Phosphorylation and Inactivation of Myeloid Cell Leukemia 1 by JNK in Response to Oxidative Stress*

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Oxidative stress induces JNK activation, which leads to apoptosis through mitochondria-dependent caspase activation. However, little is known about the mechanism by which JNK alters mitochondrial function. In this study, we investigated the role of phosphorylation of myeloid cell leukemia 1 (Mcl-1), an anti-apoptotic member of the Bcl-2 family, in oxidative stress-induced apoptosis. We found that JNK phosphorylated Ser-121 and Thr-163 of Mcl-1 in response to stimulation with H2O2, and that transfection of unphosphorylatable Mcl-1 resulted in an enhanced anti-apoptotic activity in response to stimulation with H2O2, JNK-dependent phosphorylation and thus inactivation of Mcl-1 may be one of the mechanisms through which oxidative stress induces cellular damage.

Oxidative stress has been implicated in the pathogenesis of several abnormal conditions and diseases including ischemia, cancer, and diabetes mellitus (1–3). A recent study suggests that stress-activated protein kinases such as JNK and p38 play important roles in triggering apoptosis in response to various cellular stressors including oxidative stress. We have shown that oxidative stress-induced sustained activation of JNK and p38 is required for apoptosis (4). Apoptosis signal-regulating kinase 1 (ASK1), a member of the mitogen-activated protein kinase (MAPK) kinase kinase (MAPKKK), specifically mediates the sustained activation of JNK/p38 and apoptosis in response to oxidative stress (5, 6). ASK1-dependent apoptosis is mediated by the release of cytochrome c from the mitochondrion followed by caspase 9 activation (7). It has also been reported that JNK is required for UV-induced release of cytochrome c and that new gene expression is not required for this process (8). These reports indicate that JNK induces apoptosis in part through the mitochondria-dependent caspase activation. However, the molecular mechanism by which activated JNK induces mitochondrial dysfunction is unclear.

The members of the Bcl-2 family play pivotal roles in cellular decision to undergo apoptosis. Bcl-2 has been reported to be phosphorylated by JNK in response to different stimuli (9–11). Although the significance of phosphorylation of Bcl-2 is controversial, it was suggested that phosphorylation by JNK within the unstructured loop region of Bcl-2 decreases its anti-apoptotic activity (9, 10, 12). Anti-apoptotic Bcl-2 family proteins thus may be potential mediators of JNK-induced apoptosis. However, little is known about the relation between JNK and the other anti-apoptotic members of the Bcl-2 family in the context of oxidative stress-induced apoptosis signaling.

The myeloid cell leukemia 1 (Mcl-1) (13), also known as EAT (14), is an anti-apoptotic Bcl-2 family member. Mcl-1 plays an important role in the development of various carcinomas (15–17). Similar to other Bcl-2 family members, Mcl-1 localizes in the mitochondrion as well as in other intracellular membranes (18) and can associate with other pro-apoptotic family members (19). Mcl-1 differs from Bcl-2 and Bcl-XL in structure (19), in its short half-life (13), in the regulation of its promoter (20–22), and in its ability to protect cells from a variety of cytotoxic stimuli (23, 24). Little is known regarding posttranslational modification and regulation of Mcl-1. In this study, we investigated the potential involvement of phosphorylation regulation of Mcl-1.

**EXPERIMENTAL PROCEDURES**

Cell Culture and Transfection—HEK293 cells were grown under 5% CO2 in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 4.5 g/liter glucose, and 100 units/ml penicillin. Porcine aortic endothelial (PAE) cells were grown under 5% CO2 in F12 medium (Invitrogen) supplemented with 10% fetal bovine serum, 10 mm HEPES, and 100 units/ml penicillin. Transfection with various constructs in pGEX-Neo was performed using 2 µg of plasmid and 8 µl of Tfx 50 (Promega). Transfected cells were selected in the presence of 1 mg/ml Gentamicin for 2 weeks, and drug-resistant single-cell colonies were chosen and maintained in growth medium containing 0.4 mg/ml Gentamicin.

Antibodies and Reagents—Rabbit polyclonal antibody to Mcl-1 was purchased from BD Biosciences. Phospho-JNK (Thr-183/Tyr-185) and p38 (Thr-180/Tyr-182) were purchased from New England Biolabs. Phospho-ERK (Thr-183/Tyr-185) was purchased from Promega. The antibodies to Myc tag (clone 9E10), HA tag (clone 3F10), and FLAG tag were purchased from Calbiochem, Roche Molecular Biochemicals, and Sigma, respectively. SB203580 was purchased from Calbiochem.

**Western Blot Analysis**—Cells were lysed in a lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 1 mM PMSF.
HA-MAPKs were immunoprecipitated with an anti-HA monoclonal antibody. In vitro, B229 was inserted into the pGEX-2T grown in 293 cells and purified. Nearly 100% infection of PAE cells by recombinant adenoviruses were isolated, and high titer stocks of recombinant adenoviruses were generated by homologous recombination. This was cotransfected into the E1 transcomplementing 293 cell clone. The Green fluorescent protein-tagged MKK4 mutant cDNA was subcloned into the pcDNA3 vector and replaced by Arg using a PCR-based site-directed mutagenesis method.

*Construction of Expression Plasmids and an Adenovirus Vector*—A cDNA clone containing the full-length of the Mcl-1 coding region was inserted into pcDNA3.0 vector. To replace Ser-121 and/or Thr-163 with Ala, a PCR-based site-directed mutagenesis method was used. The Myc tag was inserted at the NH2 termini of wild type and mutant Mcl-1. pcDNA3-HA-ERK, pcDNA3-HA-JNK, pcDNA3-HA-p38, pcDNA3-HA-ASK1, pcDNA3-FLAG-ASK1, and pcDNA3-FLAG-ASK1 have been described previously (6, 26–28). Recombinant adenoviruses were constructed as described elsewhere (29, 30). MKK4 cDNA was subcloned in pcDNA3 by PCR. Lys-116 was replaced by Arg using a PCR-based site-directed mutagenesis method.

Green fluorescent protein-tagged MKK4 mutant cDNA was subcloned into the ScaI site of pAdex1pCAw cassette cosmid. Each cosmid bear the expression unit and adenovirus DNA-terminal protein complex was cotransfected into the E1 transcomplementing 293 cell clone. The recombinant adenoviruses generated by homologous recombination were isolated, and high titer stocks of recombinant adenoviruses were grown in 293 cells and purified. Nearly 100% infection of PAE cells by recombinant adenoviruses can be achieved at a m.o.i. of 100 as determined by green fluorescent protein fluorescence (data not shown).

*In Vitro Kinase Assay*—A cDNA encoding the human Mcl-1 protein corresponding to amino acids 31–229 was inserted into the pGEX-2T expression vector (Amersham Biosciences). Mcl-1-GST protein was induced in Escherichia coli BL21 cells by adding 0.5 mM isopropyl-β-D-thiogalactopyranoside and purified with glutathione-Sepharose 4B (Amersham Biosciences). The immune complex kinase assay was done as described previously (26). The indicated plasmids were co-transfected into 293 cells by Tfx50 (Promega). Cells were lysed in a lysis buffer containing 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 5 mM EGTA, 1% Triton X-100, 1% deoxycholate, 12 mM β-glycerophosphate, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 1.5% aprotinin. Cell extracts were clarified by centrifugation and re-suspended in sample buffer and boiling for 3 min.

To investigate whether Mcl-1 is regulated by phosphorylation in response to oxidative stress, PAE cells were exposed to 1.5% aprotinin. Cell extracts were clarified by centrifugation and resolved on SDS-PAGE followed by electroblotting onto polyvinylidene difluoride membrane. After blocking with 5% skim milk in Tris-buffered saline with Tween 20 (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, and 0.05% Tween 20), the membranes were probed with antibodies. The antibody-antigen complexes were detected using the ECL system (Amersham Biosciences). The immune complex kinase assay was performed using GST-Mcl-1 as the substrate. The beads were washed twice with washing buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 5 mM EGTA, and 1 mM DTT), and then incubated with GST-Mcl-1 as the substrate for 20 min at 30 °C in 30 µl of kinase buffer (20 mM Tris-HCl, pH 8.0, 20 mM MgCl2, and 0.3 µCi of [γ-32P]ATP). The kinase reaction was stopped by adding SDS sample buffer and analyzed by SDS-PAGE and a Fuji BAS2000 Image analyzer.

**RESULTS**

To investigate whether Mcl-1 is regulated by phosphorylation in response to oxidative stress, PAE cells were exposed to...
H2O2 and the electrophoretic mobility of Mcl-1 was assessed by immunoblotting analysis. We detected endogenous Mcl-1 of PAE cells as a double band under non-stressed conditions (Fig. 1A, top, lane 1). The mobility of both bands was delayed by H2O2 treatment in a time-dependent manner (Fig. 1A, top, lanes 2–6). The treatment of cell lysates prepared from H2O2-stimulated (Fig. 1A, top, lane 7) and unstimulated (data not shown) PAE cells with /H9261-protein phosphatase resulted in the acceleration of the mobility of those bands. These findings suggest that endogenous Mcl-1 is partially phosphorylated under non-stressed conditions and that additional phosphorylation occurred after H2O2 treatment.

To identify the kinase responsible for Mcl-1 phosphorylation in response to H2O2, we examined the activation state of three classes of MAPK, namely, ERK, JNK, and p38, which are all known to be activated by H2O2 (4, 31). The kinetics of activation of JNK correlated with the extent of mobility of Mcl-1 (Fig. 1A), suggesting that JNK might be involved in the H2O2-induced phosphorylation of Mcl-1. To examine which MAPK can phosphorylate Mcl-1 directly, we carried out an in vitro kinase assay using recombinant Mcl-1 as the substrate. JNK and p38 strongly phosphorylated Mcl-1 in vitro, whereas the phosphorylation of Mcl-1 by ERK was marginal (Fig. 1B). The co-transfection of ASK1, a MAPKKK that activates JNK and p38 MAPK cascades in vivo, strongly enhanced the phosphorylation of Mcl-1 by JNK and p38 (Fig. 1C, top, lanes 1–5). ASK1 itself phosphorylated Mcl-1 very weakly (Fig. 1C, top, lane 6). These findings suggested that Mcl-1 may serve as a specific substrate for JNK and p38 at least in vitro. We next examined whether Mcl-1 could be phosphorylated by JNK and p38 in mammalian cells. When Mcl-1 was co-transfected with JNK or p38 alone, the phosphorylation of Mcl-1 was undetectable as determined by the band shift analysis (Fig. 1D, lanes 2 and 4). In contrast, Mcl-1 became a shifted doublet by the co-expression of the activated allele of ASK1 (ASK1ΔN) together with JNK or p38 (Fig. 1D, lanes 3 and 5).

**Fig. 2.** Mcl-1 is phosphorylated on serine 121 and threonine 163. **A**, schematic representation of Mcl-1 and the conserved MAPK phosphorylation sites. The sequence of human and mouse Mcl-1 containing the conserved MAPK phosphorylation sites are shown. We constructed NH2-terminally Myc-tagged plasmids encoding wild type (WT), a single alanine substitution mutant (S121A and T163A) and a double alanine substitution mutant (S121A/T163A) of Mcl-1. **B**, phosphorylation sites of Mcl-1 by JNK and p38. 293 cells were transfected with WT or mutants S121A, T163A, and S121A/T163A of Mcl-1 in combination with ASK1ΔN-HA, JNK-HA, and p38-HA as indicated. Cells were incubated in the medium without phosphate for 3 h and labeled with [32P]orthophosphate for another 3 h. Mcl-1 was then immunoprecipitated with an anti-Myc antibody and separated by 8.5% PAGE. Top, phosphorylated Mcl-1 was detected by an image analyzer; middle, Western blotting of Myc-Mcl-1 in the lysate; bottom, Western blotting of HA-MAPKs and HA-ASK1 in the lysate. The intensity of Mcl-1 phosphorylation relative to amount of Mcl-1 protein was calculated, and the intensity was shown as fold increase relative to control. C, the absence of gel mobility shift of S121A/T163A Mcl-1 on H2O2 treatment. PAE clones stably expressing WT or S121A/T163A MCL-1 were treated with 0.5 mM H2O2 for the indicated periods of time. Cells were lysed in a lysis buffer for phosphatase treatment. Aliquots of the samples were incubated with or without /H9261-protein phosphatase at 30 °C for 30 min. Western blots were performed using the indicated specific antibodies. Asterisk indicates nonspecific band.
Phosphorylation of Mcl-1 by JNK

 treatment shifted the doublet down to the basal status (Fig. 1D, lanes 6–10), indicating that activated JNK and p38 could phosphorylate Mcl-1 in vivo.

A sequence comparison of human and mouse Mcl-1 revealed that Mcl-1 possesses two conserved sites, Ser-121 and Thr-163, in humans that conforms to the consensus motif for the substrate of JNK and p38 (Fig. 2A). These sites are located in the PEST (proline, glutamic acid, serine, and threonine) domain of Mcl-1 (Fig. 2A) and correspond to the so-called unstructured loop region in Bcl-2, which regulates the anti-apoptotic function of Bcl-2 (32). To examine which sites are phosphorylated by JNK or p38, we constructed three alanine substitution mutants of Mcl-1 (S121A, T163A, and S121A/T163A). The Myc-tagged wild-type (WT) or alanine-substituted mutant of Mcl-1 was co-transfected with JNK or p38 plus ASK1ΔN. Cells were metabolically labeled with [32P]orthophosphate and analyzed by autoradiography after immunoprecipitation using anti-Myc antibody. WT and single alanine substitution mutants (S121A and T163A) of Mcl-1 were clearly phosphorylated by the co-expression of activated JNK and p38 (Fig. 2B, top, lanes 5–7 and 9–11). In contrast, little phosphorylation was detected in the double-alanine mutant (S121A/T163A) of Mcl-1 (Fig. 2B, top, lanes 8 and 12). These findings suggested that when overexpressed, activated JNK and p38 can phosphorylate both Ser-121 and Thr-163 of Mcl-1 and that these two amino acids are the major phosphorylation sites of Mcl-1 in vivo.

To investigate the involvement of Ser-121 and Thr-163 in oxidative stress-induced phosphorylation of Mcl-1 as observed in Fig. 1A, we generated PAE cell clones stably expressing Myc-tagged WT and S121A/T163A mutant of Mcl-1. When these cells were treated with H2O2, the activations of endogenous JNK and p38 were clearly observed in both cells in a time-dependent manner (Fig. 2C, middle and bottom panels). In parallel with JNK activation, gel mobility of Mcl-1 was retarded in WT but not in S121A/T163A mutant-expressing cells (Fig. 2C, top), and the retardation was canceled by treatment with λ-protein phosphatase (Fig. 2C, lane 7). We have examined three independently selected clones of WT and mutant Mcl-1 and obtained essentially the same results in independent clones (data not shown). These results suggest that both Ser-121 and Thr-163 of Mcl-1 are phosphorylated in response to oxidative stress.

The overexpression of either activated JNK or p38 phosphorylated Mcl-1 in vivo (Figs. 1D and 2B), and both kinases were activated by H2O2 treatment (Figs. 1A and 2C). However, time course analysis indicated that the activation of JNK coincided with Mcl-1 phosphorylation following H2O2 stimulus much better than that of p38 (Figs. 1A and 2C). To examine which signaling pathway is physiologically required for Mcl-1 phosphorylation in response to H2O2, we used the p38 inhibitor SB203580 and a recombinant adenosine encoding dominant negative MKK4. Although p38 was specifically inactivated by SB203580 (data not shown), the treatment of PAE cells with SB203580 before H2O2 stimulation did not alter H2O2-induced Mcl-1 mobility shift (Fig. 3A). In contrast, the expression of the dominant negative MKK4 significantly reduced the gel mobility shift of Mcl-1 upon H2O2 treatment (Fig. 3B). JNK but not p38 activation was specifically reduced by adenosine encoding dominant negative MKK4. Taken together, Mcl-1 appears to be phosphorylated mainly via the JNK pathway in response to oxidative stress.

Finally, we assessed the functional importance of Mcl-1 phosphorylation in oxidative stress-induced apoptosis. To this end, the susceptibility to H2O2-induced apoptosis was examined in PAE clones stably expressing WT and those expressing S121A/T163A Mcl-1. PAE clones were treated with 0.5 mM H2O2 for 3 h, and cell death was determined by the trypan blue exclusion assay. Results are the mean ± S.E. of three independent experiments (*, p < 0.05).

H2O2 for 3 h, and cell death was determined by the trypan blue exclusion assay (Fig. 4). WT Mcl-1 conferred only minimal resistance compared with the vector control. However, S121A/T163A Mcl-1 showed substantially stronger anti-apoptotic activity than WT Mcl-1 following H2O2 treatment. We have examined three independently selected clones of WT and mutant Mcl-1 and obtained essentially the same results in independent clones (data not shown). These data indicated that eliminating phosphorylation sites increased anti-apoptotic activity of Mcl-1. In other words, Mcl-1 appears to be negatively regulated through phosphorylation of Ser-121 and Thr-163 by JNK following H2O2 stimulation.

DISCUSSION

In this study, we demonstrated that Mcl-1 was phosphorylated at Ser-121 and Thr-163 through the JNK pathway and inactivated following H2O2 treatment. We also demonstrated that both JNK and p38 could phosphorylate Mcl-1 in vitro, whereas ERK induced little phosphorylation. A recent study also suggested using an ERK inhibitor that ERK was involved in 12-O-tetradecanoylphorbol-13-acetate-induced Mcl-1 phosphorylation (33). Further investigations will be needed to determine whether the ERK pathway is also involved in Mcl-1 phosphorylation depending on stimuli.

We identified two phosphorylation sites, which regulate the anti-apoptotic function of Mcl-1 in response to H2O2. Although human Mcl-1 possesses five potential phosphorylation sites that can be phosphorylated by JNK and p38, we could not detect any phosphorylation in the S121A/T163A mutant, sug-
H2O2-induced phosphorylation. Bcl-2 has been shown to be a potential target of JNK that regulates cytochrome c release of cytochrome c. JNK acts on mitochondria and induces apoptosis through the JNK and ASK1 pathways. We detected another shift of gel mobility of the S121A/T163A mutant of Mcl-1 when it was co-expressed with JNK and ASK1 in 293 cells (data not shown). It remains to be determined whether other phosphorylation sites contribute to the regulation of the anti-apoptotic activity of Mcl-1.

The mechanisms by which phosphorylation of Bcl-2 regulates anti-apoptotic function are poorly understood. Several studies have shown that phosphorylated Bcl-2 does not heterodimerize with Bax, and thus, apoptosis is promoted by an increase in the interaction of Bax and Mcl-1 before or after the phosphorylation of Mcl-1 (data not shown). However, in the phosphorylation sites of Mcl-1 located in the PEST motif, there was no difference in the half-life of WT and S121A/T163A mutant of Mcl-1 after H2O2 stimulation (data not shown). Further studies will be needed to elucidate the mechanism of phosphorylation-mediated inactivation of Mcl-1.

The JNK signaling pathway is essential for exocytotoxic stress-induced apoptosis in neurons and UV-induced apoptosis in mouse embryonic fibroblast (8, 36). It seems that activated JNK acts on mitochondria and induces apoptosis through the release of cytochrome c (7, 8). The mechanism of cytochrome c release by JNK is not known at all. Although the Bcl-2 family is a potential target of JNK that regulates cytochrome c, several discrepancies have been pointed out. For example, Bcl-2 phosphorylation has been suggested to increase rather than decrease anti-apoptotic function (37). Stimuli that cause JNK-phosphorylation has been suggested to increase rather than decrease anti-apoptotic function (37). Phosphorylation and inactivation of Mcl-1 thus may be one of the mechanisms by which JNK induces apoptosis in response to oxidative stress.

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