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MAP Kinase-Independent Induction of Proto-oncogene *c-fos* mRNA by Hemin in Human Cells

Yoshiro Masuya,*,† Isamu Kameshita,‡ Hitoshi Fujisawa,‡ Hirao Kohno,†
Koshiro Hioki,* Rikio Tokunaga,† and Shigeru Taketani†,[1]

**Second Department of Surgery and †Department of Hygiene, Kansai Medical University, Moriguchi, Osaka 570-8506, Japan; and ‡Department of Biochemistry, Asahikawa Medical College, Asahikawa 078-8510, Japan*

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Treatment of HeLa cells or human skin fibroblast cells with hemin led to a time- and dose-dependent rapid induction of *c-fos* mRNA. This induction was absent in the cells treated with actinomycin D, indicating that the *c-fos* induction by hemin occurs at the level of transcription. Metalloporphyrins, including zinc-, cobalt-, and tin-protoporphyrin, ferric ion, and protoporphyrin also induced *c-fos* mRNA. Transient reporter assay with the reporter constructs of the human *c-fos* gene promoter up to −404 bp connected to the luciferase gene showed high activity but no induction by hemin, suggesting that *cis*-acting elements, including the serum response element located about −310 bp upstream of the human *c-fos* gene promoter, may not contribute to the heme-dependent induction. With in-gel assay of protein kinases, the activity of the mitogen-activated protein (MAP) kinases such as extracellular signal-regulated kinase ½ or p38 MAP kinase in hemin-treated HeLa cells was not stimulated. Stimulation of c-Jun *N*-terminal kinase by hemin was nil. Furthermore, PD58059 and SB203580, inhibitors for MAP kinases, did not affect the hemin-dependent *c-fos* induction. Of the inhibitors for protein kinases so far tested, KN-62, a specific inhibitor for calmodulin-dependent protein kinase II (CaMK II), inhibited the induction of *c-fos* mRNA by hemin. Phosphorylation of CaMK II in hemin-treated cells increased. With gel mobility assay, the DNA AP-1 binding activity transiently increased when treating HeLa cells with hemin. Therefore, induction of *c-fos* led to an activation of AP-1 in the presence of hemin. We suggest that calmodulin-dependent protein kinase II rather than the MAP kinase family regulates the induction of the human *c-fos* gene expression by hemin.

***Key Words: c-fos;* hemin; AP-1; MAP kinase; calmodulin-dependent protein kinase II.**

Heme acts as a redox-active cofactor in cytochromes and as an oxygen carrier in globin. Heme has been shown to function as an effector molecule that can regulate various biological processes, including transcription, translation, protein translocation and cell differentiation (1). Once heme interacts with molecular oxygen, it can catalyze oxygen radical formation, damaging lipids and proteins, events which enhances the consequence of oxidative damage. The best known oxidative stress-inducible molecule is HO-1, the rate-limiting enzyme of heme degradation (2). The expression of HO-1 is markedly induced in response to the substrate heme and a variety of environmental conditions (heat shock and hypoxia) and agents, including heavy metals, TPA and UV irradiation (3–5). A recent study with mice lacking HO-1, as the result of targeted disruption demonstrated that HO-1 functions as an adaptive mechanism to protect cells from oxidative damage during stress (6). HO-1, therefore, is thought to provide antioxidant defense mechanisms, on the basis of its marked activation in stressed cells (6, 7).

The transcription factor AP-1 is composed of members of the Fos family (c-Fos, Fos B, Fra-1, Fra-2) and Jun family (c-Jun, Jun B, Jun D) that form restricted homo- or heterodimers (8). AP-1 is predominantly induced at the level of transcription by *de novo* synthesis of its subunits. This induction is controlled predominantly by cis-acting SRE elements and AP-1 binding sites in the promoters of several AP-1 genes. A large number of mitogenic and proinflammatory signals lead to activation of AP-1 gene (8, 9). Similarly changes in the cellular redox status activate AP-1 (10), which has been shown to increase the expression of several proto-

[1] To whom correspondence should be addressed. Fax: 06-6992-3522. E-mail: taketani@takii.kmu.ac.jp.

Abbreviations used: HO-1, heme oxygenase-1; SRE, serum response element; DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; BSA, bovine serum albumin; SDS, sodium dodecylsulfate; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; ERK, extracellular signal regulated protein kinase; MAP kinase, mitogen activated protein kinase; MEK: mitogen-activated protein kinase kinase/extracellular signal-regulated protein kinase kinase; JNK, c-Jun *N*-terminal kinase; CaMK II, calmodulin-dependent protein kinase II; bp, base pair(s); kDa, kilodaltons.

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oncogene by activating transcription. The *c-fos* gene is a well studied early response gene and activation of *c-fos* by mitogens and changes in the intracellular redox level are controlled by cis-acting SRE in the *c-fos* gene (11–13). Furthermore, stressors including heat shock, arsenite and cadmium can stimulate SRE by activation of a ternary complex factor through the MAP kinase pathway, which includes ERK, JNK and p38 MAP kinase (14–16). Thus, many HO-1 inducers, including UV-irradiation, hydrogen peroxide and heavy metals, stimulate the expression and/or activity of c-Fos and c-Jun (8). Conversely AP-1 proteins mediate transcriptional activation of the HO-1 gene by various agents (17, 18). Although little attention has been directed to heme-mediated oxidant injury, there are several overlaps between the activation of AP-1 and induction of the HO-1 expression. Therefore, the activation of HO-1 by hemin prompted us to investigate transcription factors responsible for induction of the AP-1 gene.

Here we report the induction of human *c-fos* mRNA with hemin in HeLa and skin fibroblast cells. This induction was accompanied by a rapid and transient increase in AP-1 DNA binding. The *c-fos* induction by hemin was independent of the activation of MAP kinase. As an inhibitor for CaMK II inhibited the hemin-dependent induction of *c-fos* mRNA and stimulation of CaMK II occurred by hemin, signal transduction through CaMK II may be involved in activation of the *c-fos* gene.

MATERIALS AND METHODS

Materials. [α-^{32}P]dCTP (3000 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were obtained from NEN. Restriction endonucleases and nucleic acid-modifying enzymes were obtained from Toyobo Co. Human *c-fos* cDNA and the DNA fragment of the human *c-fos* gene promoter between −404 and +41 bp (19) were kind gifts of Dr. J. Fujisawa of Kansai Medical University. The DNA fragment containing nucleotide position −404 bp to +41 bp of the human *c-fos* gene was also inserted into SacI/NheI site of pGL2-Basic plasmid (Promega Co.) to generate pGL-FOS404. Calphostin C, genestein, herbimycin A and KN-62 were products of Wako Pure Chemicals. H-7, H-89, K252a, and KT5823 were from Calbiochem. Co. Anti-phospho-JNK and anti-phospho-CaMK II antibodies were from New England BioLabos Inc. and Promega Co., respectively. Anti-JNK and anti-CaMK II antibodies were from Transduction Laboratories. Hemin was dissolved in dimethylsulfoxide at a concentration of 20 mM, then diluted 20-fold with PBS containing 10 mg/ml BSA. Zinc-, cobalt-, and tin-protoporphyrins were kind gifts of Dr. S. Sassa of The Rockefeller University. All other chemicals used were of analytical grade.

Cell cultures. HeLa cells and human skin fibroblast cells which obtained from Japan Cell Bank were maintained in DMEM supplemented with 10% FCS, 50 U/ml penicillin and 50 μg/ml streptomycin. To expose the cells to hemin, arsenite or cadmium, the cells were washed twice with PBS, incubated in DMEM containing 1 mg/ml BSA for 16 h, followed by incubation in the presence of insulting agents at 37°C for the indicated period. For treatment with protein kinase inhibitors, the cells were incubated with inhibitors at 37°C for 30 min prior to the addition of hemin. After incubation of the cells with hemin, the cells were washed twice with PBS.

Northern blots. Total RNAs from the cells were prepared by the guanidium isothiocyanate method (20). RNA preparations were electrophoresed in a 1% agarose gel, under denaturing conditions, then transferred onto a nylon filter. Conditions of hybridization and washing were as described (20). The mRNA concentration was quantified using an Advantec DMU-33c densitometer.

Transient transfection and reporter assay. HeLa cells (1×10^6) were suspended in serum-free DMEM medium and transfected with 1 μg of pGL-FOS404 and 4 μg of β-galactosidase reference plasmid (Promega), using a transfection reagent lipofectamine (GIBCO-BRL) for 5–6 h, according to the manufacturer's recommendation. The cells were incubated in DMEM supplemented with 10% FCS and antibiotics for 24 h, and then incubated in serum-free DMEM for 16 h. After incubation of cells with hemin, the cells were collected and washed twice with PBS. The cells were then lysed in 100 μl of Reporter lysis buffer (Promega), centrifuged and the supernatants were assayed for luciferase activity and β-galactosidase activity (21). Luciferase assay was done, according to protocol for the Luciferase Assay System (Promega), and β-Galactosidase Assay System (ICN). Normalization of transfection efficiency was done on the basis of β-galactosidase activity.

In-gel protein kinase assay. HeLa cell pellets were suspended in 100 μl of an extraction buffer consisting of 20 mM Tris–HCl buffer, pH 7.4, 20 mM NaCl, 1 mM sodium orthovanadate, 10 mM NaF, 5 mM sodium pyrophosphate, 0.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 0.2 μM okadaic acid, and 10 μg/ml each of leupeptin, antipain, chymostatin, and pepstatin A at 0°C. The cells were disrupted by five passages through a 25-gauge needle, and homogenized four times by sonication for 30 s with 30-s intervals. The homogenates were centrifuged at 15,000*g* for 10 min, and supernatants were used as crude cell extracts. In-gel assay of MAP kinases and protein kinase A was carried out essentially as described (22, 23). Protein concentration of cell extracts was estimated by the method of Bradford (24).

Western blotting. Cells were lysed in a Laemmli's sample buffer (25), sonicated and boiled for 1 min. Proteins were analyzed by 10% polyacrylamide gel in the presence of 0.1% SDS, and transferred onto nitrocellulose membranes. Immunoblotting was done using anti-phospho CaMK II and anti-phospho JNK antibodies as the primary antibody, as described (21).

Nuclear extract preparation. Nuclear extracts were prepared, using methods similar to those reported (21). Briefly, HeLa cells (5×10^6) untreated or treated with hemin (20 μM) were washed twice with 10 mM Tris–HCl buffer, pH 7.4, containing 150 mM NaCl and then the cells were lysed with 10 mM Hepes, pH 7.9, containing 0.6% Nonidet P-40, 10 mM KCl, 1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonyl fluoride. After centrifuging the homogenates at 1000*g* for 1 min, the nuclear pellets were suspended in 20 mM Hepes, pH 7.9, containing 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonyl fluoride. The nuclear extracts were obtained by centrifugation at 10,000*g* for 5 min and stocked at −70°C.

Electrophoretic mobility shift assay. Double-stranded consensus oligonucleotides for AP-1 (5′-CGCTTGATGAGTCAGCCGGAA-3′) (Promega) and NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′) (Promega) were end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Nuclear extracts were incubated with ^{32}P-labeled probes (20,000 cpm) in reaction buffer containing 25 mM Hepes, pH 7.9, containing 0.5 mM EDTA, 50 mM KCl, 10% glycerol, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 100 μg/ml poly(dI-dC/dI-dC), with or without competitor. After a 10-min incubation at room temperature, electrophoresis using 4% polyacrylamide gels was done, as described (21). The gels were fixed, dried, and exposed to a X-ray film at −70°C.

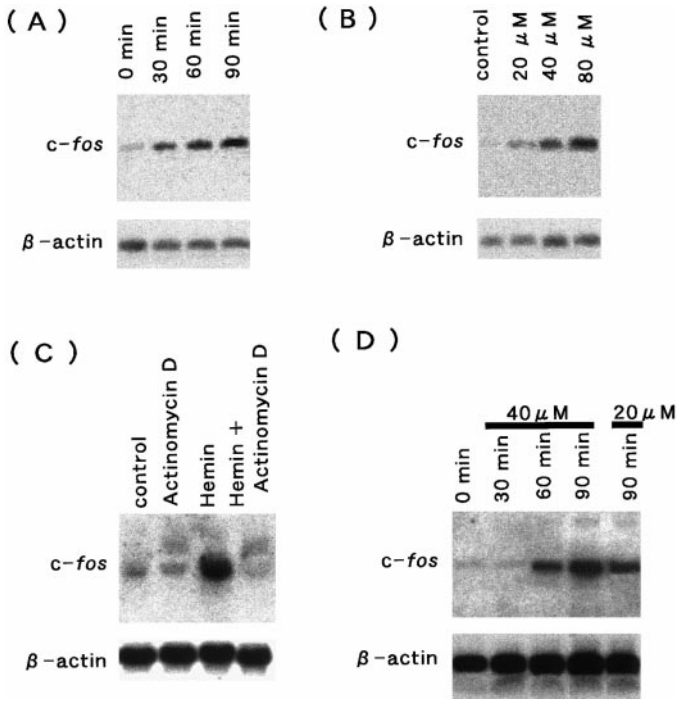

FIG. 1. Induction of *c-fos* mRNA by hemin in HeLa cells and human skin fibroblast cells. (A) Time course of *c-fos* mRNA induction by hemin. HeLa cells were treated with 20 μM hemin for the indicated time. Total RNA (10 μg) was electrophoresed on a formaldehyde/agarose gel and then transferred to a nylon membrane. The filter was hybridized with ^{32}P-labeled DNA fragment of *c-fos* (upper panel) or β-actin (lower panel). (B) Dose-dependence induction of *c-fos* mRNA. HeLa cells were untreated or treated with indicated amounts of hemin for 90 min. RNA blots were carried out as above. (C) Effect of actinomycin D on the hemin-dependent *c-fos* mRNA induction. HeLa cells incubated without or with actinomycin D (0.5 μg/ml) for 15 min prior to the addition of 20 μM hemin were then incubated with for 90 min. Equal amounts of total RNA (10 μg) were electrophoresed and analyzed by RNA blotting. (D) Induction of *c-fos* mRNA in hemin-treated human skin fibroblasts. The cells were treated with indicated amounts of hemin and RNA blotting was performed.

RESULTS

Induction of c-Fos mRNA in HeLa cells and fibroblast cells by hemin. Total RNA was isolated from HeLa cells incubated in the presence of hemin, and the level of *c-fos* mRNA was determined by Northern blot analysis. A 1.8-kbp band corresponding to *c-fos* mRNA in HeLa cells treated with 20 μM hemin appeared after 30 min and increased with time up to 90 min (Fig. 1A). By increasing the concentrations of hemin, the amount of *c-fos* mRNA increased in a dose-dependent manner (Fig. 1B). When exposing cells to actinomycin D, no induction by hemin was observed, indicating that the *c-fos* induction occurred at the level of transcription (Fig. 1C). When human skin fibroblast cells were incubated with hemin, the *c-fos* mRNA increased in a time- and dose-dependent manner (Fig. 1D). To further test inducers of *c-fos* mRNA induction, HeLa cells were incubated with heme-related compounds, metalloporphyrins including cobalt-, zinc- and tin-protoporphyrin at 50 μM markedly induced *c-fos* expression (Fig. 2). Protoporphyrin IX (50 μM) as well as ferric-nitrilotriacetate (100 μM) also induced the expression. Thus, metalloporphyrins as well as iron compounds can act as inducers of *c-fos* expression.

Possible mechanisms involved in the induction of c-fos mRNA. To examine mechanisms involved in changes in the level of *c-fos* mRNA, chimeric genes fused to the 5′-flanking region of the human *c-fos* gene to the luciferase gene were transfected into HeLa cells and reporter activity was measured. pGL-FOS404 carrying native *cis*-acting elements, including SRE of the *c-fos* promoter, exhibited high activity but change in the reporter activity in the presence of heme was nil (data not shown). We suggest that SRE at −320 to −300 bp of the human c-fos promoter which responds to signal transduction through MAP kinase is not involved in activation of the *c-fos* gene by heme. To further determine if MAP kinase is associated with activation of the *c-fos* gene by hemin, activities of ERK1/2 isoforms and p38 MAP kinase in hemin-treated HeLa cells were measured, using in-gel protein kinase assay. As shown in Fig. 3, two bands with molecular masses of 44 and 42 kDa corresponding to ERK1 and 2 were detected in untreated cells and densities of the bands increased in case of treatment with arsenite (10 μM) or cadmium (2 μM), but not with hemin (40 μM). A band at the molecular mass of 38 kDa (the position of p38 MAP kinase) in untreated or hemin-, arsenite-, or cadmium-treated cells was never appeared, implying that p38 MAP kinase was not activated by these treatments. Stimulation of JNK activities in hemin-treated cells was not observed by in-gel kinase assay as determined, using c-Jun as a substrate (data not shown). Immunoblot analysis with anti-phospho-JNK confirmed that JNK was not activated in the cells treated with hemin (data not shown).

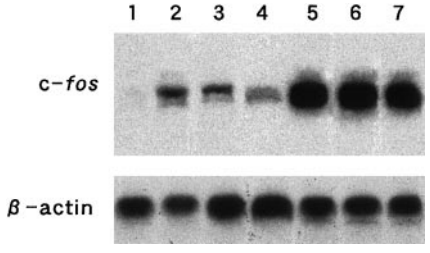

FIG. 2. Effect of metalloporphyrins, protoporphyrin and ferric ion on the induction of *c-fos* mRNA in HeLa cells. The cells were incubated without (lane 1) or with 20 μM hemin (lane 2), 50 μM protoporphyrin IX (lane 3), 100 μM ferric nitrilotriacetate (lane 4), 50 μM cobalt-protoporphyrin (lane 5), 50 μM zinc-protoporphyrin (lane 6) and 50 μM tin-protoporphyrin (lane 7) for 90 min. Total RNA (10 μg) from the indicated cells was electrophoresed and analyzed by RNA blotting, as shown in the legend to Fig. 1.

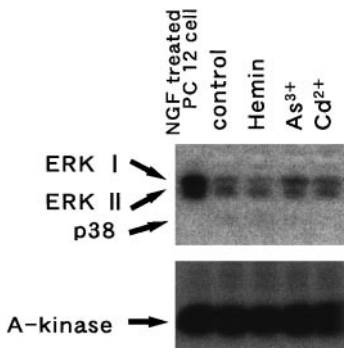

FIG. 3. Activities of MAP kinases in hemin-, arsenite-, and cadmium-treated HeLa cells. HeLa cells were untreated or treated with 40 μM hemin, 10 μM sodium arsenite and 2 μM cadmium chloride for 30 min. Cellular proteins were analyzed by a polyacrylamide in the presence of SDS and the activity of MAP kinase was measured, as described under Materials and Methods (upper panel). The ERK1/2 activities from nerve growth factor-treated rat pheochromocytoma PC 12 cells are shown, as a positive control (23). Quantity of each sample was normalized with the activity of protein kinase A (lower panel).

Effect of protein kinase inhibitors on the induction of *c-fos* by hemin was then examined. As shown in Fig. 4A, H-7 (100 μM) and K252a (100 nM), inhibitors for broad spectrum protein kinases abolished the expression of *c-fos* mRNA. KN-62, a specific inhibitor for CaMK II, at 0.5 and 2 μM inhibited the induction by 40 and 60%, respectively (Fig. 4B). Of other inhibitors for protein kinases, an inhibitor for protein kinase A, H-89 (100 μM) or a cAMP analogue, Rp-cAMPS (500 μM) did not affect the induction. Calphostin C (10–100 μM), an inhibitor for protein kinase C or KT5823 (1–5 μM), an inhibitor for protein kinase G did not inhibit the induction by hemin. PD58059 (100 μM), an inhibitor for MEK, or SB203580 (10 μM), an inhibitor for p38 MAP kinase, did not inhibit the hemin-dependent induction of *c-fos* (data not shown). To further examine whether CaMK II is involved in *c-fos* induction by hemin, immunoblotting with anti-phospho-CaMK II antibody was carried out to assess stimulation of CaMK II. As shown in Fig. 5, the level of phospho-CaMK II rapidly increased by hemin-treatment. These results suggest that CaMK II and not the MAP kinase family may contribute to activation of the *c-fos* gene by hemin.

Activation of AP-1 DNA binding activity in hemin-treated HeLa cells. To determine if induction of *c-fos* by hemin is accompanied by AP-1 transcription factor activity, we measured DNA-AP-1 binding activity in hemin-treated HeLa cells, using gel mobility shift assay. Figure 6 shows an increase in AP-1-DNA binding activity in HeLa cells within 30 min after exposure to 20 μM hemin, and the activity gradually reverted to basal levels. Formation of the DNA-protein complex was completely inhibited by coincubation of nuclear extracts with an unlabeled excess AP-1 oligonucleotide. The transcription factor NF-κB-DNA binding activity of nuclear extracts from hemin-treated HeLa cells remained unchanged for 4 h.

DISCUSSION

The present study is apparently the first to show that *c-fos* mRNA is induced by hemin in HeLa cells and human skin fibroblast cells. The c-fos induction was rapid and was abolished in actinomycin D-treated cells, indicating that the *c-fos* mRNA induction by hemin is an event related to transcription. Heme-related metalloporphyrins including tin-, cobalt-, and zinc-protoporphyrins as well as protoporphyrin increased *c-fos* mRNA. Furthermore, ferric-nitrilotriacetate also induced *c-fos* mRNA. The stimulation of *c-fos* expression by heme-related compounds seems to be a common mechanism for diverse types of cellular stress. In other studies (13, 15, 16) stressors such as cadmium and

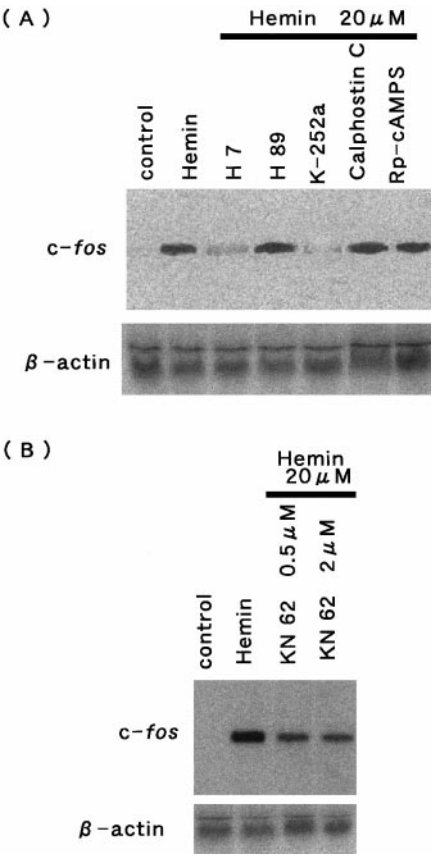

FIG. 4. Effect of inhibitors for serine/threonine protein kinase on the *c-fos* mRNA induction by hemin in HeLa cells. (A) Inhibition by broad spectrum protein kinase inhibitors, H-7 or K252a, of the hemin-dependent *c-fos* mRNA induction. The cells were incubated without or with 20 μM hemin, and 20 μM hemin plus H-7 (100 μM), K252a (100 nM), H-89 (100 μM), calphostin C (100 μM) and Rp-cAMPS (500 μM) for 90 min. Equal amounts of total RNA (10 μg) were electrophoresed and analyzed by Northern blotting. (B) Inhibition by KN-62. The cells were untreated or treated with 20 μM hemin or 20 μM hemin plus the indicated concentration of KN-62.

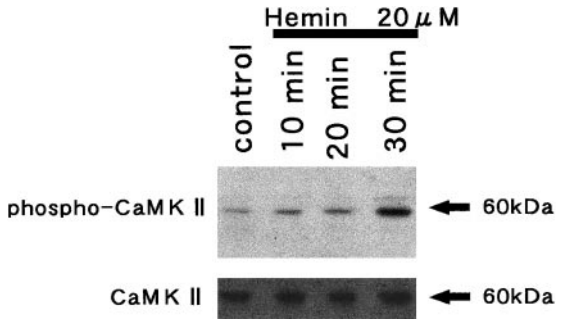

FIG. 5. Stimulation of CaMK II in hemin-treated HeLa cells. The cells were incubated without or with 20 μM hemin for the indicated period. Cellular proteins were analyzed by 10% polyacrylamide gel in the presence of 0.1% SDS. Immunoblotting was performed with anti-phospho CaMK II and anti-CaMK II antibodies as the primary antibody. Quantity of each sample was normalized with that of CaMK II (lower panel).

arsenite proved to be potent inducers of *c-fos*. Of various stress-induced proteins, HO-1 is markedly induced by hemin, heavy metals and oxidative stress. Thus, hemin is an inducer of both genes although mechanisms involved in the HO-1 induction by hemin are unclear. Alam *et al.* (18) reported that all inducers, including hemin, cadmium, H_2O_2 and TPA, require intact AP-1 binding sites for activation of the mouse HO-1 gene. Based on the present data that hemin-dependent *c-fos* mRNA induction was accompanied by a rapid increase in the AP-1 DNA binding activity, the stimulated AP-1 transcriptional activity may lead to activation of the HO-1 gene. Since HO-1 is an antioxidant defense enzyme (6, 7), the increase in AP-1 activity may afford protection from oxidative damage by depleting oxidant heme.

The present observations made in a gel mobility shift assay show that a rapid and transient activation of the AP-1 transcriptional complex occurred in the presence of hemin. This observation is consistent with previous studies where stressors such as arsenite and cadmium in various cells were shown to increase the transcription of *c-fos* mRNA as well as AP-1 activity (12, 26). In contrast to a transient increase in AP-1 activity, hemin did not cause an increase in NF-κB-DNA binding activity during hemin-treatment of HeLa cells for up to 4 h. These findings differ from observations of Lavrovsky *et al.* (27) that NF-κB binding activity rather than AP-1 activity was activated when human erythroleukemia K562 cells were treated with hemin. The reason of this discrepancy is unclear but it may be that cellular events in hemin-treated HeLa cells differ from those in K562 cells since hemin-dependent differentiation of K562 cells occurs by activation of various transcriptional factors including HSF-1, HSF-2, and NF-E2 (28, 29).

Hemin treatment led to stimulation of AP-1 activity, without the activation of JNK. Since the AP-1 complex is typically composed of the Fos (c-Fos, FosB, Fra1, and Fra2) and the Jun (C-Jun, JunB, and JunD) transcriptional factor families (8), heme may stimulate activity of the AP-1 complex through a change in the abundance of the components of the complex. Griffiths *et al.* (30) recently reported that insulin stimulates expression of *c-fos*, *fra1*, and *c-jun* with concomitant activation of the AP-1 complex, without increasing c-Jun phosphorylation or activating JNK. The present data show that c-fos mRNA induced by hemin is responsible for an increase in AP-1 activity, but it remains to be established what role other factors play in regulating AP-1 complex activity by hemin.

Other investigators (14, 15, 31) reported that various insults such as arsenite, cadmium and hypoxia led to activation of MAP kinase and this proposal was supported by the present observation that activities of ERK1/2 isoforms were stimulated by cadmium or arsenite. In contrast, the activity in HeLa cells remained unchanged in the presence of hemin. Stimulation in JNK or p38 MAP kinase in hemin-treated HeLa cells was nil, which was consistent with observations by Elbirt *et al.* (32) that hemin did not induce activity of ERK, JNK, or p38 MAP kinase in chicken hepatoma cells. There are reports that arsenite and cadmium activate the c-fos gene by stimulating the SRE via MAP kinase pathway (14, 15). A transient assay using the luciferase reporter construct carrying −404 bp upstream of the human *c-fos* gene promoter showed that SRE could not be involved in the activation of the *c-fos* gene since the activity was not affected by hemin-

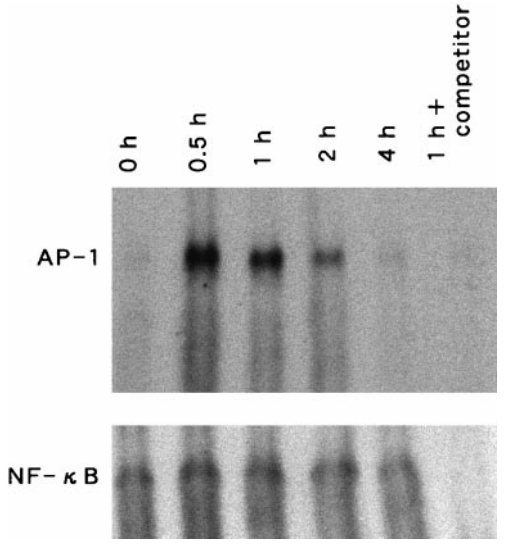

FIG. 6. Electrophoretic mobility shift assay of the AP-1-DNA interaction. HeLa cells were incubated with 20 μM hemin for the indicated time. Total cell extracts were analyzed in a gel mobility shift assay with ^{32}P-labeled AP-1 (upper panel) and NF-κB (lower panel) oligonucleotides. The labeled probes were also incubated with cell extracts from cells with 20 μM hemin for 1 h and the corresponding oligonucleotides at 100-fold excess as competitors.

treatment. Together with the failure of the MAP kinase inhibitors, PD98059 or SB203580, to inhibit *c-fos* mRNA induction, we consider that these data rule out either primary or secondary activation of MAP kinase as underlying *c-fos* induction by hemin in HeLa cells.

Induction of *c-fos* mRNA by hemin was sensitive to the broad spectrum protein kinase inhibitors K252a and H-7, but was insensitive to inhibitors for protein kinase A, protein kinase G and protein kinase C. On the other hand, KN-62, a specific inhibitor for CaMK II inhibited *c-fos* mRNA induction by only about 50%. Among isoforms of calmodulin-dependent protein kinase, CaMK II is present as a major form and plays an essential role in cell growth in HeLa cells (33, 34). CaMK II may regulate *c-fos* gene transcription through its potential to phosphorylate serum response factor (35), cAMP responsive element-binding protein (36) and activating transcription factor 1 (37). Therefore, the hemin-dependent *c-fos* induction in HeLa cells and fibroblast cells possibly depends on the activation of CaMK II. Autophosphorylation of CaMK II may potentiate its response by a brief elevation of cytoplasmic calcium ion. The calcium ionophores, ionomycin and A23187, also caused the induction of *c-fos*, without activating ERK (38). This induction was suppressed by heparin which inhibits the development of autonomous CaMK II activity, indicating that the *c-fos* induction by ionomycin is mediated by CaMK II pathway (39). The present data showed that phosphorylation of CaMK II increased by hemin-treatment. Based on the observations that reporter activity of PGL-FOS404 carrying a native *cis*-acting elements of the *c-fos* gene promoter was not stimulated by hemin, SRE may be insufficient for the hemin-dependent *c-fos* induction or that alternative pathways where CaMK II activates *cis*-acting element(s) separated from SRE are involved in the *c-fos* induction by hemin. Since KN-62 partially inhibited the hemin-dependent *c-fos* induction, (an)other signalling pathways in addition to CaMk II, may be involved in the activation of the *c-fos* gene by hemin. Although precise mechanisms underlying hemin-dependent *c-fos* induction are unclear, this unique regulation by multiple pathways on *c-fos* mRNA induction by hemin may provide a pertinent model to clarify molecular mechanisms involved in heme-dependent gene activation.

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