Several Dual Specificity Phosphatases Coordinate to Control the Magnitude and Duration of JNK Activation in Signaling Response to Oxidative Stress*5

Mitogen-activated protein kinases (MAPKs) are important mediators that integrate signaling from upstream pathways in response to various environmental cues. In order to control appropriate gene expression through phosphorylation of transcription factors, the activity of MAPKs must be tightly regulated by the actions coordinated between protein kinases and phosphatases. In this study, we explore the underlying mechanism through which the oxidative stress-activated c-Jun N-terminal kinases (JNKs), members of MAPKs, are regulated by dual specificity phosphatases (DUSPs). DUSPs are a group of enzymes that belong to the superfamily of protein-tyrosine phosphatases. They are able to recognize phospho-Ser/Thr and phospho-Tyr residues in substrates. Using quantitative real time PCR, we found that stimulation of human embryonic kidney 293T cells with H2O2 or xanthine/xanthine oxidase led to rapid and transient induction of DUSP1 and DUSP10 to be essential for determining the appropriate magnitude of JNK activation in response to oxidative stress. The transcription factor ATF2, which is phosphorylated and activated by JNK, is a critical mediator for inducible expression of DUSP1 and DUSP10 in this signaling pathway. We further demonstrated that DUSP4 and DUSP16, both showing significant late phase induction, dephosphorylate JNK effectively, causing the downregulation of the signaling cascade. Thus, this study provides new insights into the role of several DUSPs that coordinate with each other to control the magnitude and duration of JNK activity in response to oxidative stress.

Certain signaling events are necessary for cells to respond appropriately to environmental cues like physiological stimuli and stresses. In doing so, cells employ signaling modules that integrate input from upstream stimuli used to control appropriate cell functions, including proliferation, differentiation, migration, and death. One of the most important signaling modules is composed of a highly conserved cascade of three kinases, in which mitogen-activated protein kinases (MAPKs) are the primary component (1). The activation of MAPKs requires phosphorylation of both the Thr and Tyr residues located within the TXY signature motif by dual specificity MAPK kinases (MEKs or MKKs) (2, 3). The activation of MEKs requires Ser/Thr phosphorylation by upstream MAPK kinases (MEKs or MKKKs) (2, 3), which are composed of a large number of enzymes from diverse families.

To date, ~20 mammalian MAPKs have been identified and characterized (4). These enzymes are classified into two major categories based on their activation in response to different external stimuli. The extracellular signal-regulated kinases (ERKs), which are characterized by the TEY consensus sequence in the activation loop (4), are activated by a variety of physiological agonists that trigger cell proliferation and differentiation (4, 5). The second category of MAPKs, namely stress-activated protein kinases, includes the c-Jun amino-terminal kinases (JNKs, with the TPY consensus sequence in the activation loop) and p38 MAPKs (p38s, with the TGY consensus sequence in the activation loop) (4). The stress-activated protein kinases, and members of the JNK family in particular, are effectively activated in response to cellular stresses caused by proinflammatory cytokines, radiation, osmotic stress, and oxidative stress (6). Despite the heterogeneity of upstream signaling inputs that activate various types of ERKs and stress-activated protein kinases, enzymes in the MAPK family share some common features. These characteristics are critical for the integration of complex signaling pathways that yield appropriate cellular responses. For example, both ERKs and stress-activated protein kinases are translocated to the nucleus after MEK phosphorylation of the Thr and Tyr residues at the TXY motif (4–6). Once inside the nucleus, these activated MAPKs are capable of directly phosphorylating a number of transcription factors and cell cycle regulators (4–6). Regardless of the subfamilies in which individual enzymes are assigned, it is generally accepted that the key function of all forms of MAPKs is to precisely control the activity of transcription factors in order to regulate the expression of a large number of important genes.

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1 These authors contributed equally to this work.

2 To whom correspondence should be addressed. Tel.: 886-2-27895696; Fax: 886-2-27892161; E-mail: tcmeng@gate.sinica.edu.tw.

5 The abbreviations used are: MAPK, mitogen-activated; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; PTP, protein-tyrosine phosphatase; DUSP, dual specificity phosphatase; MKP, MAPK phosphatase; ROS, reactive oxygen species; X, hypoxanthine; XO, xanthine oxidase; siRNA, small interfering RNA; HMBS, hydroxymethylbilane synthase; RNAi, RNA interference.
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(4–6). Therefore, the duration and magnitude of MAPK activity must be tightly regulated. Research thus far has demonstrated that the activation of MAPKs induced by various stimuli is normally transient (7, 8), suggesting a mechanism for down-regulation of their enzymatic activity in vivo. It is now clear that protein phosphatases participate in the control of MAPK-mediated signaling through dephosphorylation of upstream kinases or by direct targeting on MAPKs (7–9).

Although the signaling cascades leading to the activation of MAPKs have been well defined, the underlying mechanism for down-regulation of MAPKs is not. Previous studies have demonstrated that the dephosphorylation of either phosphoamino acid residue at the conserved TXY motif results in the inactivation of MAPKs (7–9). Therefore, it has been proposed that both Ser/Thr-specific phosphatases, as well as protein-tyrosine phosphatases (PTPs), can potentially dephosphorylate MAPKs. Indeed, it has been reported that some Ser/Thr phosphatases, such as PP2A (10) and PP2Co (11), can inactivate MAPKs through dephosphorylation of the Thr residue. Similarly, tyrosine-specific phosphatases, such as HePTP (12) or PTP-SL (13), can target MAPKs on the phospho-Tyr residue. Moreover, the recent identification of a subgroup of enzymes within the PTP superfamily termed dual specificity phosphatases (DUSPs; ~60 genes in the human genome (14, 15)) has led to significant advancement in our understanding of the regulation of MAPKs. DUSPs, characterized by the presence of a PTP signature motif, HEXXXGXXX(S/T), display limited sequence homology to the classical, tyrosine-specific PTPs. Initial studies showed that DUSP1, a prototypical MAPK phosphatase (MKP), is capable of dephosphorylating both the Thr and Tyr residues on the TXY motif in ERKs (16). Since then, several studies have documented a number of DUSPs that recognize various forms of MAPKs as substrates. To date, 10 typical MKPs (DUSP1/MKP-1/CL100, DUSP2/PAC-1, DUSP4/MKP-2, DUSP5/hVH3, DUSP6/MKP-3/PYST1, DUSP7/MKP-X/PYST2, DUSP8/hVH5, DUSP9/MKP-4/PYST3, DUSP10/MKP-5, and DUSP16/MKP-7) are characterized by the presence of a Cdc25 homology region 2 domain within the N-terminal region and a catalytic domain within the C-terminal region. Two additional DUSPs (DUSP3/VHR and DUSP14/MKP-6) contain only a catalytic domain. These 12 MKPs regulate the activity of MAPKs through the dephosphorylation of Thr and Tyr residues (8). Although biochemical data have revealed the distinct substrate specificities of DUSPs toward various types of MAPKs, the exact function of these enzymes in the regulation of signal transduction in vivo needs elucidation.

Human DUSP1 has been identified as an immediate early gene whose expression is rapidly induced by oxidative stress, heat shock, and growth factors (17, 18). More recent research has demonstrated that this characteristic is also shared by other DUSPs, in particular those enzymes functioning as MKPs (19–24). Based on such observations, it has been proposed that these inductively expressed DUSPs might be key players in the feedback loop mechanism, since they may terminate the signal transduction mediated by MAPKs. In the current study, we examined the signaling pathways activated by reactive oxygen species (ROS). We present evidence that the treatment of human embryonic kidney 293T cells with H₂O₂ can result in the transient activation of type 1 and type 2 JNKs, concomitant with the inducible expression of DUSPs. Our study demonstrates that several DUSPs play distinct roles in orchestrating a regulatory network that controls the precise magnitude and duration of JNK activation in response to oxidative stress.

EXPERIMENTAL PROCEDURES

Cell Culture and Transient Transfection with Small Interfering RNA—Human embryonic kidney 293T cells were routinely maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% glutamine. For direct exposure to H₂O₂ or stimulation with ROS generated by a mixture of hypoxanthine and xanthine oxidase (X/XO; both from Sigma), cells were plated in medium containing 10% fetal bovine serum for 24 h and then serum-starved for 16 h before treatment. For transient transfection with small interfering RNA (siRNA) oligonucleotides, cells were plated in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum for 16 h. The siRNA duplexes of DUSPs or the transcription factors p53, c-Jun, and ATF2 (SMARTpool siRNA oligonucleotides; Dharmacon) were introduced using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Cells were continuously incubated in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum for 8 h, followed by serum deprivation for an additional 16 h prior to stimulation with H₂O₂ or X/XO.

Immunoblotting—Antibodies used for immunoblotting were purchased from Cell Signaling: anti-phospho-JNK (Thr(P)183/Tyr(P)185), anti-JNK, anti-phospho-p38 (Thr(P)180/Tyr(P)182), anti-p38, anti-phospho-ERK (Thr(P)202/Tyr(P)204), anti-ERK, anti-phospho-p53 (Ser(P)39), anti-p53, anti-phospho-c-Jun (Ser(P)63), anti-c-Jun, anti-phospho-ATF2 (Thr(P)69), and anti-ATF2. Cells were lysed in lysis buffer containing 20 mM HEPES (pH 7.5), 1% Nonidet P-40, 150 mM NaCl, 1% glycerol, 200 μM Na₃VO₄, and protease inhibitors (10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 0.1 mM phenylmethylsulfonyl fluoride). Aliquots of total lysates (20–30 μg of protein/sample, as indicated in the figures) were subjected to SDS-PAGE and transferred to nitrocellulose membranes, which were then incubated with appropriate primary and secondary antibodies. The specific signals were visualized by an ECL detection system (Amersham Biosciences).

 Extraction of RNA and cDNA Synthesis—Total RNA was isolated from 293T cells using a Trizol reagent kit (Invitrogen) according to the manufacturer’s instructions. The cDNA was synthesized from total RNA with Transcriptor reverse transcriptase (Roche Applied Science) using oligo(dt) primers according to the manufacturer’s instructions.

 Quantitative Real Time PCR—The mRNA expression levels were quantified by real time PCR using a LightCycler instrument (Roche Applied Science) with the SYBR Green PCR Master Mix (Qiagen) in a one-step reaction according to the manufacturer’s instructions. Primers were designed using the LightCycler Probe Design software 1.0 (Idaho Technology). The sequences for 12 DUSP forward and reverse primers are shown in Table 1. The primer sequences for the transcription factors p53, c-Jun, and ATF2 as well as a housekeeping gene, hydroxymethylbilane synthase (HMBS), were as follows: p53 forward, 5’-ATGTGTAAAACGGTCTTGC-3’; p53 reverse, 5’-GCTCGCTTATGTGCTCC-3’;
c-Jun forward, 5′-CGACCTTCTATGACGATGC-3′; c-Jun reverse, 5′-GTGGTGGTGATGTGCC-3′; ATF2 forward, 5′-TACAAGTGGTCGTCGG-3′; ATF2 reverse, 5′-CGGTTACAGGGCAATC-3′; HMBS forward, 5′-AGTATTCGGGGAACT-3′; HMBS reverse, 5′-AAGCAGAGTCTCGGGA-3′. The melting curves and gel electrophoresis of the end products were obtained to confirm the PCR specificity and the correct size of the PCR band. The mRNA levels of target genes were normalized to the relative amounts of the housekeeping gene HMBS.

RESULTS

De Novo mRNA and Protein Synthesis Are Involved in Down-regulating Oxidative Stress-induced JNK Signaling in 293T Cells—In this study, we wanted to identify endogenous DUSPs involved in the regulation of MAPK signaling in response to oxidative stress. To induce such a response, human embryonic kidney 293T cells were exposed to 200 μM H₂O₂. This concentration of H₂O₂ was selected, because physiological stimuli, such as platelet-derived growth factor, can induce a transient increase in the intracellular level of H₂O₂ that is equivalent to that achieved by stimulating cells with H₂O₂ at an extracellular concentration ranging between 0.1 and 1.0 mM (25). Cells were harvested at various time points, and the phosphorylation status of MAPKs was analyzed by immunostaining using specific antibodies that recognize the bisphosphorylated forms of JNK, p38, and ERK. As shown in Fig. 1A, the phosphorylation level of type 1 and type 2 JNKs was significantly increased within 60 min after H₂O₂ treatment, peaking (~5.5-fold, compared with the no treatment sample) at 2 h. Interestingly, the activated form of JNK was rapidly dephosphorylated, as demonstrated by
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TABLE 1

| Name   | Synonyms                        | Accession number | Substrate specificity (Ref. 8–10) | Primer used for real time PCR analysis |
|--------|---------------------------------|------------------|-----------------------------------|----------------------------------------|
| DUSP1  | MKP-1, 3CH134, PTPN10, erp, HVH1, CL100 | NM_004417 | JNK ~ p38 ~ ERK                  | Forward: 5′-TTGAGGAGGCCTACCTCAGGAC-3′ |
|        |                                 |                  |                                   | Reverse: 5′-GACTGTCGCTCCCTGGGCCC-3′    |
| DUSP2  | PACI                            | NM_004418 | ERK and p38                      | Forward: 5′-AGGAGTCCTGCTGAGAAC-3′      |
|        |                                 |                  |                                   | Reverse: 5′-AGTCAGTCTGCTGAGACC-3′      |
| DUSP3  | VHR, -DSP11                     | NM_004990 | ERK and JNK                      | Forward: 5′-AGGAGTCGAGCCAAGCGG-3′      |
|        |                                 |                  |                                   | Reverse: 5′-ATGATGAGAGGGCAAACT-3′      |
| DUSP4  | MKP-2, hVH2, TYP1               | NM_001394 | JNK > p38                        | Forward: 5′-GAGAGTCGAGCCAAGCGG-3′      |
|        |                                 |                  |                                   | Reverse: 5′-ATGATGAGAGGGCAAACT-3′      |
| DUSP5  | hVH3, B23                       | NM_004419 | ERK                              | Forward: 5′-GAGAGTCGAGCCAAGCGG-3′      |
|        |                                 |                  |                                   | Reverse: 5′-ATGATGAGAGGGCAAACT-3′      |
| DUSP6  | MKP-3, PYST1, RVH6              | NM_001946 | ERK                              | Forward: 5′-GAGAGTCGAGCCAAGCGG-3′      |
|        |                                 |                  |                                   | Reverse: 5′-ATGATGAGAGGGCAAACT-3′      |
| DUSP7  | PYST2, B59, MKP-X               | NM_001947 | ERK                              | Forward: 5′-GAGAGTCGAGCCAAGCGG-3′      |
|        |                                 |                  |                                   | Reverse: 5′-ATGATGAGAGGGCAAACT-3′      |
| DUSP8  | hVH5, M3/6, HB5                 | NM_004420 | JNK ~ p38 >= ERK                 | Forward: 5′-GAGAGTCGAGCCAAGCGG-3′      |
|        |                                 |                  |                                   | Reverse: 5′-ATGATGAGAGGGCAAACT-3′      |
| DUSP9  | MKP-4, Pyst3                    | NM_001395 | ERK > JNK or p38                 | Forward: 5′-GAGAGTCGAGCCAAGCGG-3′      |
|        |                                 |                  |                                   | Reverse: 5′-ATGATGAGAGGGCAAACT-3′      |
| DUSP10 | MKP-5                           | NM_007207 | JNK ~ p38                        | Forward: 5′-GAGAGTCGAGCCAAGCGG-3′      |
|        |                                 |                  |                                   | Reverse: 5′-ATGATGAGAGGGCAAACT-3′      |
| DUSP14 | MKP-6, MKP-L                    | NM_007226 | ERK > JNK or p38                 | Forward: 5′-GAGAGTCGAGCCAAGCGG-3′      |
|        |                                 |                  |                                   | Reverse: 5′-ATGATGAGAGGGCAAACT-3′      |
| DUSP16 | MKP-7, MKP-M, VHY               | NM_030640 | JNK ~ p38 >= ERK                 | Forward: 5′-GAGAGTCGAGCCAAGCGG-3′      |
|        |                                 |                  |                                   | Reverse: 5′-ATGATGAGAGGGCAAACT-3′      |

The fact that phosphorylation of JNK had already returned to the basal level at 6 h after stimulation (Fig. 1A). The p38 and ERK proteins were also phosphorylated and activated in response to oxidative stress but to a substantially lesser than was JNK (Fig. 1B). Immunoblotting analysis further indicated that the protein expression levels for these three MAPKs remained constant after oxidative treatment (Fig. 1, A and B), suggesting that potentially post-translational modifications for the regulation of activation and inactivation of MAPKs may be involved in this signaling pathway. Based on these observations, we focused on the underlying mechanism responsible for the rapid dephosphorylation of JNK in response to H₂O₂ stimulus.

We hypothesized that JNK dephosphorylation might be mediated by the function of DUSPs. Published findings have suggested that DUSPs may be inductively expressed through transcriptional and translational processes in response to oxidative stress (19–24). Thus, we examined JNK dephosphorylation after suppression of de novo mRNA synthesis by actinomycin D or after suppression of protein synthesis by cycloheximide. As previously shown, JNK was rapidly phosphorylated and then completely dephosphorylated following treatment of cells with 200 μM H₂O₂ (Fig. 1, C and D). However, pretreatment of cells with either actinomycin D or cycloheximide prior to H₂O₂ stimulation led to considerably sustained levels of JNK phosphorylation at 6 h post-treatment (Fig. 1, C and D). These results suggest that synthesis of de novo mRNA and protein (more specifically, DUSP-inducible expression) may be involved in the negative regulation of JNK activation in response to oxidative stress.

mRNA Levels of DUSPs Are Rapidly Increased following H₂O₂ Stimulation—We tested the hypothesis that the effective dephosphorylation of JNK was due to the rapid expression of DUSPs in response to H₂O₂ stimulation. To do this, we selected 12 DUSP genes for detailed examination (listed in Table 1). They were the well characterized MKPs 1–7 and five other DUSPs that have been proposed to regulate the activity of MAPKs (8). We used quantitative real time PCR and the SYBR Green I labeling method to compare the levels of mRNA for these 12 DUSPs at various time points following H₂O₂ treatment. The specific DUSP primers used for real time PCR analysis are shown in Table 1.

These 12 DUSPs were assigned to three categories based upon the magnitude and pattern of inducible mRNA expression. Group I phosphatases, including DUSP1, DUSP8, and DUSP10, were rapidly induced in a transient manner once the cells were treated with H₂O₂ (Fig. 2A). The expression levels for DUSPs in this group peaked at 2–3 h and then rapidly decreased between 4 and 8 h (Fig. 2A). Group II phosphatases were those whose mRNA levels gradually accumulated and remained at high levels for 8 h post-treatment, DUSP4 and DUSP16 (Fig. 2B). The aforementioned DUSPs in Groups I and II showed at least a 3.5-fold induction after treatment and were therefore classified as genes that were significantly up-regulated in response to oxidative stress. In contrast, the mRNA levels for the seven remaining DUSPs, including DUSP2, DUSP3, DUSP5, DUSP6, DUSP7, DUSP9, and DUSP14, did not change or were only marginally increased (<2.5-fold) after oxidative treatment. These DUSPs were assigned to Group III, phosphatases without significant induction (Fig. 2C). Interestingly, the rapid expression of DUSPs from Groups I and II occurred over the same time period that JNK was inactivated in Group III (Fig. 1A). Thus, our results suggest that five DUSPs, namely DUSP1, DUSP4, DUSP8, DUSP10, and DUSP16, are potential negative regulators of JNK in the oxidative stress-activated signaling pathways.

Several DUSPs Participate in Regulating JNK Response to H₂O₂ Stimulation—In the next phase of the study, we investigated whether DUSPs, which have undergone a significant increase in mRNA expression following oxidative stress, participate in the regulation of JNK phosphorylation. To do this, we used the RNAi technique to suppress the inducible expression of individual DUSPs. siRNA transfectants were exposed to...
H2O2, and the levels of JNK phosphorylation were monitored over time (Fig. 3). Compared with the control cells transfected with scrambled siRNA, the mRNA levels of DUSPs in cells transfected with phosphatase-specific siRNA were substantially decreased (Fig. 3, A–E, left). The suppression by RNAi of either DUSP1 or DUSP10 led to enhanced phosphorylation of JNK at 2 h after H2O2 stimulation, followed by rapid dephosphorylation of JNK at 4–6 h (Fig. 3, A and C, right). These results suggest that the inducible expression of either DUSP1 or DUSP10 is more important to controlling the appropriate level of JNK phosphorylation at an initial stage than it is for terminating JNK signaling. We also observed that the RNAi-mediated ablation of DUSP8, the other transiently expressed DUSP, did not affect the extent and duration of JNK activation (Fig. 3B, right). To explore the effects of additional phosphatases that may recognize and dephosphorylate JNK at a later stage after H2O2 stimulation, we examined the potential function of DUSP4 and DUSP16, since both had been observed to have sustained expression of oxidative stress-inducible mRNA (Fig. 2). We found that although the level of JNK phosphorylation in siRNA transfectants was not enhanced at 2 h, it was significantly prolonged when the expression of endogenous DUSP4 (Fig. 3D) or DUSP16 (Fig. 3E) was ablated.

The regulatory role for DUSPs involved in JNK dephosphorylation was further tested using RNAi. In that experiment, the expressions of the two DUSPs were simultaneously ablated. As shown in Fig. 4A, transfection with a mixture of siRNA oligonucleotides, which caused knockdown of the expression of both DUSP1 and DUSP10, led to a robust activation of JNK at 1–2 h following H2O2 stimulation. This finding suggests that the inducible expression of functional DUSP1 and DUSP10 needed to down-regulate JNK may not take place immediately after ROS stimulation. A short exposure of H2O2 (30 min) in siRNA transfectants, in which both phosphatases were ablated, resulted in a similar degree of JNK phosphorylation compared with the control cells (Fig. S1). Interestingly, the effective dephosphorylation of JNK still occurred at a late stage (3–6 h) under these conditions (Figs. 4A and S1). On the other hand, the ablation of DUSP4 and DUSP16 had no effect on the peak activation of JNK at 2 h but significantly prevented rapid dephosphorylation of JNK that otherwise occurred between 2 and 4 h after H2O2 stimulation in control cells (Fig. 4B). Moreover, when cells were transfected with the siRNA oligonucleotides that suppressed the expression of both DUSP1 and DUSP4, the intensity and duration of JNK activation increased markedly (Fig. 4C). Thus, our results demonstrated that a coordinated action involving multiple DUSPs is required to prevent the initial hyperphosphorylation of JNK and to control the termination of JNK activation in signaling response to oxidative stress.

JNK-mediated Transcriptional Regulation Is Involved in the Inducible Expression of DUSP1 and DUSP10 and Their Down-regulation of JNK Activation in Signaling Response to Oxidative Stress—Our data had thus far demonstrated that four DUSPs function as JNK phosphatases in oxidative stress-mediated sig-

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JNK-mediated Transcriptional Regulation Is Involved in the Inducible Expression of DUSP1 and DUSP10 and Their Down-regulation of JNK Activation in Signaling Response to Oxidative Stress—Our data had thus far demonstrated that four DUSPs function as JNK phosphatases in oxidative stress-mediated sig-

FIGURE 2. Treatment with H2O2 rapidly induced mRNA expression of five DUSPs in 293T cells. Human embryonic kidney 293T cells were serum-starved for 16 h and then stimulated with 200 μM H2O2 for 0, 1, 2, 3, 4, and 8 h. Cells were harvested for total RNA preparation. The mRNA level of DUSPs at each time point was quantitated by real time PCR analysis and normalized to the level of HMBS. Based upon the degree and pattern of mRNA induction, the 12 DUSPs were divided into three groups. A, phosphatases in Group I, including DUSP1, DUSP8, and DUSP10, were transiently induced by H2O2, with the maximal level of mRNA ≥3.5-fold at 2–3 h compared with the no treatment control. B, phosphatases in Group II, including DUSP4 and DUSP16, showed continual high levels of mRNA expression (≥3.5-fold) that lasted until 8 h after stimulation. C, phosphatases in Group III, including DUSP2, DUSP3, DUSP5, DUSP6, DUSP7, DUSP9, and DUSP14, were not significantly induced (mRNA levels ≤2.5-fold) by H2O2. A–C, data are presented as -fold changes over the nontreated controls and expressed as means ± S.E. (n = 3). *, p < 0.05 when compared with controls.
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Ablation of DUSPs by RNAi enhanced JNK activation in response to H₂O₂ in 293T cells. Human embryonic kidney 293T cells were transfected with 100 nm nontargeting scrambled siRNA oligonucleotide (control) or 100 nm siRNA oligonucleotide specific to DUSP1 (A), DUSP8 (B), DUSP10 (C), DUSP4 (D), or DUSP16 (E). Eight hours post-transfection, cells were deprived of serum for 16 h and then stimulated with 200 μM H₂O₂. The mRNA level of each DUSP was determined by quantitative real time PCR (left) and normalized to the level of HMBS. Total lysates (20 μg) harvested from siRNA transfectants stimulated with H₂O₂ for the indicated times were immunoblotted with antibodies to phospho-JNK and JNK, as indicated (right). Results obtained from real time PCR analyses are presented as fold changes over the nontreated controls and expressed as means ± S.E. (n = 3). Results obtained from immunoblotting shown are representative of two independent experiments.

FIGURE 3. Ablation of DUSPs by RNAi enhanced JNK activation in response to H₂O₂ in 293T cells. Human embryonic kidney 293T cells were transfected with 100 nm nontargeting scrambled siRNA oligonucleotide (control) or 100 nm siRNA oligonucleotide specific to DUSP1 (A), DUSP8 (B), DUSP10 (C), DUSP4 (D), or DUSP16 (E). Eight hours post-transfection, cells were deprived of serum for 16 h and then stimulated with 200 μM H₂O₂. The mRNA level of each DUSP was determined by quantitative real time PCR (left) and normalized to the level of HMBS. Total lysates (20 μg) harvested from siRNA transfectants stimulated with H₂O₂ for the indicated times were immunoblotted with antibodies to phospho-JNK and JNK, as indicated (right). Results obtained from real time PCR analyses are presented as fold changes over the nontreated controls and expressed as means ± S.E. (n = 3). Results obtained from immunoblotting shown are representative of two independent experiments.

We further investigated whether a feedback regulatory loop was involved in the attenuation of JNK activation through the inducible expression of these DUSPs. The Thr/Tyr-phosphorylated form of JNK is translocated to the nucleus, where it phosphorylates and thus activates various transcription factors (4–6). Therefore, we proposed that genes encoding JNK phosphatases might be transcriptionally activated in a JNK-dependent manner. To test this hypothesis, 293T cells were pretreated with SP600125, a specific JNK inhibitor (26), prior to treatment with H₂O₂. The mRNA levels of DUSP1, DUSP4, DUSP10, and DUSP16 were examined by quantitative real time PCR under oxidative stress in the absence or presence of the JNK inhibitor SP600125. As seen in Fig. 5A, we observed that the H₂O₂-induced expression of DUSP1, DUSP10, and DUSP16 was significantly suppressed by SP600125, whereas the expression of DUSP4 was not.

We further investigated the potential role of transcription factors that may be directly phosphorylated by the active form of JNK in the transcriptional activation of DUSP genes. Of the various transcription factors activated by extracellular stresses, p53 (27–30), c-Jun (31), and ATF2 (32–34) may be direct substrates of JNK. We investigated whether these transcription factors were involved in facilitating the inducible expression of JNK phosphatases in the feedback regulation of JNK activation in its response to oxidative stress. The 293T cells were treated with H₂O₂ and then harvested at various time points to observe Ser/Thr phosphorylation of p53, c-Jun, and ATF2 by immunoblotting. We selected specific antibodies that recognize the potentially JNK-phosphorylated residues of these transcription factors. As shown in Fig. 5B, the phosphorylation levels of Ser20 of p53, Ser63 of c-Jun, and Thr69/71 of ATF2 were rapidly increased in response to H₂O₂ stimulation. To further examine whether these signaling events were induced by H₂O₂-activated JNK, we added the inhibitor SP600125 to suppress JNK activation prior to treatment with H₂O₂. The phosphorylation of c-Jun and ATF2 was significantly suppressed in the presence of SP600125 (Fig. 5C). In contrast, the phosphorylation levels of both Ser20 (Fig. 5C) and Ser15 (data not shown) of p53, two potential phosphorylation sites of JNKs (27, 28), were not.

FIGURE 3. Ablation of DUSPs by RNAi enhanced JNK activation in response to H₂O₂ in 293T cells. Human embryonic kidney 293T cells were transfected with 100 nm nontargeting scrambled siRNA oligonucleotide (control) or 100 nm siRNA oligonucleotide specific to DUSP1 (A), DUSP8 (B), DUSP10 (C), DUSP4 (D), or DUSP16 (E). Eight hours post-transfection, cells were deprived of serum for 16 h and then stimulated with 200 μM H₂O₂. The mRNA level of each DUSP was determined by quantitative real time PCR (left) and normalized to the level of HMBS. Total lysates (20 μg) harvested from siRNA transfectants stimulated with H₂O₂ for the indicated times were immunoblotted with antibodies to phospho-JNK and JNK, as indicated (right). Results obtained from real time PCR analyses are presented as fold changes over the nontreated controls and expressed as means ± S.E. (n = 3). Results obtained from immunoblotting shown are representative of two independent experiments.
To validate the results obtained from employment of the pharmacological inhibitor, we used RNAi to further investigate the role of JNK in the inducible expression of DUSPs by an RNAi technique. Before being stimulated with $H_2O_2$, the 293T cells were transfected with a mixture of siRNA oligonucleotides (Fig. 5A), including those targeting c-Jun, p53, and ATF2. As expected, the ablation of p53, whose phosphorylation level was being used (Fig. 5C), knockdown of both JNK1 and JNK2 isoforms (Fig. 5B). As found by quantitative real-time PCR (Fig. 6A), $H_2O_2$-induced expression of DUSP1 and DUSP10 was markedly suppressed. Interestingly, although the application of JNK inhibitors ablated the inducible expression of DUSP16 (Fig. 5A), knockdown of JNKs did not lead to suppression of this phosphatase (Fig. 6A), suggesting that an unidentified side effect of SP600125 may account for its influence of gene transcription in a JNK-independent manner. Taken together, the data show that the inducible expression of JNK phosphatases DUSP1 and DUSP10 is controlled by JNK-mediated signaling pathways.

Under the condition in which both JNK1 and JNK2 were ablated by RNAi, we examined the phosphorylation level of p53, c-Jun, and ATF2 in response to $H_2O_2$ stimulation. As expected, $H_2O_2$-induced phosphorylation of c-Jun and ATF2 was significantly suppressed in siRNA transfectants (Fig. 6B).

Consistent with the previous finding observed when JNK inhibitor was being used (Fig. 5C), knockdown of JNKs by RNAi had no effect on the robust phosphorylation of p53 stimulated by $H_2O_2$ (Fig. 6B). Thus, we hypothesized that JNK-mediated phosphorylation of c-Jun and ATF2 might be involved in the inducible expression of DUSPs, which recognize and dephosphorylate the active form of JNK in the signaling response to oxidative stress.

**ATF2 Plays a Key Role in the Regulation of JNK through Transcriptionally Activating DUSP1 and DUSP10 in Response to $H_2O_2$ Stimulation**—In order to explore the detailed mechanism that controls the expression of JNK phosphatases, RNAi for the transcription factors c-Jun, p53, and ATF2 was employed. As expected, the ablation of p53, whose phosphorylation level is JNK-independent (Figs. 5C and 6B), did not lead to enhanced or sustained phosphorylation of JNK following $H_2O_2$ stimulation (Fig. 7A). Surprisingly, the $H_2O_2$-induced peak JNK phosphorylation level was not increased in cells transfected with c-Jun-targeted siRNA oligonucleotides when compared with that observed in the control cells (Fig. 7B). Although the JNK activation seemed to be slightly prolonged in siRNA transfectants (Fig. 7B), the effect of c-Jun knockdown appeared to be marginal, suggesting that c-Jun may not play a key role in transcriptional activation of genes encoding JNK phosphatases. We further examined the possible involvement of ATF2 in this signaling context. We noticed that the attenuated expression of ATF2 by RNAi led to a significant increase of JNK phosphorylation at 2 h after $H_2O_2$ stimulation (Fig. 7C). Furthermore, transfection of 293T cells with a mixture of siRNA oligonucleotides targeting both c-Jun and ATF2 resulted in increased activation of JNK (Fig. 7D). The JNK activation was remarkably similar to that observed with the ablation of ATF2 alone (Fig. 7C). To further analyze the role of ATF2, 293T cells were transfected with siRNA oligonucleotides targeting ATF2. Following a 4-h treatment of transfectants with $H_2O_2$, the mRNA levels of DUSP1, DUSP4, DUSP10, and DUSP16 were quantitated by real-time PCR analysis. Although the expression of DUSP1 and DUSP10 was significantly inhibited upon ablation of endogenous ATF2 (Fig. 8), the induction of DUSP4 and DUSP16 clearly occurred independent of ATF2 (Fig. 8). Based on these observations, ATF2 is an essential mediator of immediate expression of DUSP1 and DUSP10 induced by $H_2O_2$, a finding
Several DUSPs Control ROS-induced JNK Activation

**A.**

**FIGURE 5.** Inhibition of JNK activity led to suppressed expression of JNK phosphatases and diminished phosphorylation of c-Jun and ATF2 in response to H2O2 stimulation. A, serum-deprived human embryonic kidney 293T cells were either left untreated (Control; white bars), or pretreated with 10 μM JNK inhibitor (SP600125; black bars) for 1 h and then stimulated with 200 μM H2O2 for an additional 4 h. The relative mRNA levels of DUSP1, DUSP4, DUSP10, and DUSP16 were determined by real time PCR analysis and normalized to the level of HMBS. Data are presented as -fold changes over the nontreated controls. B, serum-deprived 293T cells were exposed to 200 μM H2O2 for the indicated times. Total lysates (20 μg) were subjected to immunoblotting with antibodies to phospho-p53 (Ser(P)15), p53, phospho-c-Jun (Ser(P)63), c-Jun, phospho-ATF2 (Thr(P)69/71), and ATF2. C, serum-deprived 293T cells were either left untreated (Control) or pretreated with 10 μM JNK inhibitor (SP600125) for 1 h and then stimulated with 200 μM H2O2 for the indicated times. Total lysates (20 μg) were subjected to immunoblotting with antibodies as indicated.

that is consistent with the known function of these phosphatases in controlling JNK activation at an early stage in the signaling response to oxidative stress.

**Inducible Expression of DUSP1 and DUSP10 Controls the Level of X/XO-mediated JNK Activation—**We used H2O2 to explore the underlying mechanism behind the control of initial JNK activation through a feedback loop involving inducible expression of DUSP1 and DUSP10. We further examined the regulatory mechanism in response to different modes of oxidative stress. Recent studies have shown that exposure of various cells to xanthine oxidase (XO), which generates superoxide anions through converting xanthine or hypoxanthine (X) to uric acid, leads to a steady increase of intracellular ROS levels (35, 36). Therefore, we wanted to investigate whether DUSPs play a critical role in the regulation of JNK activity in response to challenge by X/XO. The JNK phosphorylation was significantly increased after treating 293T cells for 2 h with X/XO (Fig. 9A). We observed consistently in repeated experiments that the degree of JNK activation induced by X/XO was smaller than that stimulated by H2O2 (Fig. 9A) (data not shown), presumably due to a low dose of XO (10 milliunits/ml) utilized in the experiments. Nevertheless, in response to X/XO treatment, the mRNA levels of both DUSP1 and DUSP10 were rapidly elevated (Fig. 9B), suggesting that there were enough ROS to induce the expression of JNK phosphatases. To further test this hypothesis, we used the RNAi technique to simultaneously ablate DUSP1 and DUSP10. Interestingly, knockdown of both phosphatases by a mixture of siRNA oligonucleotides resulted in a robust activation of JNK at 2 h following exposure to X/XO (Fig. 9C). Based on this finding, we propose that there is probably a general mechanism through which DUSP1 and DUSP10 are rapidly expressed in a JNK-dependent manner that controls the activation of JNK in cells subjected to oxidative stress.

**DISCUSSION**

Completion of the human genome sequence has shown that members of the PTP superfamily comprise a large number of enzymes with diverse primary sequences, tertiary structures, tissue distribution patterns, and substrate specificities (15). A recent comprehensive survey demonstrated that there are 107 PTP genes in the human genome (14). Of these PTP genes, 81 encode active forms of phosphatases (14), comparable with 85 genes encoding catalytically active forms of protein-tyrosine kinases in humans (37), suggesting that there is sophisticated regulation of signal transduction mediated by a large number of PTPs. The in vitro data and studies of overexpression performed in cultured cells have significantly advanced our understanding of the biochemical properties and the substrate specificity of PTPs (15, 38). Furthermore, phenotypic characterization by gene deletion in mice has also found that a number PTPs have nonredundant functions (39, 40). Although it is now apparent that PTPs function as critical signaling regulators, the exact role of endogenous PTPs in the control of complicated signal transduction largely remains undefined. The task at hand is to establish the regulatory links between particular PTPs and specific signaling pathways.

In the current study, we investigated the role of DUSPs in the regulation of oxidative stress-activated MAPK signaling. As
transcriptional activation may be important for the ROS-induced expression of DUSP16 (Fig. 10B). As opposed to DUSP1 and DUSP10, which function predominantly to control the magnitude of initial JNK activation, DUSP4 and DUSP16 are responsible for the effective dephosphorylation of JNK; therefore, they play a key role in determining the duration of JNK signaling.

In addition to observing the signaling events controlled by H$_2$O$_2$, we also examined the mechanism regulating the activation of JNK in response to oxidative stress induced by the X/XO system. We specifically selected a low dose of XO (10 milliunits/ml), which produces a low level of ROS that would not have a detectable cytotoxic effect on 293T cells even after a long period of incubation (data not shown). Recent studies have shown that the enzymes NADPH oxidase and dual oxidase generate ROS in various cells in response to a diverse array of extracellular stimuli, such as growth factors, cytokines, shear stress, and calcium signals (41). Biochemical and structural analyses have further suggested that NADPH oxidase and dual oxidase enzymes are transmembrane electron transfer proteins, which, when activated, pump superoxide anions extracellularly (41). The superoxide anions may be converted to H$_2$O$_2$, thus diffusing into cells to function as second messengers triggering the activation of signal transduction. Using the X/XO system, which also generates superoxide anions extracellularly (35, 36), we showed that the inducible expression of DUSP1 and DUSP10 function as critical regulators for the control of the phosphorylation level of JNK (Fig. 9). These data, together with our understanding of the characteristics of NADPH oxidase and dual oxidase enzymes, suggest that a general regulatory network may exist that determines the level of JNK activation under various physiological and pathophysiological conditions.

We also wanted to experiment with transcription factors that may contribute to the inducible expression of DUSPs. Because of their importance as effectors in response to various environmental stresses, we selected p53, c-Jun, and ATF2 (27–29, 31–34). We observed that p53, c-Jun, and ATF2 were rapidly phosphorylated and activated in 293T cells challenged with

summarized in Fig. 10A, the initial activation of JNK plays an important role in the increased phosphorylation of the ATF2 transcription factor. The phosphorylated and therefore activated form of ATF2 plays an essential role in H$_2$O$_2$-mediated inducible expression of DUSP1 and DUSP10. These genes then act as JNK phosphatases and thereby prevent hyperphosphorylation of JNK at an early stage of the signaling event (Fig. 10A). In a later phase after H$_2$O$_2$ stimulation (Fig. 10B), there was increased expression of DUSP4 and DUSP16, as suggested by the gradual accumulation of mRNA in cells. The transcription factors involved in this particular event are yet to be identified; however, our preliminary data suggest that the JNK-mediated
Interestingly, the phosphorylation level of p53 was not affected when the activity (Fig. 5C) or the expression (Fig. 6B) of JNK was ablated, indicating that p53 is not directly phosphorylated by JNK in this particular pathway. We demonstrated that RNAi ablation of p53 had no effect on the phosphorylation level of JNK activated by H$_2$O$_2$ (Fig. 6A), suggesting that the activation of p53 may be regulated by kinases other than JNK (e.g., Polo-like kinase-3 (Plk3)) (42). As observed by real time PCR analysis in this study, ablation of p53 led to partial inhibition of DUSP1-inducible expression in cells treated with H$_2$O$_2$ (data not shown), which is consistent with a previous report that DUSP1 may be a potential transcriptional target of p53 (43). We propose that under these conditions, the levels of DUSP1 and other inducible DUSPs, which are transcriptionally regulated in a p53-independent manner, were high enough to control the phosphorylation of JNK. Although the functional role of p53 in ROS-induced signal transduction remains unclear, our data suggest that it is not directly involved in the feedback regulation of JNK activation.

Utilizing a pharmacological inhibitor or siRNA oligonucleotides to prevent H$_2$O$_2$-stimulated activation of JNK, we were able to markedly suppress the phosphorylation level of c-Jun and ATF2 (Figs. 5C and 6B). It is interesting to note that, under these circumstances, a small degree of phosphorylation on both transcription factors still remained following H$_2$O$_2$ stimulation (Figs. 5C and 6B), indicating the involvement of other activated kinases, such as p38 (2), which might share the same substrate specificity with JNK. Nevertheless, our data suggest that, when JNK activity was ablated, a low level of phosphorylation on transcription factors was not sufficient to induce expression of DUSP1 and DUSP10 in response to H$_2$O$_2$ stimulation (Figs. 5 and 6). It has been shown that phosphorylated c-Jun and ATF2 are capable of forming either homodimers or heterodimers with a partner belonging to the basic region-leucine zipper family (44). Interestingly, heterodimerization between c-Jun and ATF2 is considered to constitute an active form of AP-1 transcriptional factor for controlling the expression of target genes (45). Thus, it is important to address whether c-Jun and ATF2 contribute to the transcriptional regulation of DUSPs individually or in a coordinated manner. The RNAi-mediated knockdown of ATF2, but not c-Jun, led to enhanced phosphorylation of JNK at an early stage (2 h) after H$_2$O$_2$ stimulation (Fig. 7). Interestingly, the combined knockdown effects of c-Jun and ATF2...
resulted in the same extent of increased phosphorylation of JNK as that observed by knockdown of ATF2 alone (Fig. 7). These results suggest that ATF2-mediated transcriptional activation of DUSP genes may not require heterodimerization with c-Jun. The mRNA levels of DUSP1 and DUSP10 were significantly suppressed in cells transfected with siRNA oligonucleotides targeting ATF2 (Fig. 8). In contrast, the inducible expression of DUSPs in response to H2O2 stimulation was not affected when c-Jun was ablated by RNAi, as revealed by quantitative real-time PCR (data not shown). Thus, our results demonstrate that, in the signaling response to oxidative stress, the JNK-activated ATF2 transcription factor plays a key role in immediate expression of DUSP1 and DUSP10. This expression then functions coordinately to control the degree of JNK activation. Our preliminary analysis of the genomic sequence showed the presence of typical ATF2 binding motifs in the promoter region of DUSP1 genes. Further research is required to determine the mechanism through which the transcriptional regulation of DUSP1 and DUSP10 genes is controlled by ATF2 in ROS-mediated signaling pathways.

We have shown that the suppression of DUSP4 or DUSP16 by RNAi led to sustained JNK activation in H2O2-treated cells (Figs. 3 and 4). Interestingly, the phosphorylation level of JNK returned to the basal level ~6–8 h after H2O2 stimulation in siRNA transfectants even when both DUSP4 and DUSP16 were ablated (Fig. 4), suggesting that additional phosphatases are involved in the down-regulation of JNK activation. We have conducted a microarray analysis using an Affymetrix HG U133 chip. Preliminary data suggest that, of all the Cys-based PTPs examined, only one additional phosphatase displayed a significant increase in mRNA level in 293T cells stimulated with H2O2 for 4 h. This phosphatase was identified as Laforin, an active tyrosine phosphatase and member of a subfamily comprising atypical DUSPs. The N-terminal region of this phosphatase has a unique carbohydrate-binding domain. To date, Laforin has been linked to the accumulation of glycogen in cells (46, 47).

**FIGURE 8.** The activation of transcription factor ATF2 is involved in H2O2-induced expression of JNK phosphatases DUSP1 and DUSP10. Human embryonic kidney 293T cells were transfected with 150 nM nontargeting scrambled siRNA oligonucleotide (Control; white bars) or 150 nM siRNA oligonucleotide to ATF2 (black bars). Eight hours after transfection, cells were deprived of serum for 16 h and then stimulated with 200 µM H2O2 for 4 h. The relative mRNA level of DUSP1, DUSP4, DUSP10, and DUSP16 was determined by real-time PCR analysis and normalized to the level of HMBS. Data are presented as fold changes over the non-treated controls and expressed as means ± S.E. (n = 3). *, p < 0.05 when compared with H2O2-treated controls transfected with non-targeted scrambled siRNA oligonucleotides.

Although the in vivo substrate of this phosphatase has not been identified. Further investigation is needed to determine the biological significance of inducible expression of Laforin in the signaling response to oxidative stress.

Although ERKs are often regarded as important modules in signaling pathways induced by growth factors, they can also be activated by stress stimuli (48–50). In the present study, we consistently observed ERKs, in particular the p42 form of Erk1, to be moderately activated in H2O2-treated 293T cells (Fig. 4). The peak phosphorylation, the ERK activation appears to be more sustained over a similar time frame (Fig. 1B), suggesting that genes encoding ERK phosphatases might not be effectively

**C.-H. Teng and T.-C. Meng, unpublished results.**

**C.-H. Teng and T.-C. Meng, unpublished results.**
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A. Early event (0–2 h)

B. Late event (2–4 h)

FIGURE 10. Proposed models illustrate possible mechanisms for inducible expression of JNK phosphatases in response to oxidative stress. In response to extracellular oxidative stress, JNK is rapidly phosphorylated by upstream kinases. The phosphorylated form of JNK is translocated into the nucleus for activation of transcription factors. A, our data demonstrate that the activation of ATF2, a substrate of JNK, is a key step for inducible expression of the genes encoding DUSP1 and DUSP10. We propose that the immediate expression of DUSP1 and DUSP10 at an early stage (0–2 h) after H2O2 stimulation is critical for controlling the initial phosphorylation level of JNK. B, the H2O2-induced expression of genes encoding DUSP4 and DUSP16 occurs at a late phase (2–4 h) after stimulation and is regulated by a mechanism that has not been identified. Our data suggest that the inducible expression of DUSP4 and DUSP16 genes, which may be regulated by a yet unidentified transcription factor (T.F.), is independent of JNK activation. We showed that both DUSP4 and DUSP16 act for effectively dephosphorylating JNK, thus controlling the duration of JNK activation in the signaling response to oxidative stress.

induced. Real time PCR (Fig. 2) and microarray analysis (data not shown) revealed that the mRNA levels of DUSP5, DUSP6, and DUSP7, which have been designated as ERK-specific phosphatases (51–57), were not significantly increased in cells exposed to H2O2, concomitant with the prolonged phosphorylation of ERKs. Although the underlying mechanism behind the selective induction of a specific set of DUSPs has still not been determined, we did find data suggesting that the oxidative stress-activated signaling cascades might be important for directing the transcriptional regulation of JNK phosphatases but not ERK phosphatases.

In addition to JNK, p38 kinases are also classified as a group of stress-activated protein kinases, since these enzymes are readily activated by various environmental stimuli, such as heat shock, ionizing irradiation, UV light, and oxidative stress (3, 4). This study found that the treatment of cells with H2O2 led to the activation of p38 (Fig. 1). Interestingly, the ROS-mediated activation of p38 was transient, since the Thr/Tyr dephosphorylation of p38 occurred rapidly following the peak phosphorylation ~4–6 h after H2O2 stimulation (Fig. 1), suggesting the involvement of p38 phosphatases in this regulatory process. Previous studies have suggested that a number of MKPs may recognize both p38 and JNK kinases as substrates (7, 8). Therefore, we examined whether the DUSPs that we identified as JNK phosphatases (Fig. 3) also contributed to p38 dephosphorylation. Results from RNAi-based approaches demonstrated that the ablation of DUSP10 and, to a lesser extent, DUSP1 resulted in an increased phosphorylation level of p38 in 293T cells treated with H2O2 (Fig. S2). In contrast, other JNK phosphatases, including DUSP4 and DUSP16, did not participate in the dephosphorylation of p38 (Fig. S2). These findings suggest that under oxidative stress conditions JNK and p38 kinases are regulated by different species of MKPs. Further investigation is required to find out what mechanism determines substrate specificity of DUSPs for the regulation of MAPK activation in a particular signaling pathway.

In conclusion, we have provided evidence that both DUSP1 and DUSP10 were transiently expressed at an early stage of the oxidative stress-induced signaling pathway, corresponding to their function in controlling how much JNK is initially activated, whereas DUSP4 and DUSP16 were expressed in the late phase, concomitant with their unique role in rapid dephosphorylation of JNK. These novel findings illustrate that a sophisticated mechanism, which involves the transcriptional regulation of several DUSP genes and the coordinated action of these phosphatases, is required for precisely controlling the magnitude and duration of JNK activation in vivo. The activation of JNKs is important for a number of physiological and pathophysiological processes (58). For example, the deregulation of JNK signaling is implicated in the development of human diseases, such as cancers, heart hypertrophy and ischemia, and neurodegenerative disorders related to Alzheimer and Parkinson diseases (58). The identification and characterization of specific DUSPs that function in the control of JNK activation may provide an exciting opportunity for the development of new therapeutic interventions for these diseases.

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