Crucial Role of c-Jun Phosphorylation at Ser63/73 Mediated by PHLPP Protein Degradation in the Cheliensisin A Inhibition of Cell Transformation

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Abstract

Cheliensisin A (Chel A), as a novel styryl-lactone isolated from Goniothalamus cheliensis Hu, has been demonstrated to have an inhibition of EGF-induced C41 cell transformation via stabilizing p53 protein in a Chk1-dependent manner, suggesting its chemopreventive activity in our previous studies. However, its underlying molecular mechanisms have not been fully characterized yet. In the current study, we found that Chel A treatment could increase c-Jun protein phosphorylation and activation, whereas the inhibition of c-Jun phosphorylation, by ectopic expression of a dominant-negative mutant of c-Jun, TAM67, reversed the Chel A inhibition of EGF-induced cell transformation and impaired Chel A induction of p53 protein and apoptosis. Moreover, our results indicated that Chel A treatment led to a PHLPP downregulation by promoting PHLPP protein degradation. We also found that PHLPP could interact with and bind to c-Jun protein, whereas ectopic PHLPP expression blocked c-Jun activation, p53 protein and apoptotic induction by Chel A, and further reversed the Chel A inhibition of EGF-induced cell transformation. With the findings, we have demonstrated that Chel A treatment promotes a PHLPP protein degradation, which can bind to c-Jun and mediates c-Jun phosphorylation, and further leading to p53 protein induction, apoptotic responses, subsequently resulting in cell transformation inhibition and chemopreventive activity of Chel A.

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Introduction

Cheliensisin A (Chel A), as a novel styryl-lactone isolated from Goniothalamus cheliensis Hu, has been reported to possess the potent chemoprevention effect (1–3). Our published studies have demonstrated that chemopreventive activity is mediated by its induction of apoptosis via triggering p53 protein expression and activation (4). The pleckstrin homology domain leucine-rich repeat protein phosphatase (PHLPP), including PHLPP1 and PHLPP2, are protein phosphatases, which have been demonstrated to specifically dephosphorylate the hydrophobic motif of Akt, subsequently triggering apoptosis and suppressing tumor growth (5). However, the following studies have found that PHLPP could also act as a tumor suppressor in several types of cancer due to its ability to block growth factor–induced signaling in cancer cells (5, 6). Most recently, the studies from our group have indicated that PHLPP1 downregulation serves as cell apoptosis controller by promoting p53 protein translation via activation of Akt/p70S6K cascade (7). We found here that PHLPP was downregulated in cells treated with Chel A, which mediated chemopreventive activity of Chel A.

c-Jun, a member of the basic region leucine zipper protein family of transcription factors, in combination with itself or other proteins such as c-Fos, forms the transcription factor activator protein 1 (AP-1). c-Jun protein consists of a C-terminal DNA-binding domain and an N-terminal transactivation domain. The transcriptional activity of c-Jun is increased by phosphorylation of serines 63 and 73 in the transactivation domain (8, 9). c-Jun phosphorylation at Ser 63 and Ser 73 could be mediated by activation of JNKs upon a large variety of external or internal stimulations (10–12) or the inhibition of its phosphatase. However, to the best of our knowledge, phosphatase that targets phosphorylated c-Jun protein has not been identified yet. Upon activation, c-Jun exerts various biologic effects on cell proliferation, differentiation, cellular transformation, and apoptosis (10–12). It has been reported that inhibition of c-Jun activation by expressing a c-Jun...
dominant-negative mutant TAM67 inhibits apoptosis due to survival signal withdrawal (11). In the current study, we revealed that Chel A treatment resulted in PHLP2 protein degradation, which further mediated c-Jun phosphorylation at Ser 63 and 73 through JNK-independent manner. Moreover, we found the downregulation of PHLP2 and its mediated activation of c-Jun were essential for the induction of apoptosis as well as the inhibition of cell transformation induced by EGF.

Materials and Methods

Reagents and plasmids

Chel A was isolated from Goniothalamus cheliensis by the Kunming Institute of Botany, Chinese Academy of Sciences (Kunming, Yunnan, China) as previously described (1, 3). The chemicals cycloheximide and MG132 were purchased from Calbiochem. Luciferase assay substrate and EGF were from Promega. The antibodies specific against c-Jun, c-Jun(D), p-c-Jun Ser63, p-c-Jun Ser73, p-AKT Ser473, p-AKTThr308, AKT, p-Erk1/2, Erk1/2, p-p38, p38, p-JNK1/2, JNK1/2, PARP, cleaved PARP, caspase-3, cleaved caspase-3, p53, p-p53 Ser15, GFP, and GAPDH were purchased from Cell Signaling Technology. HA antibody was obtained from Covance Inc.. Antibodies specific against PHLPP1 and PHLPP2 were purchased from Bethyl Laboratories. Antibodies against β-actin and α-tubulin were bought from Sigma. The plasmid, HA-PHLPP1 and HA-PHLPP2 were from Addgene. The plasmids, AP-1-luciferase reporter, dominant-negative c-Jun–mutant plasmid TAM67, and GFP-c-Jun were used and are described in our previous studies (13–15).

Cell culture and transfection

Normal mouse epidermal Cl41 cells, which have been previously described (4, 16, 17), and their stable transfectants were maintained in 5% FBS Eagle’s minimum essential medium (MEM), supplemented with 1% penicillin/streptomycin and 2 mmol/L glutamine (Life Technologies) at 37°C in 5% CO2 incubator that have been described previously (4, 15, 16). Cl41 cells and their stable transfectants, were mixed with 1 mL of 0.33% agar BMEM (supplemented with 10% FBS and 20 ng/mL EGF, as well as Chel A at indicated concentrations, was layered onto each well of 6-well tissue culture plates. A total of 1 × 10⁴ Cl41 cells, and their stable transfectants, were mixed with 1 mL of 0.33% agar BMEM (supplemented with 10% FBS with or without 20 ng/mL EGF, as well as with or without Chel A), and layered on top of the 0.5% agar layer. The plates were incubated at 37°C in 5% CO2 for 3 weeks. The colonies were then counted under inverse microscopy. Colonies with more than 32 cells were scored. Each experiment was done at least three independent times. The results were presented as colonies/10⁴ seeded cells.

Flow cytometry assay

Flow cytometry assay was conducted as described previously (4, 16, 20). Cl41 cells and their stable transfectants were cultured in 6-well plates until they reached 70% to 80% confluence. Cell culture medium was replaced with 0.1% FBS medium for 36 hours. The cells were then treated with EGF (20 ng/mL) with or without Chel A at indicated concentrations in the medium containing 0.1% FBS. Cells were harvested and fixed in ice-cold 70% ethanol. The cells were stained with propidium iodide (PI) for 15 minutes and then subjected to flow cytometry (Beckman Coulter) for apoptotic analysis.

Western blotting

Cells were cultured using the same method described in flow cytometry assay, followed by pretreated with Chel A for 30 minutes, and afterwards exposed to EGF as indicated. The cells were subsequently washed on ice-cold PBS, and then extracted with lysis buffer (10 mmol/L Tris-HCl, pH 7.4, 1% SDS, 1 mmol/L Na3VO4, and proteasome inhibitor). The cell extracts were subjected to the Western blot analysis and the protein bands specifically bound to antibodies were detected using alkaline phosphatase–linked secondary antibody and ECF Western blotting system as described previously (4, 16).

Reverse transcription PCR

Total RNAs were extracted after treatment for the indicated time periods using TRIzol reagent (Invitrogen). Total cDNAs were synthesized by using oligo (dT) 20 primer by Superscript First-Strand Synthesis system (Invitrogen). PHLPP1, PHLPP2, and β-actin mRNA presented in the cells were determined by semiquantitative reverse transcription (RT)-PCR assay. The primers for mouse

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PHLPP1 (forward: 5'-ACACCGTGTAGT CTCACTCC-3', reverse: 5'-TTCAGTCACGTCTAGCCTCC-3'), mouse PHLPP2 (forward: 5'-AGGTCTCGACATCCTCTTC-3', reverse: 5'-GTCGCGCCCTTACGTGAG-3'), and mouse β-actin (forward: 5'-ATATCCTGCGCTGGTGCTGTC-3', reverse: 5'-AGCAGTCGCGTTAGGGAAC -3') were used to determine the mRNA amount of PHLPP1, PHLPP2, and β-actin, respectively. The results were imagined with Alpha Innotech SP image system (Alpha Innotech Corporation) as described previously (15).

Luciferase assay

Cl41 cells stably transfected with AP-1 luciferase reporter constructs were seeded into 96-well plates and cultured until 70% to 80% confluent. The cells were treated with various concentrations of Chel A in MEM medium containing 0.1% FBS for 12 hours and then lysed for luciferase assay using luciferase substrate as described previously (21, 22).

Immunoprecipitation

Stable transfectants of 293T cells, 293T (GFP-c-Jun, pcDNA3.0), 293T (GFP-c-Jun, HA-PHLPP1), and 293T (GFP-c-Jun, HA-PHLPP2), were cultured in 10-cm dishes until 70% to 80% confluence. Culture medium was replaced with DMEM containing 0.1% FBS for 12 hours, and the cells were then lysed in cell lysis buffer (1% Triton X-100, 150 mmol/L NaCl, 10 mmol/L Tris, pH 7.4, 1 mmol/L EDTA, 1 mmol/L EGTA, 0.2 mmol/L Na3VO4, 0.5% NP-40, and complete protein cocktail inhibitors from Roche) on ice. Lysate was incubated with normal IgG/Protein A/G plus-agarose or anti-GFP agarose (Santa Cruz Biotechnology, Inc.) at 4°C for 12 hours. The agarose beads were collected by centrifugation, followed by being washed three times with cell lysis buffer, and the beads were extracted with Western blot sample buffer and subjected to Western blot assay (23).

Statistical analysis

Student t test was employed to determine the significance of differences between the different groups in each experiment. The differences were considered significant at P < 0.05.

Results

Chel A treatment inhibited EGF-induced cell transformation with induction of c-Jun phosphorylation at Ser63/73 in Cl41 cells

Our most recent studies have indicated that Chel A could act as a chemopreventive agent for inhibition of cell transformation (2, 4). Chel A treatment consistently showed the inhibition of EGF-induced cell transformation in mouse epidermal Cl41 cells in a dose-dependent manner (Fig. 1A and B). AP-1 is a transcription factor, and its inhibition has been reported to be involved in chemopreventive effect in previous studies (15, 24). Therefore, we determined whether Chel A treatment could inhibit AP-1 activation by utilizing Cl41 cells stably transfected with AP-1-luciferase reporter. Unexpectedly, the results indicated that Chel A treatment induced AP-1-dependent transactivation in a dose-dependent manner (Fig. 1C). c-Jun is the most extensively studied protein of AP-1 components and has also been reported to be involved in the regulation of numerous cell activities, such as proliferation, survival, tumorigenesis, and apoptosis (24, 25). The transcriptional activation of c-Jun depends on its phosphorylation at Ser 63 and 73 in the transactivation domain (8, 9, 11). The phosphorylation of c-Jun has also been reported to play a role in the mediation of apoptosis under withdrawal of survival signaling (24). Therefore, the next experiment was carried out to evaluate the effect of Chel A on c-Jun phosphorylation at Ser63/73. As shown in Fig. 1D, Chel A treatment alone induced c-Jun phosphorylation at Ser63/73 in a time-dependent manner with maximum induction at 3 hours after treatment. Moreover, we found that cotreatment of cells with Chel A and EGF increased c-Jun phosphorylation at Ser63/73 (Fig. 1E and F). These results indicated that Chel A treatment led to c-Jun phosphorylation at Ser63/73 and promoted EGF-induced c-Jun phosphorylation in Cl41 cells.

c-Jun phosphorylation at Ser63/73 was crucial for the Chel A inhibition of EGF-induced transformation in Cl41 cells

To evaluate the potential role of c-Jun activation in Chel A inhibition of cell transformation, Cl41 cells stably transfected with dominant-negative N-terminal–truncated mutant of c-Jun, TAM67, and its parental vector control plasmid (Cl41 vector) had been established and well characterized in our previous publications (15, 26, 27). Verification of TAM67 expression in Cl41 stable transfectant cells was indicated in Fig. 2D. Our results revealed that ectopic expression of TAM67 dramatically attenuated the inhibitory effect of Chel A on EGF-induced cell transformation (Fig. 2A and B); this strongly indicates that induction of c-Jun phosphorylation at Ser63/73 is crucial for the chemopreventive activity of Chel A.

c-Jun exerted its chemopreventive effect via upregulation of p53 protein expression and apoptosis

To elucidate the molecular mechanisms underlying c-Jun–mediated chemopreventive activity of Chel A, the flow cytometry assay was used to assess the effect of TAM67 overexpression on cell-cycle progression and apoptotic responses, and western blot assay for p53 protein expression due to Chel A treatment. As shown in Fig. 2C, Chel A treatment led to a marked cell death in Cl41 (vector) cells. Very interestingly, EGF cotreatment slightly increased Chel A–induced cell death, rather than provide a protective effect.
Chel A Inhibits Cell Transformation via Degradation of PHLPP

Figure 1. The inhibition on EGF-induced cell transformation and the induction of c-Jun phosphorylation and AP-1 transactivation by Chel A in Cl41 cells. A and B, Cl41 cells were exposed to indicate concentrations of Chel A in combination with EGF for cell transformation assay in soft agar as described previously (4). The colony formation was photographed (A), and the number of colonies was scored and presented as colonies/10^4 seeded cells (B). *, a significant decrease as compared with that of EGF treatment alone (P < 0.05). Each bar indicates the mean and SD of three independent experiments. C, Cl41 cells (1 x 10^5) stably expressing AP-1-luciferase reporter were seeded into each well of a 96-well plate. After synchronization, cells were cultured in 0.1% FBS medium for 48 hours and then treated with various concentrations of Chel A for 12 hours, and then extracted for determination of luciferase activity as described previously (19, 41). *, a significant increase in AP-1 activity (P < 0.05). Each bar indicates the mean and SD of three independent experiments. D–F, Cl41 cells were seeded into each well of 6-well plates and cultured as described in ref. (4). Then, the cells were treated with EGF and Chel A at different concentrations (E) or for different time periods (D and F). The cell extracts were subjected to Western blotting as described in Materials and Methods. GAPDH and α-tubulin were used as a control for protein loading. The results shown are data represented from three independent experiments.

effect on cell death induced by Chel A in Cl41(vector) cells. Importantly, Chel A–induced cell death was dramatically attenuated by ectopic expression of TAM67 in Cl41 cells (Fig. 2C), suggesting that c-Jun activation was crucial for Chel A–induced cell death. Consistently, the results obtained from flow cytometry also showed that sub-G1 DNA content (cell death peak) increased significantly upon Chel A treatment in Cl41 vector cells, and stable expression of TAM67 abolished Chel A–induced increase in sub-G1 cells (Fig. 2E). Moreover, our results indicated that TAM67 abrogated Chel A–induced cleaved PARP and caspase-3 as demonstrated in Western blot analysis (Fig. 2F and G), clearly revealing that Chel A–induced cell death was an apoptotic response. As our recent studies have demonstrated that p53 protein induction was essential for Chel A–induced apoptotic responses (4), we assess the relationship between c-Jun activation and p53 protein expression by comparison of p53 protein expression due to Chel A treatment between Cl41 (vector) and Cl41 (TAM67) transfectants. As indicated in Fig. 2D, the inhibition of c-Jun activation by ectopic expression of TAM67 blocked p53 protein expression in comparison with that observed in Cl41 vector cells, demonstrating that c-Jun activation mediated p53 protein expression.

Chel A treatment promoted PHLPP protein degradation and such PHLPP protein degradation mediated c-Jun phosphorylation at Ser63 and Ser73 due to Chel A treatment

MAPK, including the ERK1/2, INK, and p38 have been reported to be responsible for the regulation of various cell functions in different experimental systems (28). Activated MAPK causes phosphorylation and activation of transcription factors in the cytoplasm and/or nucleus (29). c-Jun has been reported as one of the most important transcription factors that can be phosphorylated at Ser63/73 and activated by MAPKs, especially JNKs. Thus, to elucidate the mechanism underlying c-Jun phosphorylation upon Chel A treatment, we first examined whether Chel A treatment could induce the activation of MAPKs. The results showed
colonies were scored and presented as colonies per 10,000 seeded cells (B). Cl41 vector cells was determined in soft agar assays. The colony formation was observed under inverted microscope and photographed (A). The numbers of

that Chel A treatment alone only induced a slight increase in the activation of ERKs, p38, and JNKs at high dose (4 μmol/L) and did not promote kinase activation in dose–response studies (Fig. 3A and B). In contrast, to increase cell death in cotreatment of Cl41 cells with Chel A and EGF, as compared with treatment of cells with either one alone, Chel A cotreatment of cells with EGF slightly inhibited EGF–induced activation of ERKs and JNKs 15 minutes after treatment, as compared with EGF treatment alone (Fig. 3C). Thus, the results obtained from determination of ERKs, p38, and JNKs activation were completely inconsistent with c-Jun phosphorylation at Ser63/73 (Fig. 1D–F), suggesting that MAPK activations were not major mediators responsible for c-Jun activation by Chel A treatment. Thus, we anticipated that phosphatases might play a major role in c-Jun phosphorylation following Chel A treatment.

The recent discovery of the PHLPP Ser/Thr phosphatases added a new player to the cast of phosphate-controlling enzymes in cell signaling responses (30). PHLPPs, consisting of PHLPP1 and PHLPP2, catalyzes the dephosphorylation of a conserved regulatory motif, (the hydrophobic motif) on the AGC kinases Akt, PKC, and S6 kinase, as well
as an inhibitory site on the kinase Mst1, to inhibit cellular proliferation and induce apoptosis (30). Our most recent studies demonstrated that PHLPP1 inhibited cell apoptosis via downregulation of p53 translation (7). Hence, we first determined the effect of Chel A on PHLPP protein expression in Cl41 cells. The results showed that Chel A treatment markedly attenuated the expression of PHLPPs in a time-dependent manner (Fig. 3A). To elucidate the mechanisms underlying Chel A downregulation of PHLPPs protein expression, we first examined the effect of Chel A on phlpp1 and phlpp2 mRNA levels by RT-PCR assay. The results showed that Chel A treatment had no observable effect on mRNA levels of either phlpp1 or phlpp2 (Fig. 3D), excluding the possibility of Chel A affecting phlpp gene transcription or mRNA stability. Thus, we further assessed the potential effect of Chel A on PHLPPs protein degradation. To test whether Chel A treatment was able to promote PHLPPs protein degradation, Cl41 cells were first pretreated with proteasome inhibitor MG132 to accumulate PHLPPs protein. The MG132 was then removed from cell culture medium and the protein synthesis inhibitor cycloheximide was added to the cells alone or in combination with Chel A. The effect of Chel A on the dynamics of PHLPP protein degradation was determined during the indicated time periods. As shown in Fig. 3E, the PHLPP1 and PHLPP2 protein degradation rates were markedly increased when cells were coincubated with Chel A plus cycloheximide in comparison with the cells incubated with cycloheximide alone. Those results strongly indicated that Chel A treatment decreased PHLPP expression via promoting PHLPP protein degradation.

To evaluate the role of PHLPPs protein degradation in c-Jun activation upon Chel A treatment, we tested whether the overexpression of HA-PHLPP proteins could regulate c-Jun phosphorylation at Ser63/73 in Cl41 cells. The results revealed the c-Jun protein phosphorylation at Ser63/73 was abolished upon ectopic expression of PHLPP1 in either transfectants of Cl41 HA-PHLPP1, Cl41 HA-PHLPP2, or HCT116 HA-PHLPP2 (Fig. 4A). To further buttress this notion, Chel A–induced c-Jun phosphorylation was compared among the transfectants of Cl41 Vector, Cl41 HA-PHLPP1, and Cl41 HA-PHLPP2. As anticipated, either overexpression of HA-PHLPP1 or HA-PHLPP2 blocked c-Jun phosphorylation at Ser63/73 due to Chel A treatment as comparison with Cl41 Vector cells (Fig. 4B). It was noted that basal level of c-Jun phosphorylation at Ser63/73 was not inhibited in Cl41 HA-PHLPP1 and HA-PHLPP2 transfectants as compared with Cl41 (vector) transfectant (Fig. 4B), which is different with the results observed in Fig. 4A. This could be caused by various cell culture conditions in two experiments. The cell culture medium used in Fig. 4A is 5% FBS MEM, whereas the cell culture medium used for experiment shown in Fig. 5B is 1% FBS MEM. It is well known that low serum of cell culture causes cell stress responses. We therefore anticipated that low concentration of serum for experiment shown in Fig. 4B might cause cell stress, which could increase the basal level of c-Jun phosphorylation through activation of stress kinases, such as JNks. These results demonstrated that PHLPP1/2 could repress c-Jun phosphorylation at Ser63/73, which might be responsible for the effect of Chel A on apoptosis and cell transformation in Cl41 cells.
PHLPP protein degradation contributed to apoptotic response, p53 induction, and the inhibition of EGF-induced cell transformation by Chel A treatment

To evaluate whether PHLPP downregulation contributed to Chel A–induced apoptotic induction, Cl41 HA-PHLPP1 and Cl41 HA-PHLPP2, Cl41 vector were treated with indicated Chel A. The cultured cells showed a declined tendency to apoptosis by Chel A, B, Cl41 HA-PHLPP1 cells, Cl41 HA-PHLPP2 cells, and Cl41 vector cells were treated with Chel A for indicated concentrations and the cell extracts were applied to Western blotting to determine the expression and cleavages of PARP and caspase-3, p53, and phosphorylation at Ser15. β-Actin was used as a protein loading control. The result represents one of three independent experiments. C and D, effect of Chel A on EGF–induced cell transformation in Cl41 HA-PHLPP1 cells, Cl41 HA-PHLPP2 cells, and Cl41 vector cells was determined by soft agar assay. The colony formation was observed under inverted microscope and photographed (C). The numbers of colonies were scored and presented as colonies per 10,000 seeded cells (D). ^ {**} a significant increase in Cl41 HA-PHLPP1 cells and Cl41 HA-PHLPP2 cells as compared with those in Cl41 vector cells in response to Chel A (P < 0.05). Each bar indicates the mean and SD from three independent experiments.
PHLPP degradation caused by Chel A treatment was essential for c-Jun phosphorylation, p53 protein expression, and apoptosis, which in turn led to the inhibition of EGF-induced cell transformation in Cl41 cells.

**PHLPPs interacted with c-Jun and mediated its phosphorylation**

PHLPP1 and PHLPP2 act as the phosphatases, and have been reported to exert the function through binding with their substrates (such as AKT and PKC), and subsequently dephosphorylating those targeted proteins. Our abovementioned results revealed that the PHLPPs and c-Jun protein phosphorylation at Ser63/73 was inversely correlated in Cl41 cells after Chel A treatment. Moreover, ectopic expression of either PHLPP1 or PHLPP2 could abolish the c-Jun protein phosphorylation at Ser63/73. Those results prompted us to test whether PHLPP proteins could interact with c-Jun protein. To test this possibility, 293T cells were cotransfected with GFP-c-Jun, GFP-c-Jun plus HA-PHLPP1, or GFP-c-Jun plus HA-PHLPP2, respectively, and coimmunoprecipitation was performed to pull down GFP-c-Jun using specific anti-GFP antibodies. The stable 293T GFP-c-Jun/PHLPP1 transfectants were selected and identified as shown in Fig. 6A. The HA-PHLPP1 was observed in the immunocomplex pull down with specific anti-GFP antibodies, suggesting that PHLPP1 protein did interact with c-Jun protein in the intact cells. Similarly, the binding of GFP-c-Jun with PHLPP2 protein was also observed in transfectants of GFP-c-Jun and HA-PHLPP2 (Fig. 6C). To determine whether Chel A could regulate the interaction of c-Jun with PHLPP, the stable Cl41 GFP-c-Jun/PHLPP1 transfectants and Cl41 GFP-c-Jun/PHLPP2 were treated with Chel A and the cell

**Figure 6.** PHLPPs were responsible for c-Jun phosphorylation via interaction with c-Jun. A, 293T cells were cotransfected with GFP-c-Jun, along with HA-PHLPP1, and the cell extracts were applied to Western blotting for determination of the protein expressions using specific antibodies. B and C, coimmunoprecipitation was performed with anti-GFP antibody–conjugated agarose beads. Immunoprecipitates were then subjected to immunoblotting for detection of PHLPP1 and PHLPP2 using HA antibody. D, Cl41 cells cotransfected with GFP-c-Jun, along with HA-PHLPP1 or HA-PHLPP2, were treated with or without Chel A (4 μmol/L) for 6 hours, the cell extracts were used for coimmunoprecipitation by using anti-GFP antibody–conjugated agarose beads. Immunoprecipitates were then subjected to immunoblotting for detection of various protein expressions as indicated. E, a model for Chel A–inhibited EGF-induced cell transformation by inducing apoptosis in JB6 Cl41 cells: Chel A treatment reduced the protein level of PHLPPs which results in upregulating the activation of c-Jun and promoting p53 activation and accumulation, apoptosis, and inhibiting EGF-induced cell transformation.
induced cell transformation. The results showed that although co-immunoprecipitated HA-PHLPP1 or HA-PHLPP2 protein by pull-down assay with anti-GFP antibodies was decreased in Chel A–treated cells as compared with vehicle-treated cells, the decreased levels were consistent with the downregulated protein levels of HA-PHLPP1 and HA-PHLPP2 upon Chel A treatment (Fig. 6D). This result revealed that Chel A only inhibited HA-PHLPP protein expression, while it did not affect the binding of GFP-c-Jun with either HA-PHLPP1 or HA-PHLPP2. Thus, our studies demonstrated that Chel A treatment resulted in PHLPP protein degradation, which reduced PHLPP protein interaction with c-Jun, and in turn led to the upregulation of c-Jun protein phosphorylation, p53 protein induction, apoptotic responses, as well as the inhibition of EGF-induced cell transformation.

Discussion

Defects in apoptosis underpin both tumorigenesis and drug resistance, and most anticancer drugs exert their chemotherapeutic effect by inducing tumor cell apoptosis (31). Chel A exhibits a potent cytotoxicity in HL-60 cells, and is capable of inducing apoptosis of leukemia cells by down-regulation of Bcl-2 expression (2). In addition, our recent studies have found that Chel A exerts an inhibitory effect on EGF-induced cell transformation with the induction of apoptosis in CI41 cells through stabilization and activation of p53 (4). In the current study, we showed that Chel A significantly inhibited EGF-induced cell transformation accompanied with the induction of c-Jun protein phosphorylation at Ser63/Ser73 and PHLPP protein downregulation. Further studies showed that ectopic expression of dominant-negative c-Jun–mutant TAM67 attenuated Chel A–induced p53 protein expression, apoptotic induction, and the inhibition of cell transformation; these indicated that c-Jun phosphorylation and activation was the upstream mediator for p53 protein expression. Moreover, we found that PHLPP protein could bind to and interact with c-Jun protein and attenuated c-Jun protein phosphorylation. In addition, we revealed that Chel A treatment could induce PHLPP1 and PHLPP2 protein degradation, which further reduced the PHLPP protein interaction with c-Jun protein, resulting in the upregulation of c-Jun protein phosphorylation. Therefore, we identify a novel molecular mechanism underlying Chel A as a chemopreventive agent by activating PHLPPs/c-Jun/p53 apoptotic axis as shown in Fig. 6E.

p53 is a tumor suppressor that has a crucial role in the inhibition of cancer development (32). One of biologic functions of p53 is to trigger cell apoptosis (33–35). Our previous studies have strongly indicated that Chel A treatment leads to p53 protein accumulation and activation by preventing p53 proteins from degradation (4). c-Jun is a key member of the AP-1 family of transcription factors which bind to AP-1 elements in their target genes (36), and c-Jun/AP-1 activation is involved in the regulation of numerous cell biologic activities, such as proliferation, survival, tumor-igenesis, and apoptosis (24, 25). c-Jun appears to be both a positive and a negative regulator of apoptosis (36). The exact function of c-Jun is likely to be cell type- and stimulus-specific. Eferl and colleagues analyzed the antiapoptotic function of c-Jun using a cell culture system and found that c-Jun–deficient hepatocytes are more sensitive to TNFα-induced apoptosis and that this sensitization was rescued upon p53 deficiency, showing that c-Jun–deficient liver tumors accumulate high levels of p53 protein (37). However, elevated levels of p53 could not be detected in c-Jun–deficient hepatocytes. Our current studies found that Chel A–initiated c-Jun phosphorylation was crucial for this p53 protein accumulation and activation. It has been reported that phosphorylated c-Jun has the ability to bind to p53 promoter for the induction of p53 transcriptional activity (38). Our recent studies, however, have shown that Chel A treatment upregulates p53 expression by decreasing protein degradation level rather than transcription or mRNA stability (4). Although the detailed mechanism involved in c-Jun regulation of p53 protein expression is undergoing investigation in our group, the phosphorylation of c-Jun is a crucial factor for activation of p53 by Chel A.

PHLPP1 and PHLPP2 are the phosphatases that directly dephosphorylate Akt, promote apoptosis, and suppress tumor growth (5). PHLPP acts as a tumor suppressor in several types of cancer due to its ability to block growth factor–induced signaling in cancer cells (5, 6). However, our most recent studies have indicated that PHLPP1 down-regulation by miR-190 in arsenite responses could mediate apoptosis via promotion of p53 translation via upregulation of Akt/p70S6K pathway (7). Our current results showed that Chel A treatment promoted PHLPP protein degradation, leading to an increase in c-Jun protein phosphorylation at Ser63/73, and in turn resulting in p53 protein induction and further leading to apoptosis and inhibition of cell transformation. Given only a limited number of Ser/Thr phosphatases to balance the actions of more than 400 Ser/Thr kinases in human cells (39), PHLPPs, a family of Ser/Thr phosphatases with multiple regulatory domains, are predicted to have multiple targets in addition to Akt and PKC that have been identified. On the basis of previous studies, PHLPPs are critical for tumor suppression, whereas their loss can activate p53, leading to cellular senescence and apoptosis (40). Nevertheless, our studies showed that PHLPPs interacted with c-Jun, which inhibited c-Jun phosphorylation at Ser63/73, p53 protein expression and activation, as well as apoptosis. Very importantly, we found that ectopic expression of HA-PHLPP1 or HA-PHLPP2 could almost completely impair c-Jun phosphorylation at Ser63/73, p53 protein expression, and apoptosis, as well as reverse EGF-induced cell transformation in CI41 cells, which strongly demonstrated that Chel A–induced apoptosis via PHLPPs/c-Jun/p53 was crucial for the chemopreventive activity of Chel A. In addition, our results revealed that PHLPPs might act as phosphatases of c-Jun, which is currently under investigation in our group.

In summary, our current studies elucidated a novel effect of Chel A in regulation of PHLPP protein degradation. We
also found that PHLPP protein degradation could decrease the interaction of PHLPP protein with c-Jun protein and resulting in an increase in c-Jun phosphorylation at Ser-63/Ser-73, further promoting p53 protein expression, apoptosis, which inhibited EGF-induced cell transformation. Those results provide a new mechanistic insight into the understanding chemopreventive effect of Chel A as a cancer chemopreventive agent.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: J. Zhang, H. Huang, J. Gao, Q. Zhao
Development of methodology: J. Zhang, X. Deng
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Zhu, J. Li, H. Jin, Y. Li, X. Deng, C. Huang

References


Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): J. Zhang, H. Huang, Y. Yu, Y. Li, X. Deng, Q. Zhao, C. Huang

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