, or to ASK1 to evaluate the amount of immunoprecipitated kinases.

Immunoprecipitation—Crude cell lysates (500 μg of protein in 1 ml) were incubated for 1 h at 4 °C with 20 μl of protein A-Sepharose CL-4B, after which the resin was removed by centrifugation and the resulting supernatants were incubated for 1 h at 4 °C with 1 μg of antibodies to FLAG. Immune complexes were then isolated by precipitation with 20 μl of protein A-Sepharose CL-4B for 3 h at 4 °C, washed four times with lysis buffer, boiled in SDS sample buffer, and subjected to immunoblot analysis.

RESULTS

Construction of a dominant negative form of Prx II-2-Cys Prx enzymes, which exists as a dimer with the monomers arranged in a head-to-tail manner, and their catalytic cycle requires the formation of intermolecular disulfides between the NH₂-terminal conserved Cys (Cys^{51} of Prx II) of one subunit and the COOH-terminal conserved Cys (Cys^{172} of Prx II) of the other subunit. We reasoned that a double mutant of Prx II in which both conserved Cys residues were replaced with serine might form a catalytically inactive dimer with wild-type (WT) Prx II and thereby act in a dominant negative manner. To determine whether the Prx II(C51S,C172S), or Prx II(DN), mutant forms a dimer with endogenous Prx II(WT) in cells, we subjected NIH 3T3 or HeLa cells to transient transfection with an expression vector for FLAG-tagged Prx II(WT) or FLAG-tagged Prx II(DN) and subsequently immunoprecipitated the FLAG-tagged proteins with specific antibodies. Endogenous Prx II coprecipitated with either FLAG-Prx II(WT) or FLAG-Prx II(DN), and the mutation did not affect the amount of associated endogenous Prx II (Fig. 1A). These results thus suggested that dimerization between endogenous and FLAG-tagged Prx II proteins does not require disulfide formation. The association between the endogenous and FLAG-tagged Prx II proteins increased with time after transfection and was maximal after transfection for 36 h (Fig. 1A), which was the longest time interval examined. Even at this time point, however, the extent of coprecipitation of the endogenous protein was less than that expected from random dimerization with the FLAG-tagged proteins (Fig. 1A), probably because of the long half-life (>3 days) of Prx II (data not shown) and because dimerization occurs only between those molecules synthesized concurrently. Both FLAG-Prx II proteins also interacted with endogenous Prx I (Fig. 1A), albeit to a lesser extent than with endogenous Prx II; the amino acid sequences or Prx I and II are 87% identical. However, targeting of FLAG-Prx II proteins to mitochondria by expression from the pShooter vector (Invitrogen) did not result in a detectable association with the endogenous mitochondrial Prx III (75% identical to Prx II), even though the targeting was successful (data not shown).

To examine whether the Prx II(DN) mutant acts in a dominant negative manner in cells, we measured the intracellular concentration of H₂O₂ with the use of the oxidant-sensitive fluorescent probe DCF (36) in NIH 3T3 cells that had been transfected with a vector for FLAG-Prx II(WT) or FLAG-Prx II(DN). Consistent with previous observations (7, 8), TNF-α induced a transient increase in the intracellular concentration
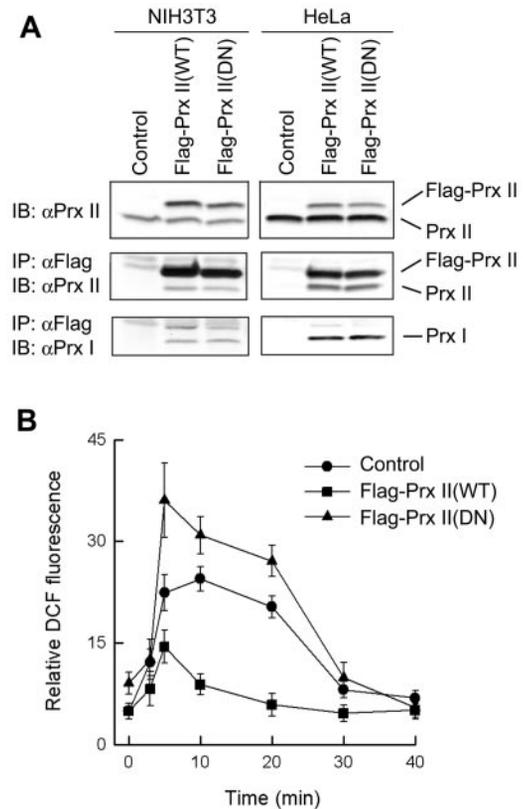

FIG. 1. **Dominant negative action of the Prx II(DN) mutant.** *A*, association of endogenous Prx II with ectopically expressed FLAG-Prx II in cells. NIH 3T3 or HeLa cells were transfected for 36 h with pCR3.1 (control), pCRIIWT-FLAGN (which encodes FLAG-Prx II(WT)), or pCRIIDN-FLAGN (which encodes FLAG-Prx II(DN)). Cell lysates were then prepared and subjected to immunoblot (*IB*) analysis with antibodies to Prx II (*αPrx II*) (*upper panels*). Proteins immunoprecipitated (*IP*) with antibodies to the FLAG epitope (*αFLAG*) were also subjected to immunoblot analysis with antibodies either to Prx II (*middle panels*) or to Prx I (*αPrx I*) (*lower panels*). Data are representative of two similar experiments. *B*, NIH 3T3 cells were transfected for 24 h as in *A*, incubated for 20 h in DMEM containing 0.5% calf serum, stimulated with TNF-α (15 ng/ml) at 37 °C for the indicated times, and then incubated with DCFH-DA (5 μg/ml) for 5 min. DCF fluorescence was monitored by confocal microscopy to estimate the amount of oxidants produced in response to TNF-α. Data are means ± S.E. of values from five groups of 20–30 cells and are representative of two similar experiments.

of H₂O₂ in control cells transfected with the empty vector, and this effect was inhibited by expression of FLAG-Prx II(WT) (Fig. 1B). In contrast, the TNF-α-induced accumulation of H₂O₂ was more pronounced in cells expressing FLAG-Prx II(DN) than in control cells (Fig. 1B), indicating that the mutant protein acts in a dominant negative manner by forming a catalytically incompetent dimer with the endogenous enzyme.

Effects Prx II(WT) and Prx II(DN) on TNF-α-induced MAPK Activation—We investigated the effects of ectopic expression of Prx II(WT) or Prx II(DN) on TNF-α-induced MAPK activation in HeLa cell lines stably transfected with the corresponding vectors. The level of Prx II expression in all such cell lines obtained was ~1.5- to 2.5-fold that of control cells transfected with the empty vector (Fig. 2A). Although we performed several rounds of transfection and G418 selection, we were not able to isolate cell clones expressing the recombinant protein at higher levels. Two clones of each type (CT2 and CT4 for control cells, WT4 and WT12 for Prx II(WT)-expressing cells, and DN10 and DN15 for Prx II(DN)-expressing cells) were chosen for use in subsequent experiments. Untagged Prx II, rather than FLAG-Prx II, was expressed in the WT and DN cell lines, because modification of the NH₂- or COOH-terminal regions of the
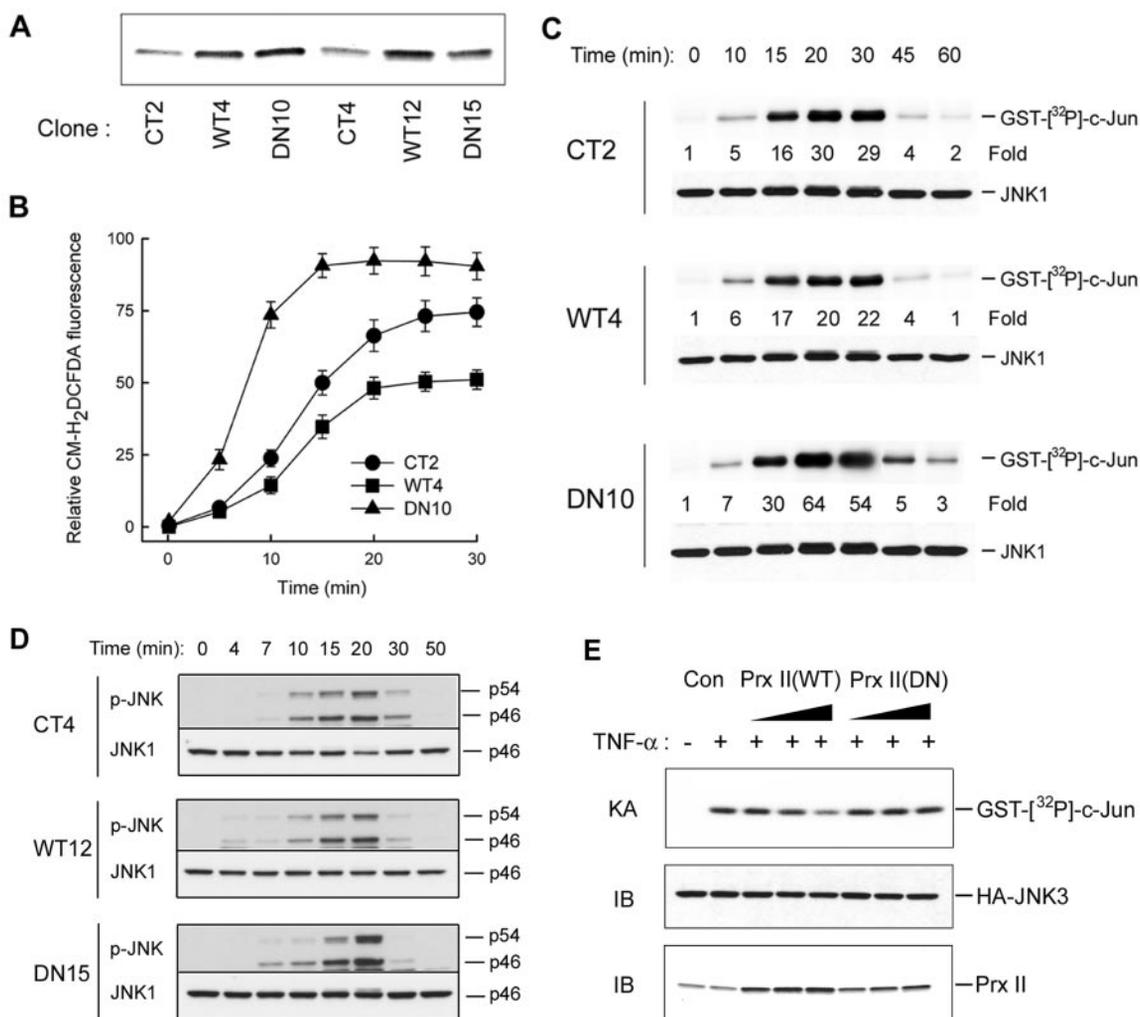
FIG. 2. **Effects of expression of Prx II(WT) or Prx II(DN) on TNF-α-induced JNK activation.** *A*, immunoblot analysis with antibodies to Prx II of lysates prepared from two independent clones each of HeLa cells stably transfected with pCR3.1 (*CT2* and *CT4*), pCRIIWT (*WT4* and *WT12*), or pCRIIDN (*DN10* and *DN15*). *B*, the stably transfected CT2, WT4, and DN10 cells were loaded with CM-H₂DCFDA (5 μg/ml) and then immediately stimulated with TNF-α (15 ng/ml) for the indicated times. DCF fluorescence was monitored as described in Fig. 1*B*. *C*, TNF-α-induced activation of JNK1 in CT2, WT4, and DN10 cells. The stably transfected HeLa cells were stimulated with TNF-α (15 ng/ml) for the indicated times, after which JNK1 was immunoprecipitated from cell lysates with antibodies to this protein. The immunoprecipitates were either assayed for kinase activity with GST-c-Jun-(1–79) as substrate (*upper panels*) or subjected to immunoblot analysis with the same antibodies to JNK1 (*lower panels*). The *numbers between the panels* represent the amount of ³²P radioactivity incorporated into GST-c-Jun-(1–79) after normalization by the corresponding JNK1 immunoblot intensity and expression as -fold increase relative to the corresponding value for time zero; they are means of values from two independent experiments. *D*, TNF-α-induced JNK phosphorylation in CT4, WT12, and DN15 cells. The stably transfected HeLa cells were stimulated with TNF-α (15 ng/ml) for the indicated times, after which cell lysates were subjected to immunoblot analysis with antibodies to phospho-JNK (*p-JNK*). The same membranes were reprobed with antibodies to JNK1 to verify equal loading of samples. Data are representative of two independent experiments. *E*, TNF-α-induced activation of JNK3 in HeLa cells transiently expressing Prx II(WT) or Prx II(DN). HeLa cells were transfected for 24 h with a vector for HA-tagged JNK3 (4 μg/ml) and with either pCR3.1 (6 μg/ml) or various amounts of pCRIIWT or of pCRIIDN (2, 4, or 6 μg/ml). They were then incubated for 24 h in DMEM containing 0.5% fetal bovine serum before stimulation with TNF-α (15 ng/ml) for 20 min. HA-JNK3 was immunoprecipitated with antibodies to HA and either assayed for kinase activity (*KA*) as in *C* (*top panel*) or subjected to immunoblot analysis with the same antibodies (*middle panel*). Cell lysates were also subjected to immunoblot analysis with antibodies to Prx II (*bottom panel*). Data are representative of two independent experiments.

protein inactivates its peroxidase activity. We were thus not able to estimate the extent of heterodimerization between endogenous Prx II and Prx II(DN). Given that mutation of the two conserved Cys residues did not affect heterodimerization and that the abundance of Prx II(DN) was higher than that of endogenous Prx II, more than half of the endogenous protein in DN10 or DN15 cells was likely present in an inactive (heterodimeric) form.

We attempted to assess TNF-α-induced changes in H₂O₂ levels in HeLa cells. Although we treated the cells with up to 100 ng/ml of TNF-α, the increase in DCF fluorescence was too small to allow the assessment of Prx effect (not shown). This was probably because H₂O₂ is more rapidly eliminated in HeLa cells than in NIH3T3 cells such that not enough H₂O₂ is accu-

mulated for DCF oxidation. In the commonly used DCF assay method such as that employed for the NIH cells above (see Fig. 1*B*), because of the limit of sensitivity, it is necessary to allow accumulation of H₂O₂ for several minutes after agonist stimulation before DCF is introduced to the cells to measure the H₂O₂ produced. In fact, the concentrations of Prx enzymes are much higher in HeLa cells than in NIH 3T3 cells (not shown). Therefore, we modified the assay to capture the effect of Prx II: HeLa cells were incubated with DCF and TNF-α simultaneously, with DCF oxidation monitored at different time points, in hopes that the H₂O₂ produced in response to TNF-α would be quenched by DCF before being eliminated by antioxidant enzymes. The shortcoming of the modified procedure is that H₂O₂ cannot be measured at various times after TNF-α
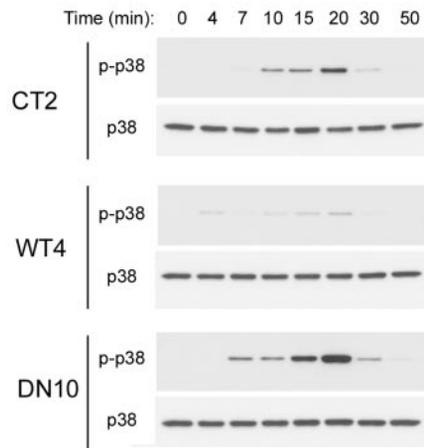
FIG. 3. **TNF-α-induced p38 phosphorylation in HeLa cell lines stably expressing Prx II(WT) or Prx II(DN).** CT2, WT4, or DN10 cells were stimulated with TNF-α (15 ng/ml) for the indicated times, after which cell lysates were subjected to immunoblot analysis with antibodies to phospho-p38 (*p-p38*). The same membrane was reprobed with antibodies to p38 to verify equal loading of samples. Data are representative of three independent experiments.

treatment, because DCF and TNF-α are applied simultaneously. Nevertheless, the experiment revealed that DCF oxidation was inhibited by the expression of Prx II(WT) but enhanced by the expression of Prx II(DN) (Fig. 2*B*).

We examined the TNF-α-induced activation of JNK1 in CT2, WT4, and DN10 cell lines. The kinase activity of immunoprecipitated JNK1 was measured with a GST-c-Jun-(1–79) fusion protein as substrate. The TNF-α-induced activation of JNK1 was inhibited by ~30% in WT4 cells, but was increased about 2-fold in DN10 cells, compared with that apparent in CT2 cells (Fig. 2*C*). When similar experiments were carried out with CT4, WT12, and DN15, JNK1 activity was reduced by ~40% in WT12 cells but was increased by ~60% in DN15 cells compared with that in CT4 cells (data not shown). Given that JNK activity is correlated with the extent of phosphorylation of Thr[183] and Tyr[185], we also assessed TNF-α-induced JNK activation in CT4, WT12, and DN15 cells by immunoblot analysis with antibodies specific for JNK1 or JNK2 phosphorylated at these two sites (Fig. 2*D*). The extent of phosphorylation of both p54 and p46 isoforms of JNK1 and JNK2 did not differ substantially between CT4 and WT12 cells but was increased about 2-fold in DN cells.

We also studied JNK activation in HeLa cells transiently expressing Prx II(WT) or Prx II(DN) together with HA-tagged JNK3. The kinase activity of HA-JNK3 immunoprecipitated with antibodies to HA was thus measured after treatment of the cells with TNF-α. Overexpression of Prx II(WT) resulted in a small, but dose-dependent, decrease in JNK activity, whereas transient expression of Prx II(DN) had no effect on JNK activity (Fig. 2*E*). This lack of effect of Prx II(DN) is likely attributable to the fact that, unlike in stably transfected cells, only a small fraction of endogenous Prx II is able to form heterodimers with newly synthesized mutant molecules in the transiently transfected cells (Fig. 1*A*), and the TNF-α-induced increase in H$_2$O$_2$ was therefore not sufficient to enhance JNK activation.

We also examined the activation of p38 in response to TNF-α in the stably transfected cell lines CT2, WT4, and DN10. Immunoblot analysis with antibodies specific for p38 phosphorylated on Thr[180] and Tyr[182] indicated that TNF-α-induced p38 activation was almost abolished in WT4 cells and was increased ~2.5-fold in DN10 cells (Fig. 3). These results suggest that the TNF-α-induced activation of p38 is more sensitive than is that of JNK to the decrease in H$_2$O$_2$ accumulation
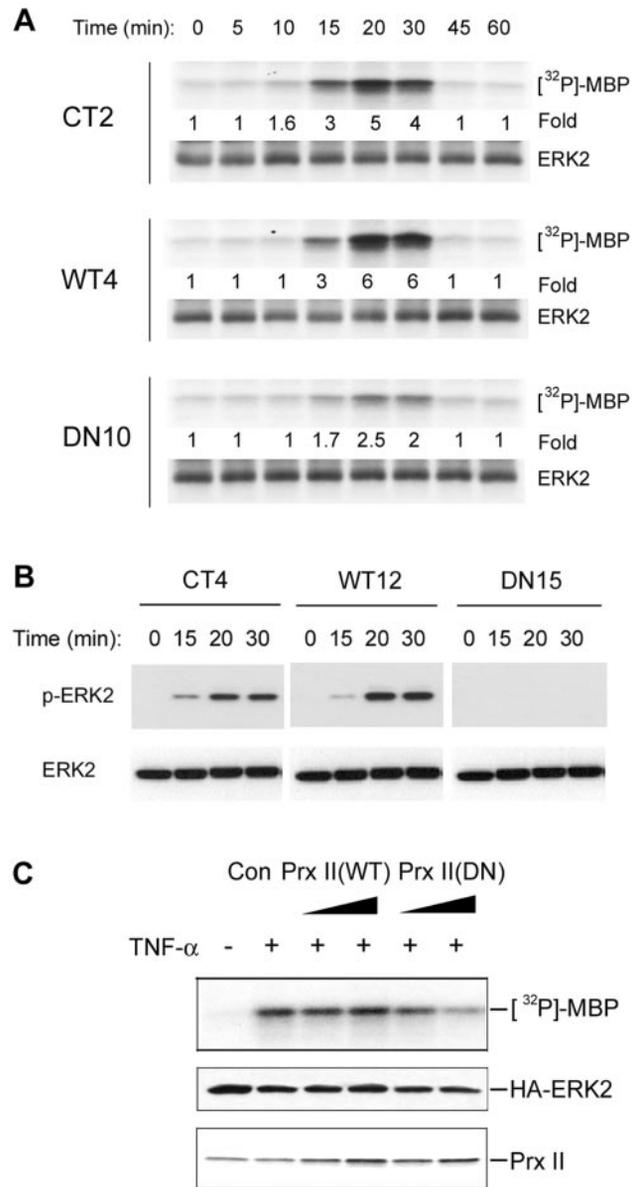

FIG. 4. **Effects of expression of Prx II(WT) or Prx II(DN) on TNF-α-induced ERK activation.** *A*, TNF-α-induced ERK2 activation in CT2, WT4, and DN10 cells. The stably transfected HeLa cells were stimulated with TNF-α (15 ng/ml) for the indicated times, after which ERK2 was immunoprecipitated from cell lysates with antibodies to ERK2. The resulting precipitates were either assayed for kinase activity with MBP as substrate (*upper panels*) or subjected to immunoblot analysis with the same antibodies to ERK2 (*lower panels*). The *numbers between the panels* represent the amount of ^{32}P radioactivity incorporated into MBP after normalization by the corresponding ERK2 immunoblot intensity and expression as -fold increase relative to the corresponding value for time zero; they are means of values from two independent experiments. *B*, TNF-α-induced ERK phosphorylation in CT4, WT12, and DN15 cells. The stably transfected HeLa cells were stimulated with TNF-α (15 ng/ml) for the indicated times, after which cell lysates were subjected to immunoblot analysis with antibodies to phospho-ERK1/2. The same membrane was reprobed with antibodies to ERK2. Data are representative of two independent experiments. *C*, TNF-α-induced ERK2 activation in HeLa cells transiently expressing Prx II(WT) or Prx II(DN). HeLa cells were transfected for 24 h with a vector for HA-ERK2 (3 μg/ml) together with either pCR3.1 (6 μg/ml) or two different amounts of pCRIIWT or pCRIIDN (3 or 6 μg/ml). They were then incubated in DMEM containing 0.5% fetal bovine serum for 24 h before stimulation with TNF-α (15 ng/ml) for 20 min. HA-ERK2 was immunoprecipitated from cell lysates with antibodies to HA and either assayed for kinase activity as in *A* (*upper panel*) or subjected to immunoblot analysis with the same antibodies (*middle panel*). Cell lysates were also subjected to immunoblot analysis with antibodies to Prx II (*lower panel*). Data are representative of two independent experiments.
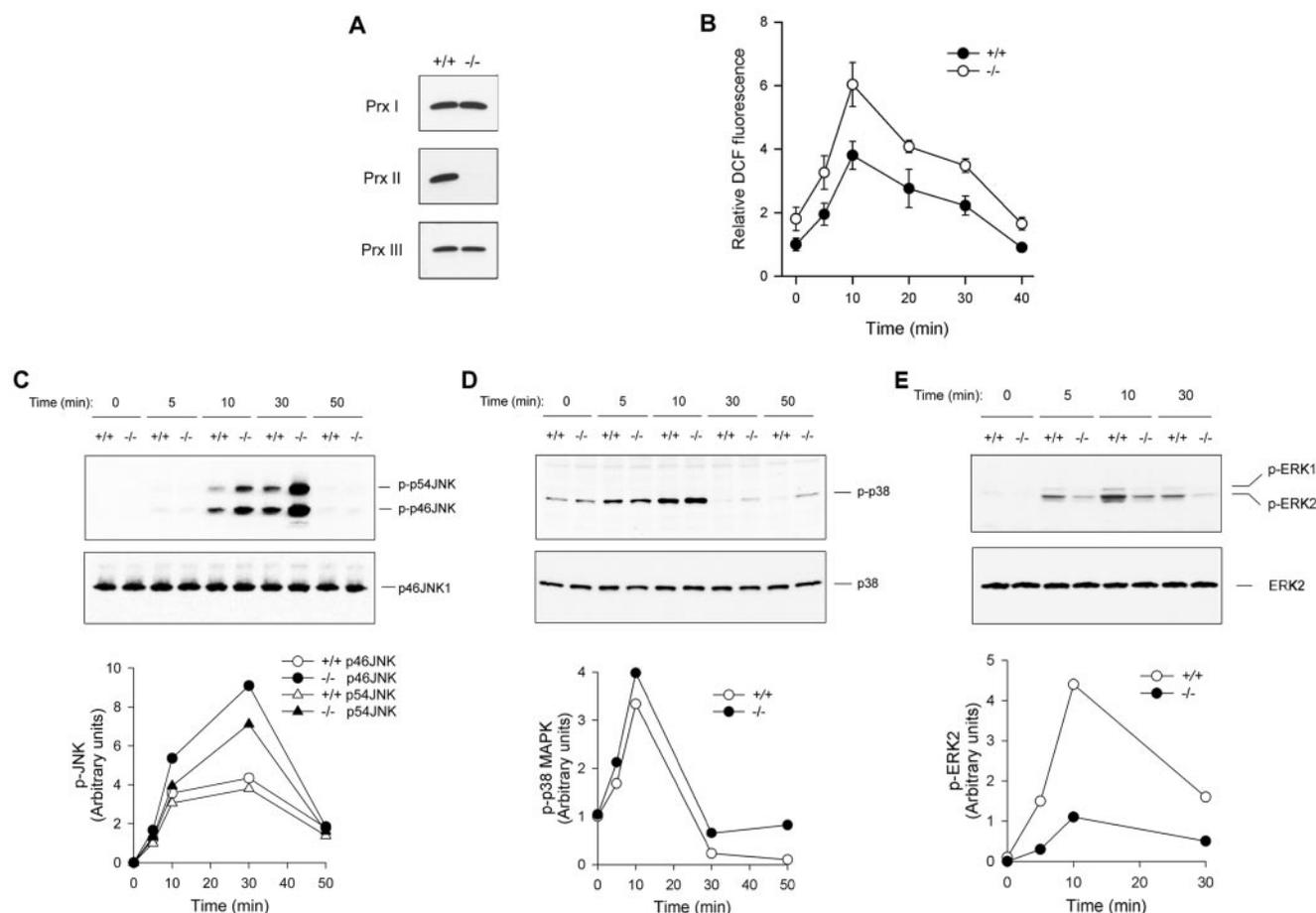
FIG. 5. **TNF-α-induced activation of JNK, p38, and ERK in Prx II⁻/⁻ and wild-type MEFs.** *A*, expression of Prx proteins in Prx II⁻/⁻ and wild-type (+/+) MEFs as detected by immunoblot analysis with isoform-specific antibodies. *B*, Prx II⁻/⁻ and wild-type (+/+) MEFs in DMEM containing 0.5% calf serum were stimulated with TNF-α (15 ng/ml) at 37 °C for the indicated times, and then incubated with DCFH-DA (5 μg/ml) for 5 min. DCF fluorescence was monitored as described in Fig. 1*B*. *C–E*, Prx II⁻/⁻ or wild-type MEFs were stimulated with TNF-α (15 ng/ml) for the indicated times, after which cell lysates were subjected to immunoblot analysis with antibodies specific for phosphorylated forms of either JNK (*C*, *upper panel*), p38 (*D*, *upper panel*), or ERK (*E*, *upper panel*). The same membranes were reprobed with antibodies to JNK1 (*C*, *middle panel*), to p38 (*D*, *middle panel*), or to ERK2 (*E*, *middle panel*), respectively. The relative intensities of the bands corresponding to the phosphorylated forms of either p54 and p46 JNK (*C*, *bottom panel*), p38 (*D*, *bottom panel*), or ERK2 (*E*, *bottom panel*) were normalized by those of the bands for p46 JNK1, p38, and ERK2, respectively, and were then plotted against time; data are means of values from two independent experiments.

associated with overexpression of Prx II(WT), whereas the activation of p38 and that of JNK are enhanced by similar extents in response to the increase in the H₂O₂ concentration associated with Prx II(DN) expression. Because of the irreproducibility of p38 immunoprecipitation achieved with available antibodies, we were not able to evaluate p38 activation by *in vitro* kinase assay.

The effect of Prx II on TNF-α-induced ERK activation was examined first with CT2, WT4, and DN10 cells. ERK2 was immunoprecipitated from TNF-α-treated cells and assayed for kinase activity with MBP as substrate (Fig. 4*A*). The kinase activity derived from WT4 cells was ~30% higher, and that from DN10 cells was ~50% lower, than that derived from CT2 cells. When similar kinase assays were carried out with CT4, WT12, and DN15, TNF-alpha-induced ERK activation was ~40% higher in WT12 cells but was ~40% lower in DN15 cells compared with that apparent in CT4 cells (data not shown). The ERK activation profile was also assessed in these same cell lines by immunoblot analysis with antibodies to ERK phosphorylated on Thr²⁰² and Tyr²⁰⁴ (Fig. 4*B*). The extent of TNF-α-induced phosphorylation of ERK2 in WT4 cells was increased by ~30%, compared with that apparent in CT2 cells, whereas that in DN10 cells was reduced to virtually undetectable levels. The responses of ERK to changes in the intracellular concen-

tration of H₂O₂ were thus opposite compared with those of JNK or p38.

We also evaluated TNF-α-induced ERK activation in HeLa cells transiently transfected with a vector for Prx II(WT) or Prx II(DN) together with a vector for HA-ERK2. *In vitro* kinase assays performed with HA-ERK2 immunoprecipitated with antibodies to HA revealed that ERK2 activity was not substantially affected by Prx II(WT) but was inhibited in a dose-dependent manner (maximal inhibition, 50%) by Prx II(DN) (Fig. 4*C*). These results thus contrast with those obtained for the effects of the recombinant Prx II proteins on TNF-α-induced JNK3 activation (Fig. 2*E*).

Effects of Prx II Deficiency on TNF-α-induced MAPK Activation—To substantiate further the results obtained with HeLa cells expressing recombinant Prx II proteins, we isolated MEFs from Prx II knockout mice (35). Specific loss of Prx II expression in these cells was verified by immunoblot analysis (Fig. 5*A*). Similar analysis with isoform-specific antibodies revealed that the absence of Prx II did not affect the expression of Prx I or III (Fig. 5*A*). The abundance of Trx and Trx reductase, which are required to support the peroxidase activity of Prx enzymes, was also unchanged in the Prx II⁻/⁻ MEFs (data not shown). In response to stimulation with TNF-α, the intracellular concentration of H₂O₂ increased transiently, and the increase was
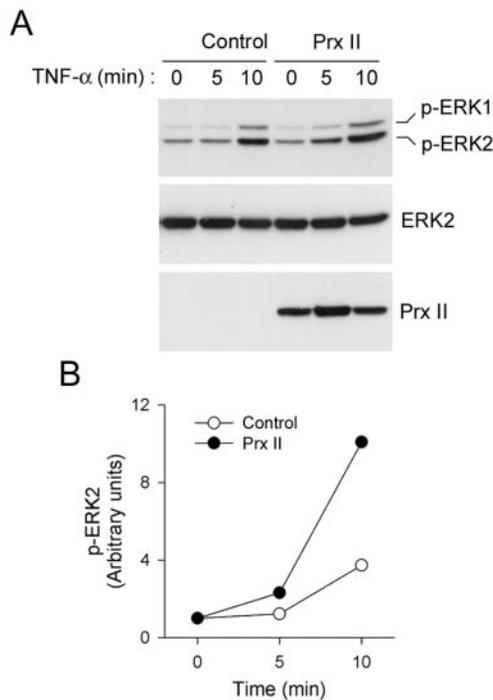
A

B

FIG. 6. **Restoration of ERK activation by retrovirus-mediated expression of Prx II in Prx II$^{-/-}$ MEFs.** Prx II$^{-/-}$ MEFs were infected twice at 12-h intervals at 5 multiplicity of infection with a Prx II-encoding retrovirus or the corresponding empty vector (control). They were then incubated with DMEM containing 0.5% fetal bovine serum for 24 h before stimulation with TNF-α (15 ng/ml) for the indicated times. Cell lysates were subjected to immunoblot analysis with antibodies to phospho-ERK; the same membrane was reprobed with antibodies to ERK2. Cell lysates were also subjected to immunoblot analysis with antibodies to Prx II. *A*, the relative intensities of the phospho-ERK2 bands were normalized by those of the ERK2 bands and plotted against time (*B*); data are means of two independent experiments.

more pronounced in the knockout cells than in wild-type cells (Fig. 5*B*). The knockout cells were then stimulated with TNF-α, and the extents of activation of JNK, p38, and ERK were determined by immunoblot analysis with antibodies specific for the phosphorylated forms of these proteins. TNF-α-induced JNK activation, as estimated from the phosphorylation of p46 and p54 JNK, was increased ~2.5- to 3-fold in the Prx II$^{-/-}$ MEFs compared with that apparent in wild-type cells (Fig. 5*C*). The activation of p38 was enhanced only slightly in the Prx II$^{-/-}$ MEFs relative to that apparent in the wild-type cells (Fig. 5*D*). In contrast, loss of Prx II expression resulted in a marked (~80%) inhibition of the TNF-α-induced activation of ERK2 (Fig. 5*E*). Expression of Prx II in the Prx II$^{-/-}$ MEFs with the use of a recombinant retrovirus restored the sensitivity of ERK2 activation to TNF-α stimulation (Fig. 6).

Effects of Prx II(WT) and Prx II(DN) on TNF-α-induced ASK1 Activation—H$_2$O$_2$ is known to activate ASK1, an MAPK kinase kinase that phosphorylates and activates MAPK kinases that in turn activate JNK and p38 by phosphorylating their TXY motifs (14, 15). To examine whether the activity of ASK1 is affected by the stable expression of Prx II(WT) and Prx II(DN), ASK1 was immunoprecipitated from CT2, WT4, and DN10 cells that had been treated with TNF-α or left untreated and assayed for kinase activity using a GST-SEK1 fusion protein as substrate. SEK1 is a MAPK kinase that is phosphorylated by ASK1 (Fig. 7). TNF-α activated ASK1 activity by 7-fold in the empty vector-transfected CT2 cells. The TNF-α-induced activation of ASK1 was inhibited by ~40% in the Prx II(WT)-expressing WT4 cells, but was increased by ~70% in the Prx II(DN)-expressing DN10 cells.
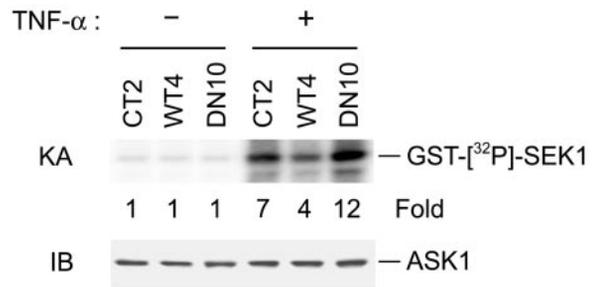

FIG. 7. **Effects of expression of Prx II(WT) or Prx II(DN) on TNF-α-induced ASK1 activation.** The stably transfected CT2, WT4, and DN10 cells were incubated in the absence ($-$) or presence ($+$) of TNF-α (15 ng/ml) for 15 min, after which ASK1 was immunoprecipitated from cell lysates with antibodies to ASK1. The resulting precipitates were either assayed for kinase activity (*KA*) with GST-SEK1 as substrate (*upper panels*) or subjected to immunoblot analysis (*IB*) with the same antibodies to ASK1 (*lower panels*). The *numbers between the panels* represent the amount of ^{32}P radioactivity incorporated into GST-SEK1 after normalization by the corresponding ASK1 immunoblot intensity and expression as -fold increase relative to the corresponding value for the cells left unstimulated; they are means of values from two independent experiments.

DISCUSSION

TNF-α is an extracellular stimulus that induces the production in cells of O$_2^-$, H$_2$O$_2$, and NO, all of which in turn are thought to participate in intracellular signaling pathways activated by this cytokine. MAPK signaling cascades are regulated both directly and indirectly by ROS, and the aim of the present study was to determine the role of H$_2$O$_2$ in the TNF-α-induced activation of individual MAPKs as well as to evaluate that of a cytosolic Prx in TNF-α signaling pathways.

Given that, among the various oxidants produced in cells, catalase removes H$_2$O$_2$ selectively, overexpression of this enzyme has been used to study the role of H$_2$O$_2$ in signaling pathways triggered by several stimulants (19, 24–27). However, catalase is localized exclusively in peroxisomes, and its effects on signaling events in the cytosol rely on the diffusion of H$_2$O$_2$ into these organelles. Furthermore, despite its high turnover number, catalase is not efficient in eliminating low levels of H$_2$O$_2$, because it is difficult to saturate with H$_2$O$_2$ and its catalytic cycle requires the interaction of two H$_2$O$_2$ molecules with a single active site, which is less likely at low H$_2$O$_2$ concentrations (37). Unlike catalase, Prx I and Prx II are cytosolic enzymes and their K_m values are in the micromolar range (30, 31). In addition, the activity of Prx I and Prx II, like that of many enzymes responsible for the elimination of intracellular messengers, appears to be modulated by phosphorylation (38). The active site Cys-SH group of Prx I and II also undergoes reversible hyperoxidation to sulfinic acid by H$_2$O$_2$ (39–41). Manipulation of the intracellular H$_2$O$_2$ concentration by altering the level of expression of cytosolic Prx would thus appear to be a more physiological approach than altering that of catalase.

We selected Prx II rather than Prx I as the target in our experiments because Prx I interacts with various proteins, including c-Abl (although the significance this association is not known) (42–44); effects of alteration of Prx I expression might thus result from actions other than that on H$_2$O$_2$ concentration. Although Prx II has not been shown to interact with other cellular proteins, we chose to examine the effects of a dominant negative mutant of Prx II in addition to those of Prx II depletion by gene targeting; expression of such a mutant is more likely to reveal whether the effects of overexpression of the wild-type protein are attributable to its H$_2$O$_2$-scavenging function.

The various approaches adopted in the present study (transient or stable expression of Prx II proteins; depletion of endogenous Prx II) all indicated that H$_2$O$_2$ produced in response

to stimulation of cells with TNF-α plays opposite roles in the activation of JNK and p38 on the one hand and ERK on the other. Thus, whereas H_2O_2 potentiates the activation of JNK and p38 induced by TNF-α, it inhibits that of ERK in both HeLa cells and MEFs. Although the qualitative results were consistent among the experimental approaches adopted, the magnitude of the observed changes in kinase activity was dependent on the specific experimental conditions. For example, in the presence of TNF-α, both JNK and p38 activities were reduced in HeLa cells stably expressing Prx II(WT) and were increased in those permanently expressing Prx II(DN); the sensitivity of JNK activity to Prx II(WT) was less marked than was that of p38 activity. In contrast, the lack of Prx II in MEFs had a more pronounced effect on TNF-α-induced JNK activation than it did on that of p38. In addition, JNK appeared to be more sensitive to a decrease than to an increase in H_2O_2 concentration, whereas the opposite appeared true for ERK. For example, in HeLa cells transiently expressing JNK3, the activity of this protein was inhibited by expression of Prx II(WT) but was unaffected by that of Prx II(DN); in contrast, ERK2 activity was not affected by expression of Prx II(WT) but was substantially inhibited by that of Prx II(DN), even though only a small fraction of endogenous Prx II molecules is expected to form heterodimers with the mutant protein. The pronounced sensitivity of ERK activity to H_2O_2 production was also evident in MEFs, in which the lack of Prx II resulted in inhibition of TNF-α-induced ERK activation by ~80%.

Exogenous H_2O_2 has been shown to activate all three types of MAPK in many cell types, including HeLa cells (4, 18, 45–50). We have also confirmed the activation of all three types of MAPK in HeLa cells by exogenous H_2O_2 (data not shown). These stimulatory effects of exogenous H_2O_2 are consistent with the positive role of H_2O_2 in TNF-α-induced activation of JNK and p38 but not with the negative role of H_2O_2 in ERK activation revealed in the present study. Furthermore, the effects of inhibition of H_2O_2 accumulation by introduction of catalase on receptor-induced MAPK activation depend on both receptor and cell type; catalase inhibited the activation of all three types of MAPK in rat vascular smooth muscle cells exposed to PDGF (19), had no effect on ERK activation in PDGF-treated bovine lung tracheal smooth muscle cells (26), inhibited the activation of p38 but had no effect on that of ERK in angiotensin II-stimulated rat vascular smooth muscle cells (24), inhibited the activation of ERK but had no effect on that of p38 in alveolar macrophages treated with zymogen (25), and inhibited ERK activation by 5-hydroxytryptamine in bovine lung artery smooth muscle cells (27). These results suggest that H_2O_2 acts on multiple targets to regulate MAPK activation in a cell type- and agonist-specific manner.

The binding of TNF-α to its cognate receptor results in receptor trimerization and recruitment of an adapter protein known as TNF receptor-associated death domain protein, which serves as a platform to recruit at least three additional adapter proteins: receptor-interacting protein 1, Fas-associated death domain, and TNF receptor-associated factor 2. TNF receptor-associated factor 2 activates several MAPKKKs, including ASK1, by unknown mechanisms to initiate the activation of JNK and p38, which is mediated either by overlapping or by independent MAPKKK and MAPKK enzymes (51, 52). A number of proteins, including RIP2, sphingomyelinases, and Raf1 have been suggested as components of TNF-α signaling that are involved in the activation of ERK (53–56). However, the mechanism responsible for the TNF-α-induced activation of ERK is largely unknown.

Among the proteins known to be involved in TNF-α signaling pathways, ASK1 is the best-characterized H_2O_2 target mole-

cule. In resting cells, ASK1 exists in a complex with reduced Trx and glutaredoxin; oxidation of Trx and glutaredoxin by H_2O_2 results in their dissociation from ASK1 and the consequent activation of ASK1 and the JNK and p38 pathways (14, 15). In agreement with the previous findings, we observed that the TNF-α-induced activation of ASK1 was inhibited in cells overexpressing wild-type Prx II but was increased in cells expressing dominant negative Prx II. This result suggests that Prx II participates in the TNF-α-induced activation of JNK and p38 at least in part by modulating the upstream kinase ASK1.

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Cytosolic Peroxiredoxin Attenuates The Activation Of Jnk And P38 But Potentiates That Of Erk In Hela Cells Stimulated With Tumor Necrosis Factor- α

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