Suppression of AP-1 Activity by Cycloprodigiosin Hydrochloride

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Cycloprodigiosin hydrochloride (cPrG·HCl), a compound isolated from a marine bacterium, acts as an immunosuppressant and an anti-cancer drug. We have previously reported that cPrG·HCl suppressed the transcriptional activation of nuclear factor (NF)-κB. Here we studied the effect of cPrG·HCl on activation of another transcription factor, activator protein 1 (AP-1). cPrG·HCl potently suppressed AP-1 activity induced by tumor necrosis factor (TNF) α and phorbol myristate acetate (PMA). cPrG·HCl did not inhibit any of the mitogen-activated protein kinase (MAPK) families, whereas it did suppress transcriptional activation of AP-1 induced by constitutively activated mutants of MEKK1 or Ras. cPrG·HCl inhibited neither TNFα- or PMA-induced DNA-binding of AP-1 nor co-activator p300-induced activation of AP-1. Taken together, cPrG·HCl suppresses AP-1-dependent gene expression downstream of MAPK group through the inhibition of the transcription activation step of the AP-1 promoter complex.

Key words prodigiosin; activator protein 1; nuclear factor (NF)-κB; mitogen-activated protein kinase

Activator protein 1 (AP-1) is involved in cellular proliferation and transformation, and is a possible molecular target of chemotherapy for cancer and inflammatory diseases.[1)] AP-1 is a transcriptional factor comprising members of the Fos family (c-Fos, FosB, Fra-1, and Fra-2) and the Jun family (c-Jun, JunB, and JunD).[2)] The activation of c-Jun NH_2-terminal protein kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 are activated by growth factors and serum, which then induces transcriptional activation of AP-1.[3)]

cPrG·HCl, a member of the prodigiosin family, is a red pigment produced by the marine bacterium *Pseudoalteromonas denitrificans*.[4)] cPrG·HCl as well as other prodigiosins act as H^+/Cl^- symporters, which in turn may induce acidification of the cytosol,[5)] since Cl^- ions are generally less abundant in the cytosol than in the extracellular milieu. It has been demonstrated that cPrG·HCl induces apoptosis in cancer cells concomitantly with intracellular acidification.[6)] However, we previously demonstrated that cPrG·HCl together with TNFα induced apoptosis in cancer cells through suppression of NF-κB, but not by H^+/Cl^- symporter activity-mediated intracellular acidification.[7)]

Here we report that cPrG·HCl also suppresses the activation of AP-1 without affecting the upstream MAPK group; the suppression was not related to H^+/Cl^- symporter activity.

MATERIALS AND METHODS

Cells HeLa cells, U373 cells, and 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 50 μg/ml kanamycin.

Plasmid and Materials The AP-1 and NF-κB reporter plasmids were obtained from Clontech Laboratories Inc. A Renilla-luciferase expression vector, pBKRLuc, was obtained from Toyobo. pIL-8 luc was constructed, which encoded the human IL-8 promoter region (−390 to +48) upstream of a luciferase gene in reporter vector pGL2 (Promega). pcDNA-F-MEKK1 DA and pEFBOS-Ha-RasV12 expressed constitutively activated MEKK1 and Ha-Ras, respectively. The anti-phospho-p38 was obtained from New England BioLabs. The anti-p38, anti-ERK1, and anti-JNK antibodies were obtained from Santa Cruz. cPrG·HCl was prepared described previously.[4)] PMA was obtained from Sigma Aldrich. Recombinant human TNFα was obtained from Upstate Biotechnology.

Luciferase Gene Reporter Assay Cells were plated in 24-well plates, and transfected with the indicated reporter plasmid (0.05 μg) and control reporter plasmid pBKRLuc (0.05 μg) together with or without Ha-RasV12, MEKK1-DA, and p300 expression vector (0.2 μg) using Lipofectin (GIBCO BRL). After 24—48 h, the luciferase activity and Renilla-luciferase activity were measured using the dual-luciferase reporter assay system (Promega) and Luminometer (EG&BERTHOLD). The luciferase activity was normalized to the Renilla-luciferase activity.

***In Vitro* Kinase Assay and Western Blot Analysis** The cells were solubilized in ice-cold buffer (20 mM Tris pH 7.4, 10 mM EGTA, 10 mM $MgCl_2$, 1 mM benzamidine, 60 mM β-glycerophosphate, 1 mM Na_3VO_4, 20 mM NaF, 1 mM DTT, protease inhibitor cocktail, 1% Triton X-100) and then centrifuged at 15000 rpm for 20 min. The supernatant was used as the cell extract. The extracts were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to an ECL membrane (Amersham Pharmacia). Western blot analysis was performed using a Western Blotting Detection System (Amersham Pharmacia) according to the manufacturer's instructions.

For, *in vitro* kinase assay, JNK or ERK-1 was recovered from the cell extract by immunoprecipitation, and then the precipitated complexes were incubated in 10 μl of a reaction mixture (20 mM HEPES pH 7.4, 10 mM $MgCl_2$, 50 mM NaCl, 100 mM Na_3VO_4, 20 mM β-glycerophosphate, 1 mM DTT, 100 μM ATP, 0.05 μCi [γ-^{32}P]-ATP, 5 μg of GST-c-Jun or pu-

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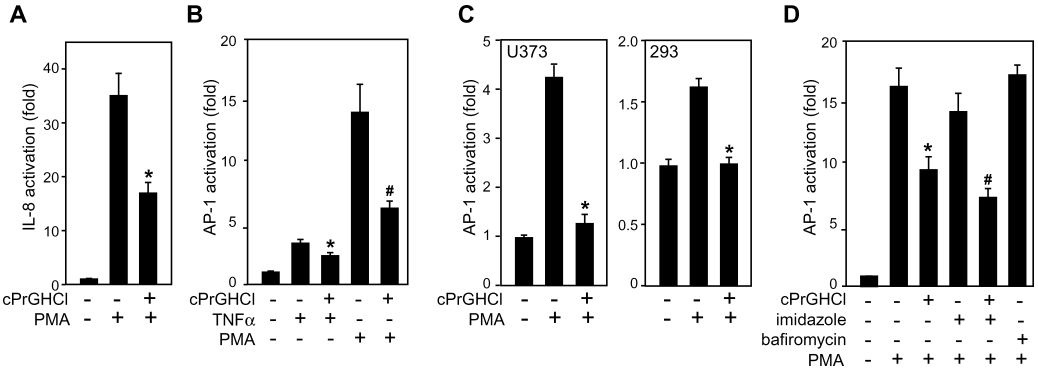

Fig. 1. cPrG·HCl Suppresses TNFα- or PMA-Induced Activation of AP-1

(A) HeLa cells were cotransfected with pIL-8 Luc and pBKRLuc (internal control). After 24 h of transfection, the cells were pretreated with cPrG·HCl (1 μM) for 1 h and were then stimulated by PMA (1 μg/ml) for 5 h. Bars show the S.D. of three independent assays. $*p<0.01$ *versus* PMA-treated cells without cPrG·HCl. (B) HeLa cells were cotransfected with pAP-1 Luc and pBKRLuc. After 24 h of transfection, the cells were pretreated with cPrG·HCl for 1 h and were then stimulated by TNFα (20 ng/ml) or PMA (1 μg/ml) for 5 h. $*p<0.01$ *versus* TNFα-treated cells without cPrG·HCl. $^{\#}p<0.01$ *versus* PMA-treated cells without cPrG·HCl. (C) U373 cells and 293 cells were transfected with pAP-1 Luc and pBKRLuc (internal control). After 24 h of transfection, the cells were pretreated with cPrG·HCl for 1 h and were then stimulated by PMA for 5 h. $*p<0.01$ *versus* PMA-treated cells without cPrG·HCl. (D) HeLa cells were transfected with pAP-1 Luc and pBKRLuc. After 24 h of transfection, the cells were pretreated with cPrG·HCl in the presence or absence of imidazole (10 mM) for 1 h and were then stimulated by PMA for 5 h. $*p<0.01$ *versus* PMA-treated cells without cPrG·HCl. $^{\#}p<0.01$ *versus* PMA and imidazole-treated cells without cPrG·HCl.

rified myelin basic protein [MBP] as a substrate) at 30 °C for 20 min. After SDS-PAGE, the phosphorylation of GST-c-Jun or MBP was measured by autoradiography.

Electrophoretic Mobility Shift Assay Nuclear extracts were prepared and electrophoretic mobility shift assay (EMSA) was performed as described previously.[7] Briefly, cells were solubilized with a buffer (10 mM HEPES pH 7.2, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.4% NP-40, protease inhibitor cocktail, 1 mM DTT) and then centrifuged at 10000 rpm for 15 min. The pellets were resuspended in a buffer (20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, protease inhibitor cocktail, 1 mM DTT) and then centrifuged at 15000 rpm for 15 min. The supernatant was used as the nuclear extract. ^{32}P-labeled DNA probes of the AP-1 binding site (underlined), AGCTTCAGAGGGGACTTTCCGAGAGGTCGA, were prepared as described previously.[7] The nuclear extract (10 μg of protein) was incubated with the ^{32}P-labeled probes (100000 cpm) in 20 μl of buffer (20 mM HEPES pH 7.9, 5% glycerol, 1 mM EDTA, 100 μg/ml poly dI-dC) for 20 min at room temperature. The samples were subjected to 8% polyacrylamide gel electrophoresis and exposed to an imaging plate (Fuji Film).

RESULTS AND DISCUSSION

IL-8 is an inflammatory cytokine that contributes to tumor progression.[8] To examine the mechanism of immunosuppression or cell death induced by cPrG·HCl, we initially analyzed the effect of cPrG·HCl on IL-8 promoter, which includes the binding sites for AP-1 and NF-κB. The luciferase reporter gene assay revealed that IL-8 promoter activity was enhanced 35-fold by PMA and remarkably reduced by the addition of cPrG·HCl (Fig. 1A). Because we previously reported that cPrG·HCl suppressed NF-κB activation,[7] we further examined whether cPrG·HCl suppressed AP-1 using an AP-1-dependent reporter gene. As shown in Fig. 1B, cPrG·HCl suppressed both TNFα- and PMA-induced AP-1-dependent transcriptional activities in HeLa cells. cPrG·HCl also suppressed PMA-induced AP-1-dependent transcriptional activity in other cell lines such as U373 and 293 cells

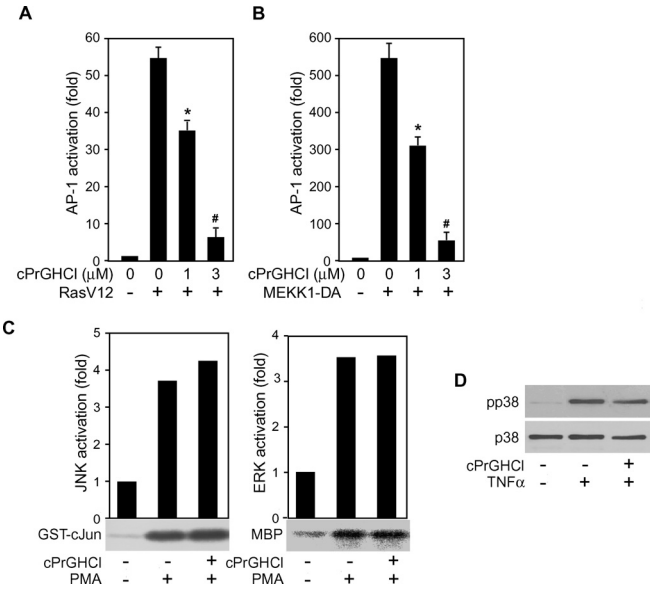

Fig. 2. cPrG·HCl Suppresses the AP-1-Dependent Gene Expression Induced by Ras, MEKK1

(A), (B) HeLa cells were transfected with expression vector of Ha-RasV12 (A) or MEKK1-DA (B) together with the reporter plasmid pAP-1 Luc and pBKRLuc. After 24 h of transfection, the cells were cultured in a medium supplied with cPrG·HCl at the indicated concentration for 7 h. Bars show the S.D. of three independent assays. $*p<0.01$ *versus* RasV12- or MEKK1-DA-expressing cells without cPrG·HCl. $^{\#}p<0.01$ *versus* RasV12- or MEKK1-DA-expressing cells with cPrG·HCl (1 μM). (C) HeLa cells were pretreated with cPrG·HCl for 1 h and were then stimulated by PMA for 15 min. The JNK (left) or ERK1 (right) was recovered by immunoprecipitation, and then subjected to *in vitro* kinase assay with GST-c-Jun (left) or MBP (right) as the substrate. The phosphorylation of GST-c-Jun or MBP was detected (bottom) and the kinase activity was estimated (upper) by Imaging analyzer. (D) HeLa cells were pretreated with cPrG·HCl for 1 h and were then stimulated by TNFα for 15 min. The phosphorylation of p38-MAPK was analyzed by Western blotting analysis using anti-phospho p38 antibody.

(Fig. 1C). Because cPrG·HCl caused intracellular acidification by its intrinsic H^+/Cl^- symporter activity,[6] we examined whether intracellular acidification contributed to cPrG·HCl-mediated suppression of AP-1-dependent transcriptional activity. Imidazole, a cell-permeable weak base, had no effect on cPrG·HCl-mediated suppression of AP-1-dependent transcriptional activity (Fig. 1D). Bafilomycin-mediated intracel-

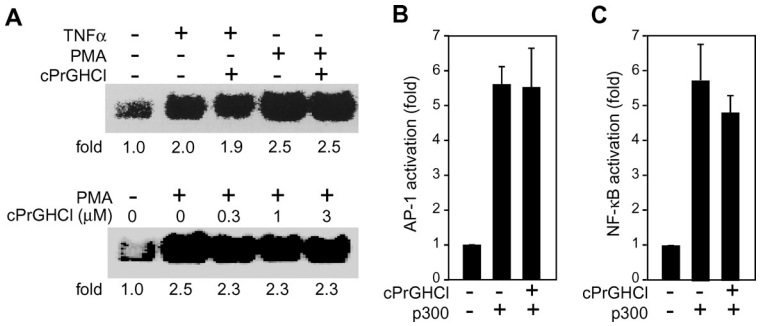

Fig. 3. cPrG·HCl Suppresses Dominant Active form RasV12- and MEKK1-Induced AP-1 Transcriptional Activity

(A) Hela cells were pretreated with cPrG·HCl at 1 μM (upper) or the indicated concentration (bottom) for 1 h and were then stimulated by TNFα or PMA for 30 min. DNA binding activity of AP-1 in the nuclear extract was analyzed by EMSA. Densitometry analysis of the EMSA gel was performed using NIH Image analysis program. (B), (C) HeLa cells were transfected with expression vector of p300 together with pAP-1 Luc (B) or NF-κB Luc (C) and pBKRLuc. After 24 h of transfection, the cells were cultured with cPrG·HCl (1 μM) for 7 h. Bars show the S.D. of three independent assays.

lular acidification through vacuolar-type ATPase inhibition also had no effect on PMA-induced AP-1-dependent transcriptional activity (Fig. 1D). Thus, the suppression of AP-1-dependent transcriptional activity by cPrG·HCl was not related to the intracellular acidification through its own intrinsic H^+/Cl^- symporter activity.

We next evaluated the effect of cPrG·HCl on constitutive AP-1-dependent transcriptional activity by introducing a constitutively activated form of Ras or MEKK. As shown in Fig. 2A, AP-1-dependent transcriptional activity was constitutively elevated more than 50-fold in cells expressing RasV12, where the addition of cPrG·HCl compromised AP-1-dependent transcriptional activity in a dose-dependent manner. Similarly, cPrG·HCl suppressed the markedly elevated AP-1-dependent gene expression in cells expressing constitutively activated MEKK1 (Fig. 2B). Because TNFα or PMA activated AP-1 through the activation of JNK and p38,[3] we examined the possible effect of cPrG·HCl on these kinases. However, cPrG·HCl did not inhibit PMA-induced activation of JNK, ERK (Fig. 2C), or TNFα-induced activation of p38 (Fig. 2D), indicating that cPrG·HCl suppressed downstream of the MAPK group to attenuate AP-1-dependent transcriptional activity.

Therefore, we next examined the effect of cPrG·HCl on the DNA binding of AP-1. Nuclear extracts from cells stimulated with TNFα or PMA in the presence or absence of cPrG·HCl were subjected to EMSA assay. cPrG·HCl had no effect on TNFα- or PMA-induced DNA binding activity of AP-1 (Fig. 3A), indicating that cPrG·HCl could work for DNA-bound AP-1 to suppress its transcriptional activity. As histon acetylation was a general regulation in transcriptional machinery, we examined whether cPrG·HCl disturbed histon acetyl-transferase-mediated AP-1-dependent transcriptional regulation after its DNA binding. However, cPrG·HCl did not affect AP-1-dependent transcriptional activity induced by ectopic expression of p300 (Fig. 3B). cPrG·HCl also had no effect on NF-κB activity induced by p300 (Fig. 3C). In the case of ectopic expression of another coactivator, CREB-binding protein (CBP), the AP-1 or NF-κB activity was not affected by cPrG·HCl (data not shown). These results showed that cPrG·HCl did not suppress p300/CBP function. It may be possible that cPrG·HCl inhibits recruitment of other coactivator or co-factor.

It has been reported that AP-1 as well as NF-κB is constitutively activated in many tumor cells.[9—11] AP-1 and NF-κB collaborate to induce gene expressions for IL-6, IL-8, uPA, and MMP9, which promote tumor proliferation or immune system diseases.[12] Therefore, anticancer therapeutics based on suppression of both AP-1 and NF-κB would be useful for extending efficacy and spectrum. cPrG·HCl as well as other prodigiosin members act as anticancer and immunosuppressant agents. Among the members, GX 15-070, a synthetic prodigiosin derivative, is in phaseI/II clinical trial as an anticancer treatment.[13] Undecyl prodigiosin analog PNU150804 also inhibits IL-2-induced but not PMA-induced AP-1 activity in human lymphocytes through attenuated DNA binding activity.[14] We showed that cPrG·HCl suppressed TNFα-, PMA-, or oncogenic-Ras-induced AP-1 activity (Figs. 1B, 2A) and remarkably reduced IL-8 promoter activity (Fig. 1A). Our results suggest that cPrG·HCl has advantageous characteristics as a possible anticancer agent among other prodigiosin compounds.

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