Suppression of MEK/ERK Signaling Pathway Enhances Cisplatin-induced NF-κB Activation by Protein Phosphatase 4-mediated NF-κB p65 Thr Dephosphorylation*

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We previously reported that suppression of the MEK/ERK pathway increases drug resistance of SiHa cells. In this study, we further characterized the underlying mechanism of this phenomenon. Pretreatment of SiHa cells with MEK/ERK inhibitor enhanced cisplatin-induced NF-κB activation. However, results of immunoblotting analysis showed that neither cisplatin nor MEK/ERK inhibitors induced marked IκB degradation, suggesting that suppression of the MEK/ERK signaling pathway may enhance cisplatin-induced NF-κB activation via mechanisms other than the conventional pathway. Previous findings that protein phosphatase 4 (PP4), a nuclear serine/threonine phosphatase, directly interacts with and activates NF-κB led us to examine the phosphorylation status of NF-κB p65. Coincident with activation of NF-κB, cisplatin induced Ser phosphorylation but decreased Thr phosphorylation of NF-κB p65. Suppression of the MEK/ERK pathway further enhanced cisplatin-induced Thr dephosphorylation but did not affect cisplatin-induced Ser phosphorylation of NF-κB p65. Further, in parallel with Thr dephosphorylation, the protein level of nuclear PP4 was increased in cisplatin-treated cells and was further increased by suppression of the MEK/ERK pathway. SiHa cells were then transfected by a sense or an antisense PP4 construct. coprecipitated complex kinase assay revealed a fragment of NF-κB p65 (amino acids 279–444) to contain potential phosphorylation sites that directly interact with PP4. Further studies by site-directed mutagenesis suggested that Thr435 was the major phosphorylation site.

Nuclear transcription factor κB (NF-κB) is widely involved in various cellular responses to extracellular stimuli. The activation of NF-κB is associated with tumorigenesis and inducible drug resistance in tumor cells (1–5). NF-κB is normally restricted to the cytoplasm by binding to an inhibitory factor, IκB. Following stimulation, IκB is phosphorylated and degraded. Unbound NF-κB then translocates into the nucleus and transactivates various downstream genes (reviewed in Refs. 6 and 7). In addition to this classical activation pathway, recent data suggest that the phosphorylation status of the NF-κB p65 subunit is also involved in NF-κB activation. For example, protein kinase A phosphorylates p65 on Ser276 (8), IKK complex phosphorylates p65 on Ser276 (9), and tumor necrosis factor induces phosphorylation of p65 on Ser276 (10). The phosphorylation of subunit p65 affects neither IκB degradation nor NF-κB nuclear translocation but is required for the transcriptional activity of NF-κB, partly via increasing the interaction of NF-κB with other transcriptional cofactors (11, 12). On the other hand, protein phosphatase 2A (PP2A) has been found to directly interact with and dephosphorylate p65 in melanocytes, leading to a decrease of the transcriptional activity of NF-κB (13).

Protein phosphatase 4 (PP4; also known as protein phosphatase X) is a novel Ser/Thr protein phosphatase that shares 65% amino acid identity with PP2A (14). PP4 is induced and activated upon stimulation, IκBα degradation, suggesting that PP4 might be involved in NF-κB activation. For example, protein kinase A phosphorylates p65 on Ser276 (8), IKK complex phosphorylates p65 on Ser276 (9), and tumor necrosis factor induces phosphorylation of p65 on Ser276 (10). The phosphorylation of subunit p65 affects neither IκB degradation nor NF-κB nuclear translocation but is required for the transcriptional activity of NF-κB, partly via increasing the interaction of NF-κB with other transcriptional cofactors (11, 12). On the other hand, protein phosphatase 2A (PP2A) has been found to directly interact with and dephosphorylate p65 in melanocytes, leading to a decrease of the transcriptional activity of NF-κB (13).

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** The abbreviations used are: NF-κB, nuclear transcription factor κB; PP2A, protein phosphatase 2A; PP4, phosphatase 4; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/ERK kinase; EMSA, electromobility shift assay; IKK, IκB kinase.
with NF-κB activation. The results showed that suppression of ERK2 increased the protein level of PP4 and thereby enhanced cisplatin-induced NF-κB activation via decrease of the Thr phosphorylation status of the NF-κB p65 subunit.

EXPERIMENTAL PROCEDURES

Cell Culture and Chemicals—Human cervical carcinoma SiHa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and incubated in a humidified incubator with 5% CO₂ at 37 °C. MEK/ERK inhibitors, PD98059 and U0126, were purchased from Calbiochem, and other chemicals were obtained from Calbiochem or Sigma.

Immunoblotting, Reprobing, and Immunoprecipitation—The cells after various treatments were subjected to cell fractionation as previously described (23). Cytosolic and nuclear lysates were prepared, and the protein concentration was determined using the Bio-Rad determination kit. Aliquots (15 μg) of lysates were subjected to immunoblotting. For reprobing, the membrane was washed with a stripping buffer (2% SDS, 7 μl of β-mercaptoethanol in 100 ml of Tris-buffered saline containing 0.2% Tween 20) for 30 min at room temperature and then subjected to another immunoblotting. Antibodies, including anti-Thr(P), anti-phospho-ERK2, anti-ERK2, anti-NF-κB p65, anti-1×αt, anti-p65 antibody. Immunoblotting, Reprobing, and Immunoprecipitation—The cells after various treatments were subjected to cell fractionation as previously described (23). Cytosolic and nuclear lysates were prepared, and the protein concentration was determined using the Bio-Rad determination kit. Aliquots (15 μg) of lysates were subjected to immunoblotting. For reprobing, the membrane was washed with a stripping buffer (2% SDS, 7 μl of β-mercaptoethanol in 100 ml of Tris-buffered saline containing 0.2% Tween 20) for 30 min at room temperature and then subjected to another immunoblotting. Antibodies, including anti-Thr(P), anti-phospho-ERK2, anti-ERK2, anti-NF-κB p65, anti-1×αt, anti-p65 antibody.

Electromobility Shift Assay (EMSA)—The activation of NF-κB was determined by EMSA as previously described (23). 32P-Labeled oligodeoxynucleotide probe, 5′-GGATTGGGACCTTCCCTTCTG-3′, was incubated with 10 μg of nuclear extract at room temperature for 30 min, and the electromobility of the probe was analyzed in 5% native polyacrylamide gel. The NF-κB-DNA complex was visualized by autoradiography. The supershift experiment was performed by adding 1 μg of anti-p65 antibody.

Plasmid Construction, Site-directed Mutagenesis, and Gene Transfection—The full-length of P44 gene was a gift from Dr. M. C. T. Hu (Baylor College of Medicine). The HindIII/BamHI-digested P44 fragment was subcloned into the pBKCMV (sense) or pCDNA3 (antisense) HindIII/BamHI site. Purified plasmid was transfected into SiHa cells with Lipofectant 2000 (Invitrogen) according to the manufacturer’s instructions. The cells were selected by 500 μg/ml G418. To identify the possible phosphorylation sites of NF-κB p65, the full-length p65 gene was cloned from SiHa cells by reverse transcriptase-PCR. A SacI/PstI fragment of p65 (921–1411 fragment encoding amino acids 279–444; p65/S) was in-frame cloned into pSSET vector (His6 tag Escherichia coli expression vector; Invitrogen). Substitution of Thr435 to Ala of p65S/P by site-directed mutagenesis was performed by a two-round PCR method as previously described (24). The mutation was confirmed by DNA sequencing.

Coproxipitated Complex Kinase Assay—E. coli expressed p65/S/P wild type or mutant proteins were adsorbed to nickel-agarose (Clontech) in native binding buffer (25 mM sodium phosphate buffer, pH 8.0, 500 mM NaCl, 10 mM imidazole) according to the manufacturer’s instructions. The nickel-agarose-p65/S/P complexes were mixed with nuclear lysates prepared from cells with various treatments as shown in the figures at 4 °C for 2 h. The precipitated complex was washed twice with binding buffer and once with kinase buffer (40 mM Hepes, pH 8.0, 2 mM dithiothreitol, 5 mM MgCl₂). The complex was incubated with kinase assay buffer containing 5 μCi of [γ-32P]ATP at 30 °C for 30 min and then subjected to SDS-PAGE. The images were visualized by exposure to x-ray film.

RESULTS

Inhibition of MEK/ERK Enhances Cisplatin-induced NF-κB Activation—To elucidate the relationship between ERK2 and NF-κB in the cellular response to cisplatin, SiHa cells were treated with 20 μM cisplatin (IC₅₀) for 1–5 h with or without a 1-h pretreatment with 10 μM U0126. As shown in Fig. 1A, ERK2 was activated by cisplatin, and U0126 suppressed this cisplatin-induced ERK2 activation. Under the same treatment, the NF-κB activation was determined by EMSA. NF-κB was activated by cisplatin, and pretreatment with U0126 further enhanced this activation. Similar results were obtained by pretreatment with PD98059 (data not shown). The NF-κB-DNA complex was confirmed by supershift experiment, which showed that the band was specifically shifted by anti-p65 antibody (Fig. 1B). The activation of NF-κB was further determined by NF-κB-driven luciferase reporter assay. NF-κB-driven luciferase reporter gene stably transfected SiHa cells have been described (23). As shown in Fig. 1C, the transcriptional activity of NF-κB was induced 1.5-fold by 20 μM cisplatin-induced NF-κB activation.
platin is a DNA alkylating agent that acts primarily in the cell nucleus. PD98059 was used in the subsequent experiments. Cisplatin markedly decreased the level of Thr phosphorylation of NF-κB p65, and cytosolic IκBα was determined by immunoblotting (IB).

Neither Cisplatin nor MEK/ERK Inhibitors Induce IκBα Degradation or NF-κB Nuclear Translocation—To characterize the pathways that lead to NF-κB activation, the protein levels of cytosolic IκBα and nuclear NF-κB after various treatments were determined by immunoblotting. As shown in Fig. 2, cisplatin with or without U0126 did not induce remarkable IκBα degradation or NF-κB nuclear translocation. These results suggest that suppression of the MEK/ERK signaling pathway might regulate NF-κB activity through an alternative pathway. PD98059 was shown to exert an effect similar to U0126 (data not shown).

PD98059 Decreases the Level of Thr but Not Ser Phosphorylation of NF-κB p65—Because suppression of MEK/ERK by either PD98059 or U0126 showed similar effects in SiHa cells, only PD98059 was used in the subsequent experiments. Cisplatin is a DNA alkylating agent that acts primarily in the nucleus. Whether cisplatin-induced NF-κB activation is subjected to further regulation in the nucleus by inhibition of ERK2 was examined by Western blotting of immunoprecipitated nuclear NF-κB p65 with anti-Thr(P) or anti-Ser(P) antibody. As shown in Fig. 3, cisplatin increased the Ser phosphorylation of NF-κB p65 in a time-dependent manner. On the contrary, cisplatin decreased the Thr phosphorylation of NF-κB p65. Although PD98059 alone had little effect on the phosphorylation status of NF-κB p65, cotreatment of PD98059 with cisplatin markedly decreased the level of Thr phosphorylation of NF-κB p65. By contrast, this cotreatment slightly increased the Ser phosphorylation of NF-κB p65. These results suggest that suppression of ERK2 enhanced cisplatin-induced NF-κB activity mainly via Thr dephosphorylation of NF-κB p65.

PP4 Is Involved in the Regulation of NF-κB p65 Thr Phosphorylation—Because PP4 is a nuclear Ser/Thr protein phosphatase and directly associates with and activates NF-κB (16), its possible involvement in mediating the downstream signal of ERK2 suppression to NF-κB nuclear activation was explored. As shown in Fig. 4, cisplatin slightly increased the protein level of PP4 in a time-dependent manner, and PD98059, either alone or in combination with cisplatin, resulted in a marked increase of PP4 protein. The role of PP4 in NF-κB activation was further characterized by transfection of PP4 (either sense or antisense; SiHa/PP4 or SiHa/AS-PP4) into SiHa cells. PP4 was constitutively expressed in SiHa/PP4 cells and was barely detectable in SiHa/AS-PP4 cells (data not shown).

The Thr phosphorylation status of NF-κB p65 in these transfected cells was examined. As shown in Fig. 5A, the Thr phosphorylation of NF-κB p65 was undetectable in SiHa/PP4 cells. By contrast, NF-κB p65 was generally Thr-phosphorylated in SiHa/AS-PP4 cells, and cisplatin with or without PD98059 could not decrease the level of Thr phosphorylation of NF-κB p65 (Fig. 5B).

Overexpression of PP4 Increases Basal and Cisplatin-induced NF-κB DNA Binding Activity—Cisplatin-induced NF-κB DNA binding activity in PP4 (sense or antisense) transfectants was examined by EMSA. As expected, SiHa/PP4 cells had higher levels of basal and cisplatin-induced NF-κB activation. By contrast, in SiHa/AS-PP4 cells, cisplatin with or without PD98059 barely activated NF-κB (Fig. 6A). For a direct comparison, the drug-induced NF-κB activation in SiHa, SiHa/PP4, and SiHa/AS-PP4 cells was examined in parallel. As shown in Fig. 6B, SiHa/PP4 cells had the highest basal and drug-induced NF-κB DNA binding activity.

PP4 Does Not Change Cisplatin-induced ERK2 Activation—ERK2 is regulated by kinases and phosphatases (25). Whether PP4 has an effect on the activity of ERK2 was examined. As shown in Fig. 7, the activation of ERK2 by cisplatin was not affected, and PD98059 retained the ability to inhibit cisplatin-induced ERK2 activation in both SiHa/PP4 and SiHa/AS-PP4 cells. In addition, IκBα degradation or NF-κB nuclear translocation was not induced by cisplatin with or without PD98059 in both SiHa/PP4 and SiHa/AS-PP4 cells (data not shown).

PP4 Targets NF-κB p65 on Thr<sup>355</sup>—To identify the Thr residue of NF-κB p65 for PP4 targeting, the possible Thr phosphorylation sites of p65 protein were derived by an Internet available program (NetPhos 2.0, developed by Technical University of Denmark; www.cbs.dtu.dk). Thr<sup>355</sup> was predicted to be the most probable residue for phosphorylation. A SacI/PstI restriction fragment (p65S/P, 921–1411 fragment encoding amino acids 279–444) containing Thr<sup>355</sup> was thus in-frame cloned into a His<sub>6</sub> tag expression vector (pREST; Invitrogen). In addition, Thr<sup>355</sup> substituted with Ala mutant was also constructed in pREST vector. E. coli-expressed p65S/P wild type or mutant proteins were adsorbed by nickel-agarose and then used as bait.
to pull down the associated nuclear proteins. The pull-down complex was then subjected to kinase assay. As shown in Fig. 8A, p65S/P wild type protein was phosphorylated by associated nuclear proteins from untreated SiHa cells. The level of p65S/P phosphorylation decreased in nuclear lysates obtained from cisplatin- or PD98059/cisplatin-treated cells. The p65S/P T435A mutant protein was not phosphorylated under the identical experimental conditions. The nuclear lysates prepared from similarly treated SiHa/PP4 or SiHa/AS-PP4 cells were also examined. As expected, p65S/P proteins were not phosphorylated by the nuclear lysates from SiHa/PP4 cells but were constitutively phosphorylated by the nuclear lysates from SiHa/AS-PP4 cells (Fig. 8B).

P65S/P Protein Interacts with PP4—The interaction between PP4 and NF-κB p65 was examined by Western blotting of p65 immunoprecipitated complex with anti-PP4 antibody. As shown in Fig. 9A, NF-κB p65 was found to physically interact with PP4. To test whether p65S/P is the region response for PP4 interaction, p65S/P wild type or T435A mutant protein coprecipitated complexes were Western blotted with anti-PP4 antibody. As shown in Fig. 9B, p65S/P interacted with PP4, and mutation of T435 did not alter this interaction.

DISCUSSION

We have previously reported that suppression of the MEK/ERK signaling pathway by PD98059 results in cisplatin resistance of the cervical carcinoma cell line, SiHa (23). In this study, we found that inhibition of MEK/ERK enhances cisplatin-induced NF-κB activation via a pathway different from conventional IKK/IκB/NF-κB signaling. We demonstrated that suppression of ERK2 increases the level of a nuclear protein phosphatase, PP4, which decreases the Thr phosphorylation status of NF-κB p65. Specifically, we identified that amino acids 281–444 of NF-κB p65 are responsible for the interaction with PP4 and that Thr^{435} is a PP4 targeting residue, which may play a critical role in NF-κB activity.

Several kinases, including cAMP-dependent protein kinase (9, 11), IKK (9, 26), phosphatidylinositol 3-kinase (27), and...
AS-PP4 cells were used in precipitated complex kinase assay. NF-κB SacI/PstI restriction fragment encoding amino acids 279–444 of NF-κB p65 was in-frame cloned into a His6 tag expression vector. Thr435 substitution with Ala mutant was created by site-directed mutagenesis. E. coli expressed p65SS/P wild type or mutant proteins were adsorbed to nickel-agarose and used as bait to precipitate nuclear proteins from SiHa cells treated with 20 μM cisplatin for 5 h with or without 10 μM PD98059 (PD) pretreatment as indicated. The precipitated complex was subjected to kinase assay. B, with the same experimental design, SiHa/PP4 and SiHa/AS-PP4 cells were used in precipitated complex kinase assay.

Fig. 8. A, p65 Thr435 is a phosphorylation site for PP4 targeting. A SacI/PstI restriction fragment encoding amino acids 279–444 of NF-κB p65 was in-frame cloned into a His6 tag expression vector. Thr435 substitution with Ala mutant was created by site-directed mutagenesis. E. coli expressed p65SS/P wild type or mutant proteins were adsorbed to nickel-agarose and used as bait to precipitate nuclear proteins from SiHa cells treated with 20 μM cisplatin for 5 h with or without 10 μM PD98059 (PD) pretreatment as indicated. The precipitated complex was subjected to kinase assay. B, with the same experimental design, SiHa/PP4 and SiHa/AS-PP4 cells were used in precipitated complex kinase assay.

Fig. 9. A, PP4 directly interacts with NF-κB p65. SiHa or SiHa/PP4 cells were treated as indicated. The NF-κB p65 subunit was immunoprecipitated (IP) from nuclear lysates, then immunoblotted (IB) with anti-F444 antibody, and reprobed with anti-p65 antibody. B, p65SS/P wild type or T435A mutant protein-coprecipitated complex was Western blotted with anti-P4 antibody. The blot was probed with anti-His tag antibody to show the amount of His6 tag p65SS/P protein. PD, PD98059.

casein kinase II (28) have been reported to activate NF-κB by phosphorylation of NF-κB p65. On the other hand, two phosphatases, PP2A and PP4, have been found to dephosphorylate NF-κB p65 (13, 16). Interestingly, although PP2A suppresses NFκB activity, PP4 activates it. These results indicate that the activity of NF-κB is under the control of multiple phosphorylations on different sites of the NF-κB p65 subunit; whereas phosphorylation on some sites activates NF-κB, phosphorylation on other sites attenuates its function.

Although several phosphorylation sites of NF-κB p65, including Ser276 (8), Ser529 (10), and Ser536 (9), have been identified, whether these sites are affected by PP2A and PP4 has not been characterized. In this study, although the precise Ser phosphorylation sites have not been determined, our results clearly demonstrated that cispalatin induced Ser phosphorylation of NF-κB p65, which was not affected by suppression of MEK/ERK. By contrast, the level of Thr phosphorylation of NF-κB p65 decreased to nearly undetectable levels in SiHa/PP4 cells but remained unchanged in SiHa/AS-PP4 cells, suggesting that PP4 was the main phosphatase involved in dephosphorylation of threonine residues of NF-κB p65. Further, based on prediction using Netphos 2.0 software, p65 Thr435 was identified as a phosphorylation site by coprecipitated complex kinase assay and site-directed mutagenesis. Although it cannot be excluded that other Thr residues are also involved in the regulation of NF-κB activity, Thr435 seems to be a major site, because peptide (amino acids 279–444 of p65) containing Thr435 was found to directly interact with PP4. The mechanism by which NF-κB activity is regulated by Ser/Thr phosphorylation remains to be established. It is possible that Ser phosphorylation and Thr dephosphorylation of NF-κB p65 increase the binding affinity of NF-κB with DNA and/or other transcriptional factors.

It is of particular importance to note that ionizing radiation and anticancer drugs, which primarily target DNA, may activate NF-κB via mechanisms different from the conventional IKK/IκBα/NFκB pathway. Several nuclear kinases, such as DNA-PK and ATM, have been reported to activate NF-κB in response to DNA-damaging agents (29, 30). Our results also showed that cispalatin-induced NF-κB activation was accompanied by the increase of Ser phosphorylation of NF-κB p65. However, the inhibitory effect of kinases on NF-κB activity has not been observed, and the kinase responsible for p65Thr435 phosphorylation remains to be identified. It is possible that DNA-damaging agents directly activate pre-existing nuclear NF-κB via nuclear kinases and/or phosphatases. These observations may be important for the understanding of NF-κB-relevant responses of some tumor cells with an intrinsically high level of nuclear expression of NF-κB (31–33). Modulation of the conventional IKK/IκBα/NFκB pathway might have less effect on these tumor cells in response to DNA-damaging agents.

In this study, U0126 did not induce observable NF-κB DNA binding activity; however, there was a slight increase of NF-κB transcriptional activity observed in reporter assay (Fig. 1, B and C). The reason for this discrepancy may be due to differences in the assay methods. EMSA assay detects DNA binding activity at a definite time point; by contrast, the results of reporter gene assay actually represent the accumulation of reporter gene expression for a period of time.

In this study, suppression of ERK2 by either PD98059 or U0126 increased the transcriptional activity of NF-κB by approximately 2-fold (Fig. 1C). Although this increase of NF-κB transcriptional activity seems small, our previous study showed that suppression of ERK2 may increase drug resistance toward cispalatin (23). One possible explanation is that cispalatin activates both apoptotic and anti-apoptotic signals (7, 34, 35), and the overall cytotoxicity of cispalatin is determined by the balance of these two opposite pathways. It is possible that even a small change of the balance of these two forces will alter the sensitivity of cells to cispalatin.

Although PD98059 increased the protein level of PP4 (Fig. 4), it alone did not induce NF-κB p65 Thr dephosphorylation (Fig. 3). Several possible mechanisms may be responsible for
this phenomenon. First, the level of PP4 protein may be insufficient for dephosphorylation of NF-κB p65 threonine residues to occur. Second, cisplatin may change the nuclear environment and increase molecular interaction between PP4 and NF-κB. Finally, cisplatin may induce conformational change of NF-κB and thereby facilitate PP4 targeting. The latter speculation is partly supported by a recent report suggesting that p65 is a target of Pin 1 (36). Additionally, the results of coprecipitated complex kinase assay suggest that p65S/P also interacts with an unknown kinase. The observation of constitutive phosphorylation of p65S/P in SiHa/AS-PP4 cells indicates this kinase was not affected by PD98059 or cisplatin treatment. The molecular interactions among NF-κB and possible kinases and phosphatases remain to be elucidated.

The molecular mechanisms by which suppression of the MEK/ERK signaling pathway leads to enhanced expression of PP4 protein remain to be elucidated. However, the biological significance of this unconventional pathway of NF-κB activation appears to be important in that it might function as an alternative mechanism of stepwise regulation of NF-κB activity that can integrate signals from different stresses.

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