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Involvement of the Extracellular Signal-Regulated Protein Kinase (ERK) Pathway in the Induction of Apoptosis by Cadmium Chloride in CCRF-CEM Cells

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ABSTRACT. When CCRF-CEM cells were incubated with 5–40 μM $CdCl_2$, apoptosis was observed most clearly at 10 μM. Prior to the development of apoptosis, mitogen-activated protein kinases (MAPKs), i.e. extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK, were activated with different sensitivity to $CdCl_2$ exposure. ERK and p38 MAPK were phosphorylated with incubation of 1 μM $CdCl_2$, but higher than 20 μM $CdCl_2$ was required for the clear phosphorylation of JNK. In the time–course study, ERK and p38 MAPK were phosphorylated earlier than JNK after $CdCl_2$ exposure. The *in vitro* activities of MAPKs also increased in response to $CdCl_2$ exposure. Pretreatment with an intracellular Ca^{2+} chelator, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA/AM), suppressed almost completely $CdCl_2$-induced phosphorylation of JNK and p38 MAPK, but not ERK phosphorylation, indicating that the activation of JNK and p38 MAPK depends on the intracellular Ca^{2+} but that of ERK does not. On the other hand, treatment with a MAPK/ERK kinase (MEK) inhibitor, U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene), suppressed $CdCl_2$-induced ERK activation and the apoptosis as well. The inhibition of p38 MAPK activity with SB203580 (4-[4-fluorophenyl]-2-[4-methylsulfinylphenyl]-5-[4-pyridyl]1*H*-imidazole) did not protect cells from apoptosis. The present results showed that the activation of ERK, JNK, and p38 MAPK is differently regulated in CCRF-CEM cells exposed to $CdCl_2$, and that the ERK pathway seems to be responsible for the induction of apoptosis by $CdCl_2$ exposure in this human T cell line. BIOCHEM PHARMACOL **60**;12:1875–1882, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. cadmium; ERK; JNK; p38 MAPK; apoptosis; CCRF-CEM cells

Cadmium is an important occupational and environmental pollutant. It has a biological half-life exceeding 20 years, and may accumulate in various organs such as liver and kidney [1]. Cadmium has been shown to be incorporated into cultured peripheral blood cells including lymphocytes [2]. It has been reported that cadmium induces apoptosis in leukemia and lymphoma cells [3–5], renal epithelial cells [6], proximal tubule cells [7], lung epithelial cells [8], liver [9], testis [10, 11], and ventral prostate [11]. However, the signal transduction pathways leading to apoptosis or subsequent regeneration in cells exposed to cadmium have not been clarified.

MAPKs§ are a family of Ser/Thr protein kinases that transmit extracellular signals into the nucleus [12, 13]. In mammalian systems, five distinguishable MAPK modules have been identified so far: ERK (ERK1/2), JNK (also known as stress-activated protein kinase), p38 MAPK, ERK3, and ERK5 [12]. MAPK cascades have been shown to participate in a diverse array of cellular functions such as cell growth, cell differentiation, and cell death [12, 13]. We have found that the exposure to cadmium induces the phosphorylation of JNK and results in the accumulation of phosphorylated c-Jun protein in LLC-PK_1 porcine renal epithelial cells [14]. Furthermore, cadmium has been reported to activate JNK in Rat-1 fibroblasts [15], and ERK as well as JNK in rat mesangial cells [16]. On the other hand, in LMH chicken hepatoma cells [17] and 9L rat brain tumor cells [18], ERK and p38 MAPK but not JNK were activated in response to cadmium exposure, suggesting that the members of the MAPK family are differentially activated by cadmium exposure, depending on the cell type. However, whether or not cadmium activates MAPKs in lymphocytes and, if it does, their possible contribution to apoptosis have not been determined. CCRF-CEM cells are a human T cell line that shows the characteristic findings of apoptosis in response to 10–100 μM $CdCl_2$ exposure [4].

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§ *Abbreviations*: MAPKs, mitogen-activated protein kinases; ERK, extracellular signal-regulated protein kinase; JNK, c-Jun N-terminal kinase; MEK, MAPK/ERK kinase; BAPTA/AM, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra(acetoxymethyl) ester; and TPEN, *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine.

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We therefore determined the phosphorylation status and *in vitro* activities of ERK, JNK, and p38 MAPK in this T cell line exposed to 1–40 μM $CdCl_2$, and also examined the effects of intracellular Ca^{2+} chelation on their phosphorylation. The possible role of the activation of MAPKs in cadmium-induced apoptosis was examined using a potent inhibitor of both activated and non-activated forms of MEK1/2, U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene) [19], and a p38 MAPK inhibitor, SB203580 (4-[4-fluorophenyl]-2-[4-methylsulfinylphenyl]-5-[4-pyridyl]1*H*-imidazole) [20]. The results presented herein demonstrated that the activation of MAPKs by cadmium exposure is differently regulated and contributed to subsequent apoptosis in CCRF-CEM cells.

MATERIALS AND METHODS

Cell Culture and Treatments

CCRF-CEM human T lymphoblastoid cells (Health Science Research Resources Bank) were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin (GIBCO BRL) in a humidified atmosphere of 5% CO_2, 95% air at 37°. Exponentially growing CCRF-CEM cells were diluted at 4×10^6 cells in 25 cm^2 culture flasks (Iwaki Glass Co., Ltd.) and subsequently used for the experiments.

The stock solution of $CdCl_2$ (Sigma) was diluted with water and sterilized by filtration. CCRF-CEM cells were incubated with serum-free medium containing 1, 5, 10, 20, or 40 μM $CdCl_2$ for 1 hr at 37°. In the time–course study, cells were incubated with 10 or 20 μM $CdCl_2$ for 15 min to 4 hr. Untreated control cells were incubated with serum-free medium without $CdCl_2$, and treated identically to the cells exposed to $CdCl_2$.

An intracellular Ca^{2+} chelator, BAPTA/AM (Calbiochem), and a cell permeable chelator, TPEN (Dojindo), were dissolved in DMSO. Cells were preincubated with serum-free medium containing either 0.03% DMSO, 10 μM BAPTA/AM, or 10 μM TPEN for 30 min. After washing twice with PBS, cells were incubated with or without 10 μM $CdCl_2$ for 1 hr at 37°.

U0126 (Promega) and SB203580 (Calbiochem) were dissolved in DMSO. Cells were preincubated with serum-free medium containing either 0.1% DMSO, 0.1 to 50 μM U0126, or 50 μM SB203580 for 30 min, and then incubated with 10 μM $CdCl_2$ for 1 or 10 hr.

Flow Cytometric Analysis

CCRF-CEM cells (2×10^6) incubated with 10 μM $CdCl_2$ for 10 hr were washed twice with PBS and fixed in 70% ethanol on ice for 30 min. The fixed cells were washed twice with PBS, incubated with 250 μg/mL of RNase at 37° for 1 hr, washed again twice, and then stained with 50 μg/mL of propidium iodide solution. The DNA content of cells (1×10^4 for each sample) was determined with a Coulter EPICS XL (Coulter Electronics). Apoptotic cells were estimated by the percentage of cells in the sub-G_1 peak.

Western Immunoblotting

After incubation with $CdCl_2$, CCRF-CEM cells were washed twice with PBS and lysed with SDS-polyacrylamide gel Laemmli sample buffer. Cell lysates were collected, sonicated, and boiled for 5 min. Aliquots equivalent to 4×10^5 cells were subjected to SDS/PAGE on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane (Hybond-ECL, Amersham Pharmacia Biotech). The membrane was blocked with 5% non-fat milk or 1% BSA in Tris-buffered saline containing 0.1% Tween 20 for 1 hr at room temperature. The antibodies used were phospho-p44/42 MAPK (Thr202/Tyr204) antibody, phosphorylation state-independent p44/42 MAPK antibody, phospho-SAPK/JNK (Thr183/Tyr185) antibody, phosphorylation state-independent SAPK/JNK antibody, phospho-p38 MAPK (Thr180/Tyr182) antibody, and phosphorylation state-independent p38 MAPK antibody (New England Biolabs, Inc.). The membrane was incubated overnight at 4° with the primary antibody diluted 1:1000 or 1:500 in 5% BSA in Tris-buffered saline containing 0.1% Tween 20. Protein was detected with a Phototope-HRP Western blot detection kit (New England Biolabs, Inc.). The blots were exposed to Hyperfilm (Amersham Pharmacia Biotech) for 1–5 min.

In vitro *Kinase Assay*

The *in vitro* activities of ERK, JNK, and p38 MAPK were measured using a p44/42 MAPK assay kit, an SAPK/JNK assay kit, and a p38 MAPK assay kit (New England Biolabs, Inc.), respectively. After washing twice with PBS, CCRF-CEM cells exposed to $CdCl_2$ were lysed in cell lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 μg/mL of leupeptin, 1 mM phenylmethylsulfonyl fluoride). Then cell lysates equivalent to 4×10^6 cells were incubated with immobilized phospho-p44/42 MAPK (Thr202/Tyr204) monoclonal antibody, GST-c-Jun (1–89) fusion protein, and immobilized phospho-p38 MAPK (Thr180/Tyr182) monoclonal antibody overnight at 4° to precipitate ERK, JNK, and p38 MAPK, respectively. After washing the pellet, *in vitro* kinase reaction was carried out at 30° for 30 min in kinase buffer (25 mM Tris–HCl, pH 7.5, 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM sodium orthovanadate, 10 mM $MgCl_2$) containing 200 μM ATP and GST-Elk-1 (307–428) fusion protein (for ERK), 100 μM ATP (for JNK), or 200 μM ATP and GST-ATF-2 (19–96) fusion protein (for p38 MAPK). The reaction was terminated by adding Laemmli sample buffer. After SDS/PAGE of sample equivalent to 1×10^6 cells, phosphorylation of Elk-1, c-Jun, and ATF-2 was analyzed with immunoblotting using phospho-

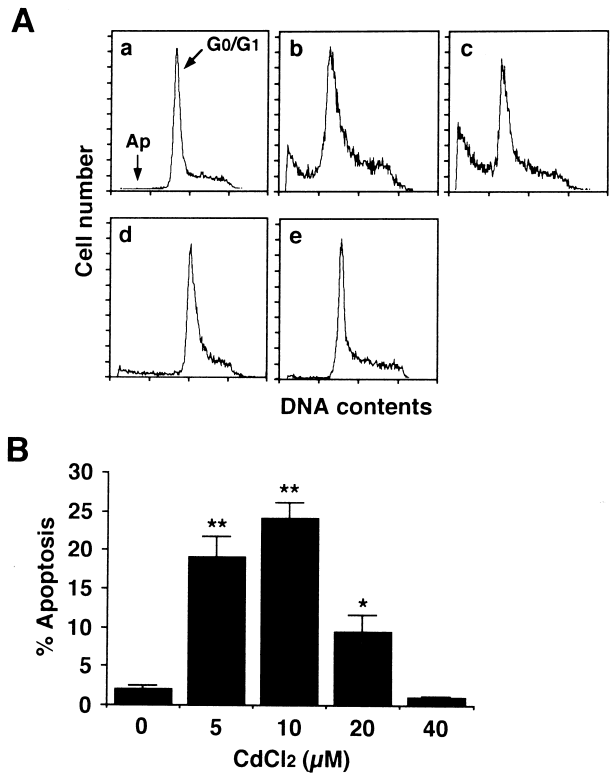

FIG. 1. Apoptosis in CCRF-CEM cells exposed to $CdCl_2$. (A) Representative flow cytometric analysis of CCRF-CEM cells incubated with 0 (a), 5 (b), 10 (c), 20 (d), or 40 μM $CdCl_2$ (e) for 10 hr at 37°. The same cell number (1×10^4) was used for each determination of the DNA content of the cells. Apoptotic (Ap) and G_0-G_1 cell populations are indicated by arrows. (B) Apoptotic cells were estimated by the percentage of cells in the sub-G_1 peak. Each column and bar represent the mean ± SEM of four independent experiments. Key: (*) $P < 0.05$, and (**) $P < 0.01$ compared with 0 μM $CdCl_2$ by Dunnett's test.

Elk-1 (Ser383) antibody, phospho-c-Jun (Ser63) antibody, and phospho-ATF-2 (Thr71) antibody, respectively. The bands on the developed films were quantified with NIH Image Version 1.61.

Statistical Analysis

Results are expressed as means ± SEM. Statistical significance was determined by one-way ANOVA followed by the Dunnett multiple comparison test. $P < 0.05$ was considered as statistically significant.

RESULTS

Apoptosis in CCRF-CEM Cells Exposed to $CdCl_2$

When CCRF-CEM cells were incubated with 5 or 10 μM $CdCl_2$ for 10 hr, significant increases in the number of apoptotic cells were found with flow cytometric analysis (Fig. 1). Fluorescence microscopic examination also showed the characteristic apoptotic alterations such as nuclear condensation and fragmentation in these cells (data not shown). On the other hand, incubation with 20 μM $CdCl_2$ caused apoptosis less markedly, and no apoptosis with 40 μM $CdCl_2$ (Fig. 1). Morphological examination revealed necrotic changes in almost all cells incubated with 40 μM $CdCl_2$ for 10 hr (data not shown).

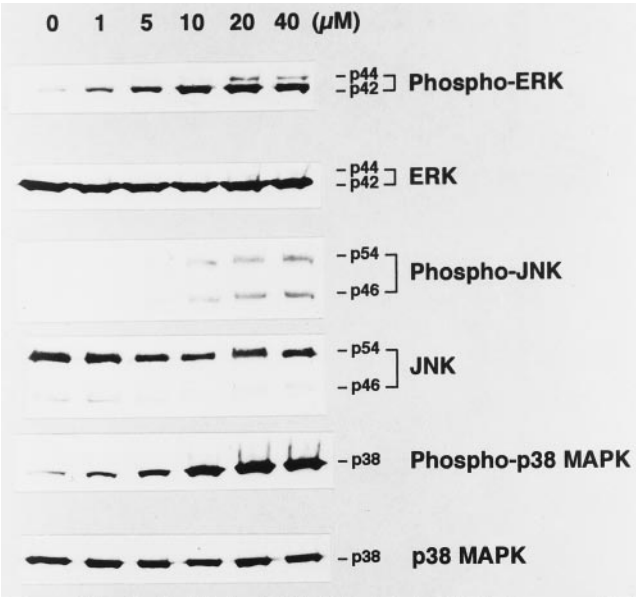

FIG. 2. Concentration effects of $CdCl_2$ on the accumulation of phosphorylated ERK, JNK, and p38 MAPK. CCRF-CEM cells were incubated with 0, 1, 5, 10, 20, or 40 μM $CdCl_2$ for 1 hr, and cell lysates were subjected to Western immunoblotting using phospho-p44/42 MAPK (Thr202/Tyr204) antibody, phosphorylation state-independent p44/42 MAPK antibody, phospho-SAPK/JNK (Thr183/Tyr185) antibody, phosphorylation state-independent SAPK/JNK antibody, phospho-p38 MAPK (Thr180/Tyr182) antibody, and phosphorylation state-independent p38 MAPK antibody (from top to bottom). Results shown are representative of four independent experiments.

Activation of MAPKs by $CdCl_2$ Exposure

We determined the phosphorylation status and *in vitro* activities of MAPKs after incubation with $CdCl_2$ for 1 hr when cell viability of CCRF-CEM cells, as evaluated with the MTT assay, was not changed (data not shown). After incubation with 1 μM $CdCl_2$ for 1 hr, phosphorylation of ERK (ERK2/p42 and ERK1/p44) and p38 MAPK was found, and the levels of phosphorylated form increased in a concentration-dependent manner (Fig. 2). While JNK (p46 and p54) was phosphorylated mildly by 10 μM $CdCl_2$ exposure, its clear phosphorylation was found only when incubated with 20 or 40 μM $CdCl_2$ for 1 hr (Fig. 2). In contrast, the levels of total (phosphorylation state-independent) ERK, JNK, and p38 MAPK were not changed by incubation with any concentration of $CdCl_2$ (Fig. 2).

Consistent with the phosphorylation of MAPKs by $CdCl_2$ exposure, the *in vitro* activities of ERK, JNK, and p38 MAPK increased depending upon the concentration of $CdCl_2$ (Fig. 3). ERK activity was elevated significantly at a concentration of 5 μM $CdCl_2$ ($P < 0.01$, Fig. 3A). While p38 MAPK activity began to increase at 10 μM $CdCl_2$

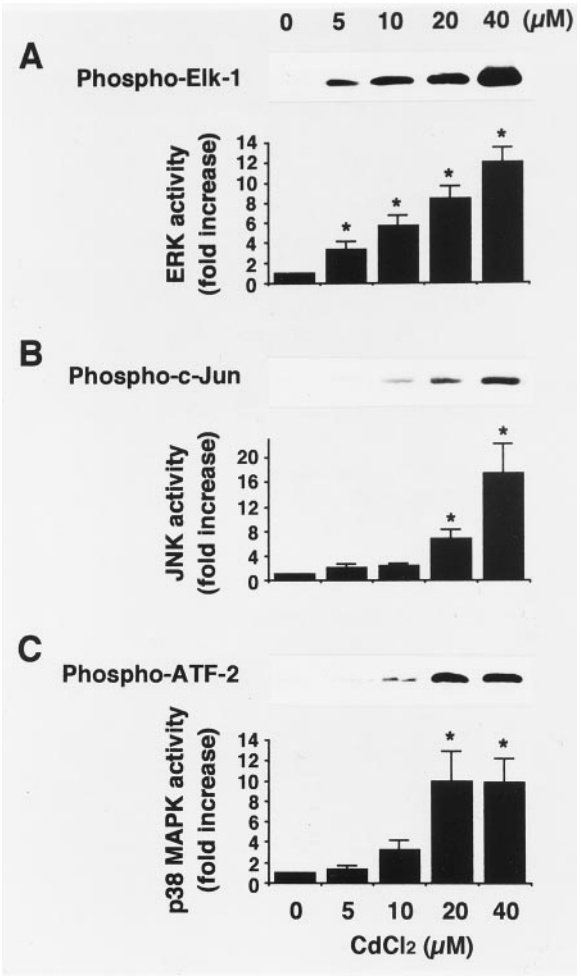

FIG. 3. Concentration effects of $CdCl_2$ on the activity of ERK (A), JNK (B), and p38 MAPK (C). CCRF-CEM cells were incubated with 0, 5, 10, 20, or 40 μM $CdCl_2$ for 1 hr, and cell lysates were used for *in vitro* kinase reaction with GST-Elk-1 (307–428) as substrate for ERK (A), GST-c-Jun (1–89) for JNK (B), or GST-ATF-2 (19–96) for p38 MAPK (C). Phosphorylation of Elk-1, c-Jun, and ATF-2 was analyzed with Western immunoblotting using phospho-Elk-1 (Ser383) antibody, phospho-c-Jun (Ser63) antibody, and phospho-ATF-2 (Thr71) antibody, respectively. Results shown are representative immunoblot and densitometric analysis of phosphorylated Elk-1, c-Jun, and ATF-2. Each value was expressed as the fold increase with respect to 0 μM $CdCl_2$. Each column and bar represent the mean ± SEM of three independent experiments. Key: (*) $P < 0.01$ compared with 0 μM $CdCl_2$ by Dunnett's test.

($P > 0.05$, Fig. 3C), the statistically significant elevation of JNK and p38 MAPK activities was found at higher than 20 μM $CdCl_2$ ($P < 0.01$, Fig. 3, B and C).

In the time–course study, the levels of the phosphorylated form of ERK and p38 MAPK increased after 30 min, and that of JNK after 1 hr in response to 10 μM $CdCl_2$ exposure (Fig. 4). The phosphorylated forms of each MAPK accumulated further as the incubation period increased, whereas their total (phosphorylated plus not phosphorylated) levels were not changed during the incubation period of 4 hr (Fig. 4). ERK and p38 MAPK were also phosphorylated earlier than JNK after 20 μM $CdCl_2$ exposure (data not shown). When CCRF-CEM cells that had been incubated with 20 μM $CdCl_2$ for 1 hr were continued to be cultured after removing $CdCl_2$ from the medium by extensive washing, the elevated levels of phosphorylated ERK, JNK, and p38 MAPK began to decrease gradually after 30 min, and returned to the basal level after 8 hr (data not shown).

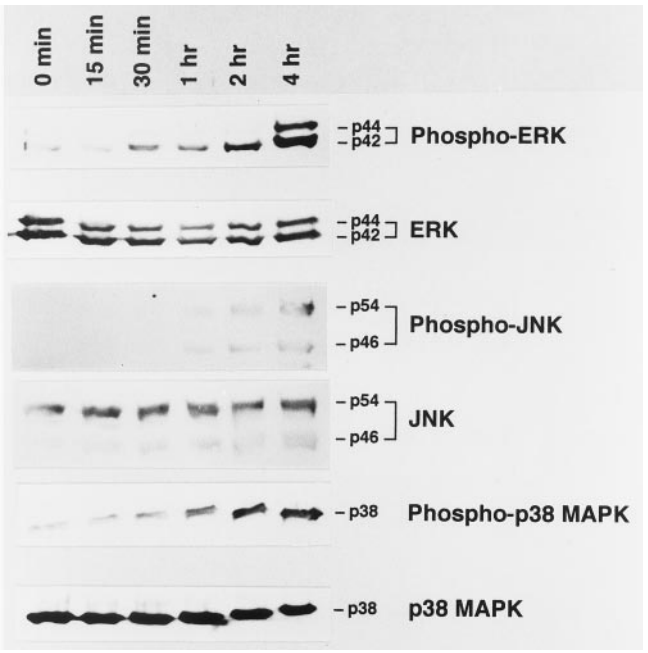

FIG. 4. Time–course of $CdCl_2$-induced accumulation of phosphorylated ERK, JNK, and p38 MAPK. CCRF-CEM cells were incubated with 10 μM $CdCl_2$ for 15 min to 4 hr, and cell lysates were subjected to Western immunoblotting using antibodies described in the legend to Fig. 2. Results shown are representative of four independent experiments.

Effects of BAPTA/AM or TPEN on $CdCl_2$-Induced MAPK Phosphorylation

Pretreatment with BAPTA/AM or TPEN alone did not change the levels of phosphorylated and total MAPKs (Fig. 5, lanes 3 and 5). In cells pretreated with an intracellular Ca^{2+} chelator, BAPTA/AM, $CdCl_2$-induced phosphorylation of JNK and p38 MAPK (Fig. 5, lane 2) was abolished almost completely without changing total protein levels (Fig. 5, lane 4). In contrast, $CdCl_2$-induced ERK phosphorylation (Fig. 5, lane 2) was suppressed only slightly by BAPTA/AM pretreatment (Fig. 5, lane 4). Pretreatment with a cell permeable chelator, TPEN, did not reduce the levels of phosphorylated MAPKs clearly (Fig. 5, lane 6).

Effects of U0126 or SB203580 on $CdCl_2$-Induced Apoptosis

Treatment with an inhibitor of MEK1/2, U0126 (50 μM), suppressed $CdCl_2$-induced elevation of ERK activity assayed using Elk-1 as substrate (Fig. 6A), as well as ERK

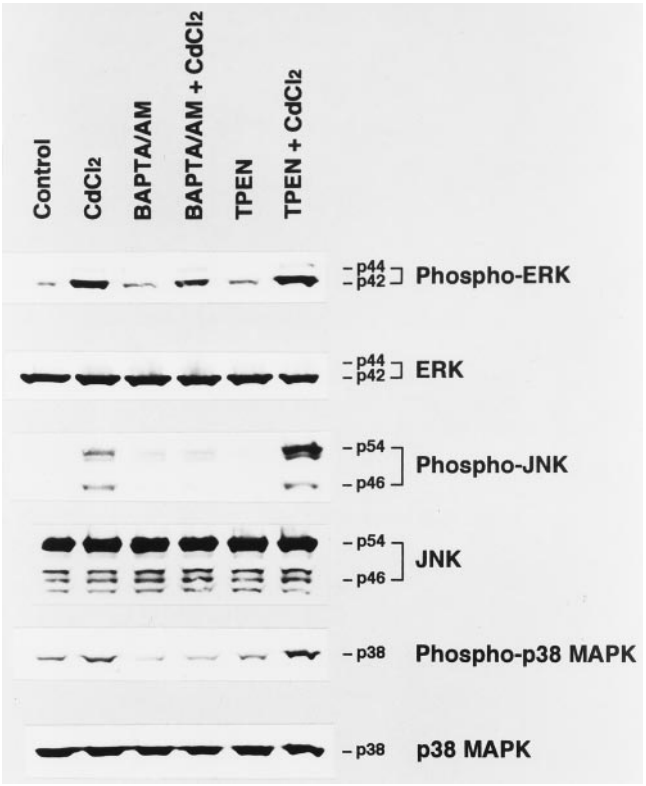

FIG. 5. Effects of pretreatment with BAPTA/AM or TPEN on $CdCl_2$-induced accumulation of phosphorylated ERK, JNK, and p38 MAPK. CCRF-CEM cells were preincubated with 0.03% DMSO, 10 μM BAPTA/AM, or 10 μM TPEN for 30 min, and then washed twice with PBS. After incubation with or without 10 μM $CdCl_2$ for 1 hr, cell lysates were subjected to Western immunoblotting using antibodies described in the legend to Fig. 2. Results shown are representative of three independent experiments.

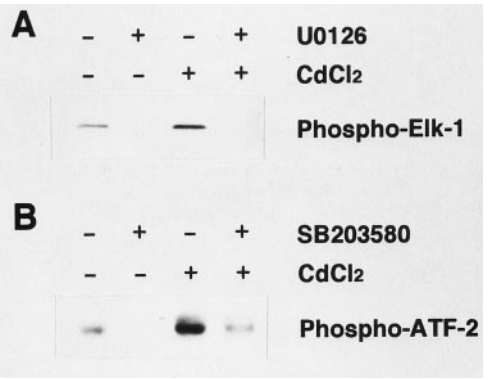

FIG. 6. Inhibition of $CdCl_2$-induced activation of ERK by U0126 (A) and of p38 MAPK by SB203580 (B). CCRF-CEM cells were preincubated with 0.1% DMSO, 50 μM U0126, or 50 μM SB203580 for 30 min, and then incubated with or without 10 μM $CdCl_2$ for 1 hr. Cell lysates were used for *in vitro* kinase reaction with GST-Elk-1 (307–428) for ERK (A) or GST-ATF-2 (19–96) for p38 MAPK (B). Phosphorylation of Elk-1 and ATF-2 was analyzed with Western immunoblotting using phospho-Elk-1 (Ser383) antibody and phospho-ATF-2 (Thr71) antibody, respectively. Results shown are representative of three independent experiments.

phosphorylation (data not shown). Treatment with a p38 MAPK inhibitor, SB203580 (50 μM), suppressed $CdCl_2$-induced elevation of p38 MAPK activity, assayed using ATF-2 as substrate (Fig. 6B), but not that of JNK activity (data not shown). In cells treated with 1, 10, or 50 μM U0126, the $CdCl_2$-induced increase in the number of apoptotic cells was reduced in a concentration-dependent manner (Fig. 7). On the other hand, SB203580 at a concentration of 50 μM, which could suppress p38 MAPK activity in cells exposed to $CdCl_2$, did not protect cells clearly from $CdCl_2$-induced apoptosis (Fig. 7).

DISCUSSION

We found that ERK, JNK, and p38 MAPK were all activated, prior to the development of apoptosis, in CCRF-CEM cells exposed to $CdCl_2$. However, the sensitivity to $CdCl_2$ was different among these members of the MAPK family. ERK and p38 MAPK were phosphorylated by incubation with $CdCl_2$ at 1 μM. On the other hand, a much higher concentration (20 or 40 μM) was required for the clear phosphorylation of JNK. Furthermore, in the time–course study, ERK and p38 MAPK were phosphorylated earlier than JNK after incubation with 10 or 20 μM $CdCl_2$. Consistent with MAPK phosphorylation by $CdCl_2$ exposure, the *in vitro* activities of MAPKs increased in a concentration-dependent manner. Among the activities of the MAPKs examined, ERK was the most sensitively elevated by $CdCl_2$ exposure. These results indicate that ERK and p38 MAPK were more sensitive pathways to $CdCl_2$ exposure than JNK in CCRF-CEM cells, as reported in LMH chicken hepatoma cells [17] and 9L rat brain tumor cells [18]. Thus, the cadmium-induced activation of ERK, JNK, and p38 MAPK may be differently regulated in lymphoid as well as in non-lymphoid cells.

It has been shown that cadmium exposure rapidly increases inositol 1,4,5-trisphosphate and then triggers Ca^{2+} mobilization in various cell types [21, 22]. And we have found previously that pretreatment with an intracellular Ca^{2+} chelator, BAPTA/AM, suppresses $CdCl_2$-induced JNK phosphorylation in LLC-PK_1 renal epithelial cells [14]. In the present study, we examined the role of intracellular Ca^{2+} in $CdCl_2$-induced activation of ERK, JNK, and p38 MAPK in CCRF-CEM cells, and found that the phosphorylation of p38 MAPK as well as JNK was abolished almost completely by BAPTA/AM pretreatment. On the other hand, $CdCl_2$-induced ERK phosphorylation was not suppressed clearly by BAPTA/AM pretreatment. Pretreatment with TPEN, a cell permeable chelator having high affinity for metals including Cd^{2+} ($\log K_{Cd} = 16.3$, $\log K_{Mn} = 10.3$, $\log K_{Fe} = 14.6$, $\log K_{Cu} = 20.5$, $\log K_{Zn} = 15.6$, $\log K_{Hg} = 25.1$, $\log K_{Pb} = 14.0$) [23] but a low affinity for Ca^{2+} ($\log K_{Ca} = 4.4$) [24], did not affect $CdCl_2$-induced MAPK phosphorylation, suggesting that intracellular Cd^{2+} is not responsible for the activation of MAPKs. Therefore, the inhibitory effects of BAPTA/AM observed do not seem to be caused by the interaction between an intracellularly

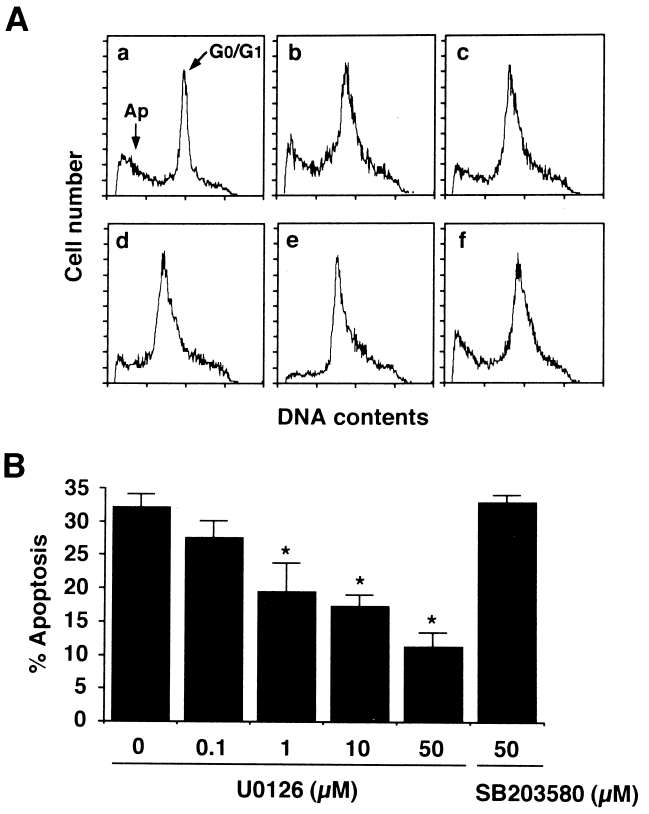

FIG. 7. Effects of U0126 and SB203580 on $CdCl_2$-induced apoptosis in CCRF-CEM cells. (A) Representative flow cytometric analysis of CCRF-CEM cells preincubated with 0 (a), 0.1 (b), 1 (c), 10 (d), and 50 μM U0126 (e), or 50 μM SB203580 (f) for 30 min and then incubated with 10 μM $CdCl_2$ for 10 hr. The same cell number (1 x 10^4) was used for each determination of the DNA content of the cells. Apoptotic (Ap) and G_0-G_1 cell populations are indicated by arrows. (B) Apoptotic cells were estimated by the percentage of cells in the sub-G_1 peak. Each column and bar represent the mean ± SEM of six independent experiments. Key: (*) $P < 0.01$ compared with 0 μM U0126 (0.1% DMSO plus 10 μM $CdCl_2$) by Dunnett's test.

hydrolyzed metabolite of BAPTA/AM, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA, log $K_{Cd} = 12.2$, log $K_{Ca} = 7.0$) [25], and Cd^{2+} within the cells. Removal of external Ca^{2+} did not suppress $CdCl_2$-induced MAPK phosphorylation (data not shown). These results indicate that $CdCl_2$-induced phosphorylation of JNK and p38 MAPK depends on the possible mobilization of Ca^{2+} from intracellular stores in CCRF-CEM cells, and that the ERK pathway is activated through an intracellular Ca^{2+}-independent mechanism.

In Jurkat human T cells, it was found that the activities of JNK and p38 MAPK increase in response to signals generated by the combination of phorbor ester (12-*O*-tetradecanoylphorbol-13-acetate) and Ca^{2+} ionophore (A23187), whereas each stimulus alone does not increase either activity [26, 27]. In contrast, ERK is fully activated by the stimulation with phorbor ester alone, and is not affected by Ca^{2+} ionophore [26, 27]. Taken together with our findings on BAPTA/AM pretreatment, the exposure of lymphoid cells to cadmium may activate another signaling pathway that can be triggered by phorbor ester in addition to Ca^{2+} mobilization. Since cadmium has been reported to activate nuclear protein kinase C by stimulating the binding of the enzyme to nuclear protein [28], and an inhibitor of protein kinase C (Ro-31-8425) could reduce the level of phosphorylated ERK in CCRF-CEM cells exposed to $CdCl_2$ (data not shown), the activation of protein kinase C may underlie, at least in part, $CdCl_2$-induced MAPK activation in lymphoid cells.

Incubation with $CdCl_2$ at a concentration of 5–20 μM induced apoptosis in CCRF-CEM cells as has been reported previously [4], whereas a higher concentration (40 μM) induced necrosis instead. We examined the effects of inhibition of the ERK and p38 MAPK pathways, which were found sensitive to $CdCl_2$ exposure in CCRF-CEM cells, on the development of apoptosis. The treatment with U0126, an inhibitor of MEK1/2, suppressed $CdCl_2$-induced ERK activation and apoptosis in a concentration-dependent manner. On the other hand, the inhibition of p38 MAPK activity with SB203580 did not protect cells from apoptosis. Therefore, the ERK pathway seems to be involved in $CdCl_2$-induced apoptosis in CCRF-CEM cells, but p38 MAPK may not play a major role. The ERK pathway is known to be responsible for cellular proliferation, differentiation, and development [29], and has been shown to have an antiapoptotic role in PC12 cells after withdrawal of nerve growth factor [30]. In Jurkat cells, activation of the ERK pathway could suppress Fas-induced [31] and immunosuppressant (FTY720)-induced apoptosis [32]. ERK has also been reported to be required for T cell receptor/CD3-mediated activation-induced cell death of T cells by regulating the expression of Fas ligand [33, 34]. These results and our findings suggest that the ERK pathway in lymphoid cells can transmit both antiapoptotic and proapoptotic signals, probably depending on the nature of co-existing signals. Consistent with our results, inhibition of p38 MAPK activity failed to suppress activation-induced cell death of T cells [35]. Therefore, there is a possibility that cadmium exposure induces apoptosis of T cells through the ERK-dependent pathway, which is activated by the stimulation of T cell receptors. However, it is still not clear whether the activated ERK by 40 μM $CdCl_2$ exposure, which induced necrosis in CCRF-CEM cells, transmits different signals from those in the apoptotic cells.

As in the case of p38 MAPK, the JNK pathway does not seem to be a prerequisite for $CdCl_2$-induced apoptosis in CCRF-CEM cells because JNK was activated only slightly by 10 μM $CdCl_2$, which induced apoptosis most markedly. Furthermore, treatment with an inhibitor of JNK/p38 MAPK pathways, macrocyclic nonaketide compound LL-Z1640-2 [36], did not suppress apoptosis in CCRF-CEM cells exposed to $CdCl_2$, while the phosphorylation of JNK and p38 MAPK was abolished (data not shown). Consistent with the unlikely involvement of the JNK pathway in the development of $CdCl_2$-induced apoptosis in CCRF-CEM cells, it has been reported that Fas-induced apoptosis

in lymphoid cells does not depend on the activation of JNK [31, 37, 38].

Activated MAPKs can phosphorylate many transcriptional factors such as Elk-1 (by ERK, JNK, and p38 MAPK), ATF-2 (by JNK and p38 MAPK), and c-Jun (by JNK) [12, 39]. The phosphorylation of Elk-1 causes increased ternary complex formation, and activates the promoter of c-*fos* at the serum response element [39, 40]. Indeed, c-Fos protein was elevated in CCRF-CEM cells exposed to $CdCl_2$ (data not shown). Although it has been shown that c-Fos protein plays a causal role in the activation of apoptosis [41], we have found that mouse 3T3-like fibroblasts lacking c-*fos* were affected more severely than the wild-type cells following exposure to $CdCl_2$, suggesting a protective role of c-*fos* against the cytotoxic effects of cadmium, at least in these fibroblasts [42]. However, it is not clear whether c-*fos* in lymphocytes functions the same way as in fibroblasts when exposed to cadmium. Therefore, the possible contributions of the expression of the c-*fos* gene and the resulting increased formation of c-Fos/c-Jun AP-1 complex in cadmium-induced apoptosis in lymphocytes remain to be determined.

In summary, while ERK, JNK, and p38 MAPK were all activated in CCRF-CEM cells exposed to $CdCl_2$, the activation of these MAPKs was differently regulated. Among the MAPKs examined, the ERK pathway, of which activation did not depend upon intracellular Ca^{2+}, was shown to be involved in $CdCl_2$-induced apoptosis in this T cell line. Cadmium, one of the immunomodulatory metals [43], may give clues to understanding the signaling pathways triggered by environmental stress in lymphocytes.

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