

# Eukaryotic Translation Initiation Factor 4E Is a Cellular Target for Toxicity and Death Due to Exposure to Cadmium Chloride\*

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Whether translation initiation factor 4E (eIF4E), the mRNA cap binding and rate-limiting factor required for translation, is a target for cytotoxicity and cell death induced by cadmium, a human carcinogen, was investigated. Exposure of human cell lines, HCT15, PLC/PR/5, HeLa, and Chang, to cadmium chloride resulted in cytotoxicity and cell death, and this was associated with a significant decrease in eIF4E protein levels. Similarly, specific silencing of the expression of the eIF4E gene, caused by a small interfering RNA, resulted in significant cytotoxicity and cell death. On the other hand, overexpression of the eIF4E gene was protective against the cadmium-induced cytotoxicity and cell death. Further studies revealed the absence of alterations in the eIF4E mRNA level in the cadmium-treated cells despite their decreased eIF4E protein level. In addition, exposure of cells to cadmium resulted in enhanced ubiquitination of eIF4E protein while inhibitors of proteasome activity reversed the cadmium-induced decrease of eIF4E protein. Exposure of cells to cadmium, as well as the specific silencing of eIF4E gene, also resulted in decreased cellular levels of cyclin D1, a critical cell cycle and growth regulating gene, suggesting that the observed inhibition of cyclin D1 gene expression in the cadmium-treated cells is most likely due to decreased cellular level of eIF4E. Taken together, our results demonstrate that the exposure of cells to cadmium chloride resulted in cytotoxicity and cell death due to enhanced ubiquitination and consequent proteolysis of eIF4E protein, which in turn diminished cellular levels of critical genes such as cyclin D1.

Cadmium, a highly toxic chemical and one of the members of the U.S. Environmental Protection Agency's "Priority List of Chemicals," has been classified by the International Agency for Research on Cancer as a human carcinogen. Cadmium is found in abundance in the environment, at specific work places, and in food and water. Therefore, human exposure to cadmium is essentially unavoidable. Higher levels of cadmium have been detected in the urine and in various organs of exposed individuals (1, 2), and the toxicological responses of cadmium exposure include kidney damage, respiratory diseases, and neurological disorders (3). Exposure to cadmium has been implicated in

cancers of the lungs, prostate, pancreas, and kidney (4–6). The mortality risk among people exposed to cadmium shows a strong correlation with the exposure level (7). Exposure of cells to cadmium leads to a complex series of events, including deregulation of gene expression resulting in changes in the cellular pathways and corresponding networks that play a critical role in the response of cells to the toxicity of the chemical (8). In the past, multiple genes, including those involved in several critical cellular functions, have been identified as potential targets for cadmium-induced cytotoxicity and cell death (reviewed in Ref. 9); however, the one or more precise mechanisms that lead to their deregulation of expression in response to cadmium exposure as well as the functional significance of such alterations with respect to cadmium toxicity are not well studied. Despite identifying several genes that may potentially function as targets for cadmium-induced cytotoxicity and cell death, the underlying mechanisms responsible for such alterations and the mechanisms that lead to cytotoxicity and cell death are not understood.

Alterations in the expression of cellular genes in response to their exposure to toxic chemicals may be due to the chemical-induced changes in cellular transcription and/or translation machinery. Although the cellular effects of chemicals, including those of cadmium, are relatively better understood with respect to transcription, there are not many studies that address how chemical exposures influence gene expression at the translation level. In eukaryotes, translation of mRNAs to synthesize the encoded proteins is a complex process carried out by several genes collectively referred to as translation factors. Three distinct phases of translation have been identified and characterized: *viz.* initiation, elongation, and termination (10). Of the more than two dozen translation initiation factors that have been cloned and characterized to date, the eukaryotic translation initiation factor 4E (eIF4E)<sup>1</sup> assumes paramount importance. eIF4E functions in the rate-limiting step of translation initiation where it binds with the 7-methylguanosine cap of mRNA and recruits the given transcript to the translational machinery. The function of eIF4E is conserved from yeast to humans (11). Overexpression of the antisense oligonucleotides to eIF4E is lethal in HeLa cells (12) suggesting that the eIF4E gene is essential for survival of cells. The eIF4E gene is a critical regulator of cellular growth (13), and its overexpression results in oncogenic transformation of immortalized cell lines (14–16). Increased levels of eIF4E are correlated with a poor

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<sup>1</sup> The abbreviations used are: eIF4E, eukaryotic translation initiation factor 4E; HO-1, hemeoxygenase-1; MT-2, metallothionein-2; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CHO, Chinese hamster ovary; siRNA, small interference RNA; ALLN, N-acetyl-Leu-Leu-nor-leucinal; MG-132, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal.

clinical outcome in a variety of human cancers, including breast cancer (17) and several non-Hodgkin's B-cell lymphomas (18). The oncogenic potential of eIF4E is attributed to its ability to inappropriately translate growth-promoting transcripts (13). However, whether eIF4E is a cellular target for chemical-induced toxicity and carcinogenesis remains to be studied.

Recently, we have reported that deregulation of translation, as evidenced from overexpression of translation initiation factor 3 (eIF3) and translation elongation factor-1 $\delta$  (eEF-1 $\delta$ ) in cadmium-transformed Balb/c-3T3 cells, is a major mechanism responsible for cell transformation and tumorigenesis induced by cadmium (19–21). Presently, we have investigated the effect of cadmium exposure on the cellular expression level of eIF4E, the rate-limiting translation initiation factor involved in eukaryotic protein synthesis. In addition, we have found that eIF4E is a novel cellular target for cadmium toxicity and the resulting cell death. The underlying mechanisms responsible for cadmium-induced deregulation of eIF4E leading to cytotoxicity and cell death are presented.

#### EXPERIMENTAL PROCEDURES

##### *Cell Culture and Cytotoxicity Studies*

Human cell lines, *viz.* HeLa (cervical adenocarcinoma, catalogue number CCL-2), HCT15 (colon adenocarcinoma, catalogue number CCL-225), PLC/PR/5 (hepatocellular carcinoma, catalogue number CRL-8024), and Chang (likely derived from HeLa cells, catalogue number CCL-13), were purchased from ATCC (Manassas, VA) and were cultured in the medium containing fetal bovine serum and all required supplements as recommended by the supplier. Exponentially growing cells were used to determine the lethal concentration 50 (LC<sub>50</sub>) for CdCl<sub>2</sub>. One hundred thousand cells each were plated in the individual wells of a 96-well cell culture plate. The cells were allowed to attach and grow overnight. Cadmium chloride (Sigma) was dissolved in sterile water to prepare a stock solution and was diluted with fresh, serum-free cell culture medium to get final concentrations ranging from 0 to 100  $\mu$ M. The cells were allowed to grow in the control as well as in the medium containing increasing concentrations of CdCl<sub>2</sub> for 24 h. Cytotoxicity was determined by quantitating the number of surviving cells as evidenced from the metabolic reduction of a tetrazolium salt (MTT assay, ATCC, Manassas, VA). The LC<sub>50</sub> for CdCl<sub>2</sub> was determined for each of the cell lines based on their response to increasing concentrations of the chemical that resulted in 0–100% cell death.

##### *Effect of CdCl<sub>2</sub> on eIF4E Expression*

The effect of CdCl<sub>2</sub> on the expression level of the eIF4E gene was determined by Western blot analysis. The cells were treated for 24 h with CdCl<sub>2</sub> at the respective LC<sub>50</sub> values obtained for each of the cell lines as determined by an independent cytotoxicity experiment. The chemically-treated cells were washed with phosphate-buffered saline, and the cell lysates were prepared from the control and the cadmium-treated cells using the mammalian protein extraction reagent (Pierce) consisting of 20 mM HEPES, pH 7.6, 75 mM NaCl, 2.5 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 0.1% Triton X-100, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 50 mM NaF, 1  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml pepstatin, and 1 mM 4-(2-aminomethyl)benzenesulfonyl fluoride. The cell lysates were centrifuged at 10,000 rpm for 10 min in the cold, and the supernatants were collected. Protein concentrations of the lysates were determined spectrophotometrically using Bio-Rad protein assay reagent (Bio-Rad). Equal amounts of protein (30  $\mu$ g each) from the control and the CdCl<sub>2</sub>-treated cells were electrophoresed on a 12% SDS-containing denaturing gel and were transferred onto a polyvinylidene membrane for Western blot analysis. The eIF4E protein reacting with a mouse anti-human eIF4E antibody (BD Biosciences) was detected using an enhanced chemiluminescent system (Amersham Biosciences). Subsequently, the blots were stripped of the eIF4E antibody, and the presence of the housekeeping gene, GAPDH, was detected by Western blot analysis. Intensity of the eIF4E, eIF4G, and GAPDH protein bands reacting with the corresponding antibodies was determined by scanning the Western blot images using a densitometer equipped with ImageQuant software (Amersham Biosciences).

##### *Quantitative Real-time PCR Analysis of eIF4E, Metallothionein-2, and Hemoxygenase-1 Gene Expression*

The expression profile of the target genes, eIF4E, metallothionein-2 (MT-2), hemoxygenase-1 (HO-1), and the housekeeping gene GAPDH, was determined by quantitative real-time PCR. Total RNA from the control and the CdCl<sub>2</sub>-treated cells was isolated using the RNeasy mini kit (Qiagen). The purity and integrity of the RNA samples were determined by UV-spectrophotometry and by agarose gel electrophoresis analysis, respectively. Total RNA was reverse transcribed using the Advantage RT-for-PCR kit (BD Biosciences). Nucleotide sequences of the primers used to amplify the various genes were: eIF4E, 5'-CTA CTA AGA GCG GCT CCA CCA C-3' (forward (F)) and 5'-TCG ATT GCT TGA CGC AGT CTC C-3' (reverse (R)); MT-2, 5'-CGG CTC CTG CAA ATG CAA AGA G-3' (F) and 5'-AGA TGC AGC CCT GGG CAC AC-3' (R); HO-1, 5'-CAT TGC CAG TGC CAC CAA GTT C-3' (F) and 5'-GGA ACG CAG TCT TGG CCT CTT C-3' (R); and GAPDH, 5'-GCC TTC TGC ACC ACC AAC TGC-3' (F) and 5'-GGC AGT GAT GGC GTG GAC TAT G-3' (R). The PCR amplification, detection of the amplified gene products, and their quantitation were performed with the SYBR green PCR kit (PE Applied Biosystems, Foster City, CA) and the ABI-PRISM 5700 sequence detection system following the instructions provided by the manufacturer. The PCR-amplified gene products were analyzed by agarose gel electrophoresis to ensure that only the intended product was amplified in each case. The expression levels of the individual genes, calculated using the formula,  $2^{-(Ct_{\text{target}} - Ct_{\text{GAPDH}})}$ , were normalized to the expression level of the housekeeping gene, GAPDH.

##### *Construction of Transgenic CHO-K1 Cell Lines and Cytotoxicity Studies*

Transgenic Chinese hamster ovary-K1 (CHO-K1) cells permanently overexpressing the eIF4E gene were generated as follows: The open reading frame of the human eIF4E cDNA (GenBank™ accession number NM\_10029) was subcloned in-frame with the V5 epitope and the His<sub>6</sub> tag of the expression vector, pcDNA3.1D/V5-His-TOPO (Invitrogen) following the instructions provided by the manufacturer. Plasmid DNA prepared using the Qiagen maxi preparation kit (Qiagen) was used to transfect CHO-K1 cells by the calcium phosphate procedure (BD Biosciences). Stable transfectants were selected by culturing the transfected cells in medium containing G418 at a final concentration of 200  $\mu$ g/ml, and cell lines were developed individually from surviving colonies. Overexpression of the cDNA-encoded protein was determined by Western blot analysis using antibody for the V5 epitope of the fusion protein as well as the eIF4E antibody (BD Biosciences).

Cytotoxicity of CdCl<sub>2</sub> in the control (transfected with the empty vector) and the eIF4E-overexpressing transgenic CHO-K1 cell lines was determined by treating the cells for 24 h with increasing concentrations of the chemical and determining the number of surviving cells using the MTT assay as described above.

##### *Silencing eIF4E Gene Expression and Cytotoxicity Determination*

The endogenous eIF4E expression in HeLa and HCT15 cells was silenced by employing the small interfering RNA (siRNA) technique. Potential siRNA target sites present in the eIF4E mRNA (GenBank™ accession number NM\_10029) were identified following the recommendations of Ambion, Inc. (Austin, TX) for siRNA design. Several potential siRNA target sites were identified and screened against the GenBank™ data base by the BLAST program to ensure that the selected target sequences did not exhibit similarity to other known gene sequences available in the data base. This step was done to prevent potential nonspecific silencing of gene expression. The potential siRNA target sequences thus selected were used to generate Silencer™ Expression Cassettes using the Silencer™ Express siRNA Expression Cassettes Kit (Ambion Inc.) following the instructions provided by the manufacturer. The Silencer™ Expression Cassette consisted of the H1 RNA polymerase promoter adjacent to a hairpin siRNA template and an RNA polymerase terminator. Three Silencer™ Expression Cassettes potentially capable of silencing the expression of the eIF4E gene were tested in HeLa and HCT15 cells. The cells were transfected with the individual Silencer™ Expression Cassettes using the Lipofectamine 2000 reagent (Invitrogen). Forty-eight hours following the transfection, cell lysates were prepared and tested for silencing of the expression of the eIF4E gene by Western blot analysis using human eIF4E antibody.

For cytotoxicity determination, exponentially growing HeLa cells were plated in individual wells of a 96-well cell culture plate and transfected with eIF4E siRNA ranging in concentrations from 0 to 2.5

nmol. Forty-eight hours following the transfection, cytotoxicity was determined by the MTT assay.

Whether silencing the expression of eIF4E will modify the response of cells to cadmium toxicity was also studied. HeLa cells plated on a 96-well cell culture plate were transfected with 0, 0.5, and 1.0 nmol each eIF4E-siRNA. Twenty-four hours following the transfection, the cells were treated with CdCl<sub>2</sub> at final concentrations of 0, 16, and 32  $\mu$ M for 24 h. At the end of the chemical exposure period, cytotoxicity was determined by MTT assay.

#### Cadmium-induced Ubiquitination of eIF4E Protein

**Induction of Ubiquitin by Cadmium Chloride**—Exponentially growing HeLa cells were treated with CdCl<sub>2</sub> at a final concentration of 32  $\mu$ M (LC<sub>50</sub>). At the end of the treatment period, cell lysates were prepared from the control and chemically treated cells. The cellular levels of ubiquitin, Nrf2, and eIF4E proteins were determined by Western blot analysis using antibodies for human ubiquitin, Nrf2 (Santa Cruz Biotechnology, Santa Cruz, CA), and eIF4E (BD Biosciences), respectively, as described above.

**Immunoprecipitation Experiments**—Immunoprecipitation experiments were carried out to determine whether the eIF4E protein underwent ubiquitination in the cadmium-treated cells. Exponentially growing HeLa cells were treated with 32  $\mu$ M CdCl<sub>2</sub> for 24 h. The cells were washed twice with ice-cold phosphate-buffered saline, and lysates were prepared. Immunoprecipitation was carried out for 16 h at 4 °C using a polyclonal rabbit anti-human ubiquitin antibody (Santa Cruz Biotechnology) and protein A-Sepharose beads (Amersham Biosciences) following procedures as described elsewhere (22). The immunoprecipitated proteins conjugated to the protein A-Sepharose beads were washed twice with lysis buffer and then boiled with denaturing SDS-PAGE loading buffer. The supernatant collected was used for Western blot analysis using human eIF4E antibody as described above.

**Proteasome Inhibitor Studies**—Using inhibitors for proteasome activity, the involvement of proteasome-mediated proteolysis of eIF4E protein in response to cadmium exposure was investigated. Exponentially growing HeLa cells were treated with the proteasome inhibitors, ALLN (25  $\mu$ M), MG-132 (10  $\mu$ M), and lactacystin (10  $\mu$ M), for 24 h. All three proteasome inhibitors used were purchased from Calbiochem. Three hours following the initiation of incubation of cells with the proteasome inhibitors, the cells were treated with CdCl<sub>2</sub> at a final concentration of 32  $\mu$ M for 21 h. The cell lysates prepared from the control and the CdCl<sub>2</sub>-treated cells at the end of the incubation period were analyzed for the expression of eIF4E protein by Western blot analysis using the human eIF4E antibody.

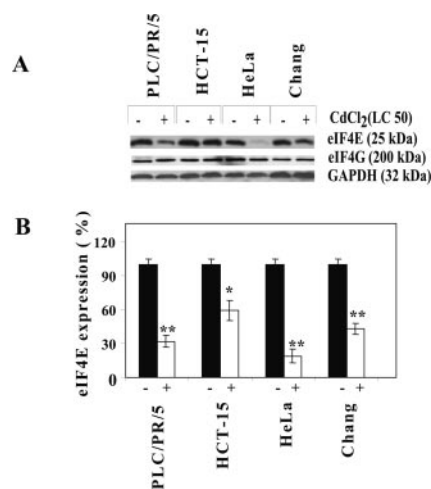
#### Statistical Analysis of the Data

The data were analyzed using SAS/STAT software, version 8.2, of the SAS System for Windows (SAS Institute, Cary, NC). Experiments were performed using a randomized complete block design structure and analyzed using mixed model analyses of variance. Post hoc comparisons were made using Fisher's least significant difference (LSD) test, and all differences were considered statistically significant at  $p < 0.05$ . Western blot and PCR data were normalized to the level of the housekeeping gene GAPDH, whereas cell survival assays were analyzed using the percentage of the control group as the dependent variable. The Pearson product moment correlation coefficients between eIF4E expression and the percentage of surviving cells based on the MTT assay were calculated in cultures treated under identical experimental conditions. Briefly, simulated data sets were generated using bootstrap sampling and matching values obtained from cultures treated under identical conditions. For each simulation, a correlation coefficient was calculated. The simulation was repeated 500 times, and the average correlation coefficient is reported.

## RESULTS

**Exposure of Cells to CdCl<sub>2</sub> Resulted in Cytotoxicity, Cell Death, and Inhibition of eIF4E Protein Expression**—Exposure of all four human cell lines, *viz.* HeLa, HCT15, PLC/PR/5, and Chang, to increasing concentrations of CdCl<sub>2</sub> resulted in concentration-dependent cytotoxicity and cell death as evidenced from results of the MTT assay. The LC<sub>50</sub> values for CdCl<sub>2</sub>, calculated from the dose-response curves of the individual cell lines were as follows: HCT15, 29.66  $\pm$  0.41  $\mu$ M; HeLa, 32.33  $\pm$  3.26  $\mu$ M; PLC/PR/5, 19.30  $\pm$  2.04  $\mu$ M; and Chang cells, 28.00  $\pm$  1.22  $\mu$ M.

Results of the experiment designed to determine the effect of



**FIG. 1. Expression of eukaryotic translation initiation factor 4E (eIF4E) is inhibited by cadmium chloride.** Exponentially growing human cell lines, PLC/PR/5, HCT15, HeLa, and Chang, were treated with cadmium chloride for 24 h at the respective LC<sub>50</sub> concentrations as determined in a separate experiment. Cell lysates equivalent to 30  $\mu$ g of total protein prepared from the control (–) and the cadmium-treated (+) cells were used to determine the expression of eIF4E, eIF4G, and the housekeeping gene GAPDH, by Western blot analysis using procedures as described under “Experimental Procedures.” The experiment was repeated four times, and the results of a representative experiment are presented in A. The Western blots were scanned using a densitometer equipped with the ImageQuant software (Amersham Biosciences), and the intensities of eIF4E, eIF4G, and GAPDH protein bands reacting with the corresponding antibodies were determined. The cellular expression levels of eIF4E and eIF4G proteins were normalized to that of GAPDH, and the percent inhibitions compared with the corresponding control cell lines were calculated using the data obtained from four independent experiments and are presented (mean  $\pm$  S.E.) in B. The expression levels of eIF4G in the cadmium-treated cells were not different from those of the corresponding controls and therefore are not presented in B. \*, statistically significant compared with the corresponding control cells (\*,  $p < 0.01$ ; \*\*,  $p < 0.001$ ).

exposure of cells to CdCl<sub>2</sub> on eIF4E expression revealed significant inhibition of eIF4E protein in all four cell lines tested. The cell lines treated with CdCl<sub>2</sub> at the respective LC<sub>50</sub> levels demonstrated significant inhibition of eIF4E at time intervals of 16 and 24 h, and the inhibition was more significant at the 24-h exposure period compared with that at the 16-h period (Western blot analysis results following the 24 h of exposure to CdCl<sub>2</sub> are presented in Fig. 1A). The highest inhibition was noticed in the case of HeLa cells (75%), whereas the lowest inhibition (40%) was noticed in the case of HCT15 cells (Fig. 1B). The specificity of the inhibitory effect of CdCl<sub>2</sub> on eIF4E is demonstrated by the lack of inhibition of eIF4G, the second member of the eIF4 family of translation factors (Fig. 1A).

Whether the cadmium-induced inhibition of eIF4E is due to the suppression of transcription of the eIF4E gene was tested by analyzing the eIF4E transcript level in the control and the CdCl<sub>2</sub>-treated cells by quantitative real-time PCR analysis. As presented in Table I, excepting for slight, but not statistically significant reductions as noticed in the HeLa and PLC/PR/5 cells, all four human cell lines treated with CdCl<sub>2</sub> exhibited eIF4E transcript level comparable to those of their corresponding control cell lines. In contrast, the expression levels of MT-2 and HO-1 genes in the CdCl<sub>2</sub>-treated cells were significantly higher compared with the control cells.

**Silencing the Expression of eIF4E Gene by Transfecting the Cells with eIF4E siRNA Resulted in Cytotoxicity and Cell Death**—Transfecting the human cell lines with eIF4E siRNA resulted in a significant inhibition of eIF4E mRNA (data not



TABLE I  
Expression of eukaryotic translation initiation factor 4E (eIF4E), hemeoxygenase-1 (HO-1), and metallothionein-2 (MT-2) mRNAs in cadmium chloride-treated cells

HCT-15, HeLa, PLC/PR/5, and Chang cells were treated with cadmium chloride for 24 h. Expression of eIF4E, HO-1, MT-2, and GAPDH were determined by real-time PCR as described under "Experimental Procedures." Data presented are mean  $\pm$  S.E. of four independent experiments.

Cell line	CdCl <sub>2</sub>	Gene expression		
		eIF4E	HO-1	MT-2
	<i>LC<sub>50</sub></i>		<i>arbitrary units</i>	
HCT-15	(-)	0.064 $\pm$ 0.006	0.025 $\pm$ 0.003	0.034 $\pm$ 0.006
	(+)	0.074 $\pm$ 0.011	0.364 $\pm$ 0.048 <sup>a</sup>	4.966 $\pm$ 0.188 <sup>a</sup>
HeLa	(-)	0.060 $\pm$ 0.022	0.039 $\pm$ 0.024	0.176 $\pm$ 0.029
	(+)	0.024 $\pm$ 0.003	1.015 $\pm$ 0.286 <sup>a</sup>	3.008 $\pm$ 0.299 <sup>a</sup>
PLC/PR/5	(-)	0.066 $\pm$ 0.024	0.047 $\pm$ 0.014	0.018 $\pm$ 0.009
	(+)	0.036 $\pm$ 0.010	1.609 $\pm$ 0.191 <sup>a</sup>	2.215 $\pm$ 0.372 <sup>a</sup>
Chang	(-)	0.019 $\pm$ 0.007	0.015 $\pm$ 0.003	0.459 $\pm$ 0.051
	(+)	0.023 $\pm$ 0.004	0.837 $\pm$ 0.141 <sup>a</sup>	2.166 $\pm$ 0.076 <sup>a</sup>

<sup>a</sup> Statistically significant compared with the corresponding controls ( $p < 0.001$ ).

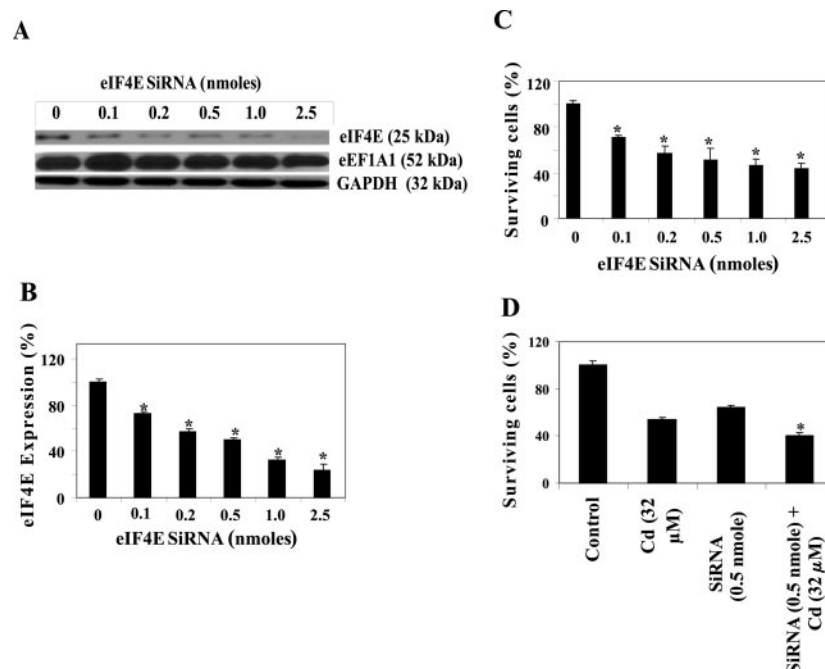
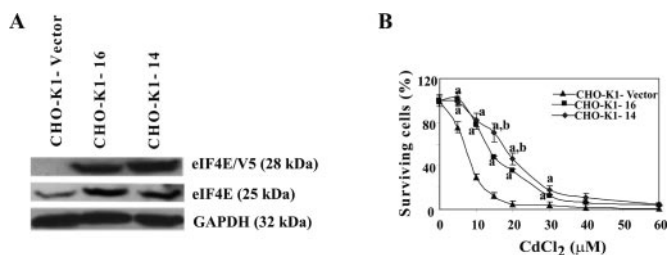


FIG. 2. **Transfection of human cell lines with eIF4E-siRNA resulted in silencing of eIF4E gene expression, cytotoxicity, and cell death.** Human cells (HCT15 and HeLa) were transfected with the indicated concentrations of eIF4E-siRNA targeting the expression of eIF4E gene. Forty-eight hours following the transfection, cell lysates were prepared from the transfected cells and the cellular levels of eIF4E, eEF1A1, and GAPDH proteins were determined by Western blot analysis using antibodies for the corresponding proteins. The cytotoxicity and cell death due to silencing of eIF4E gene expression caused by transfecting the cells with the eIF4E-siRNA were simultaneously determined by MTT assay as described in the text. In another series of experiments, HeLa cells were transfected with eIF4E siRNA, and 24 h following the transfection, the cells were exposed to CdCl<sub>2</sub> and the cytotoxicity was determined by MTT assay. The experiments were repeated four times, and the results are presented as mean  $\pm$  S.E. **A**, results of a typical Western blot analysis experiment using HeLa cells demonstrating the silencing of eIF4E protein. **B**, the summary of results from four independent experiments using HeLa cells demonstrating the silencing of eIF4E gene by eIF4E-siRNA transfection. The Western blots were scanned and the expression levels of eIF4E protein were normalized using those of GAPDH. The percent expression of eIF4E protein in the transfected cells is calculated compared with that of the control and the results are presented (mean  $\pm$  S.E.). \*, statistically significant compared with the corresponding controls ( $p < 0.01$ ). **C**, summary of the results from four independent experiments (HeLa cells) demonstrating cytotoxicity and cell death following silencing the expression of eIF4E gene by transfecting the cells with the eIF4E-siRNA. The percentage of surviving cells following the transfections compared with the control cell line was calculated, and the results are presented as mean  $\pm$  S.E. \*, statistically significant compared with the corresponding controls ( $p < 0.01$ ). **D**, summary of results from four independent experiments using HeLa cells demonstrating the enhanced cytotoxicity of CdCl<sub>2</sub> in cells transfected with eIF4E siRNA. \*, statistically significant compared with the cells treated with CdCl<sub>2</sub> or transfected with eIF4E siRNA ( $p < 0.01$ ).

presented) and eIF4E protein (Fig. 2A), and the inhibitory effect was dependent on the concentration of eIF4E siRNA that was employed in the transfection (Fig. 2, A and B). Expression of GAPDH, the housekeeping gene employed in the experiment, was not influenced by eIF4E siRNA transfection. The specific nature of inhibition of eIF4E protein with the eIF4E siRNA was further evidenced from the lack of inhibition of another translation factor, eukaryotic translation elongation factor 1A1 (eEF1A1), in the transfected cells compared with the control cells (Fig. 2A). Significant cytotoxicity and cell death were noticed among the cells transfected with the eIF4E siRNA

as evidenced from results of MTT assay (Fig. 2C). Furthermore, the cytotoxicity and cell death among the eIF4E siRNA-transfected cells exhibited a strong correlation (correlation co-efficient,  $r = 0.70$ ) with the concentration of eIF4E siRNA that was used in the transfection (Fig. 2, B and C). Similar to the results obtained with the eIF4E protein, inhibition of eIF4E mRNA was also noticed in the cells transfected with the eIF4E siRNA (data not presented). As presented in Fig. 2D, the siRNA-mediated silencing of eIF4E gene expression resulted in enhanced cytotoxicity when the cells were exposed to CdCl<sub>2</sub>.



**FIG. 3. Overexpression of eIF4E gene in transgenic CHO-K1 cells is protective against cadmium-induced cytotoxicity and cell death.** Transgenic CHO-K1 cell lines overexpressing eIF4E gene were developed by sub-cloning the open reading frame of eIF4E cDNA in the directional cloning vector, pcDNA3.1 (Invitrogen), followed by transfection of the cells with the plasmid DNA and selection of stable transfectants using G418 as described under "Experimental Procedures." **A**, lysates prepared from the cells were analyzed by Western blotting using eIF4E or V5 antibody to determine the expression of the plasmid DNA encoded recombinant eIF4E/V5 fusion protein. The blot was stripped, and the abundance of the housekeeping gene, GAPDH, was determined by Western blot analysis. **B**, the control and eIF4E-overexpressing transgenic CHO-K1 cell lines were treated with increasing concentrations of CdCl<sub>2</sub> for 24 h, and the number of surviving cells at the end of the treatment period was determined by MTT assay. The percentage of surviving cells in the CdCl<sub>2</sub>-treated cells was calculated compared with that of the control, and the results are presented as mean  $\pm$  S.E. (CHO-K1-Vector, CHO-K1 cells transfected with the empty pcDNA3.1 vector; CHO-K1-14 and CHO-K1-16 are two transgenic CHO-K1 cell lines overexpressing eIF4E gene.) *a*, CHO-K1-14 and CHO-K1-16 cell lines exhibiting a statistically significant ( $p < 0.001$ ) difference compared with the CHO-K1-Vector control cell line; *b*, CHO-K1-14 cell line exhibiting a statistically significant ( $p < 0.01$ ) difference compared with the CHO-K1-16 cell line.

**Overexpression of the eIF4E Gene in CHO-K1 Transgenic Cell Lines Was Protective against CdCl<sub>2</sub>-induced Cytotoxicity and Cell Death**—Transfection of CHO-K1 cells with the recombinant pcDNA3.1D/v5-His TOPO DNA containing the eIF4E cDNA resulted in a significant overexpression of the V5-eIF4E fusion protein as evidenced from the results of Western blot analysis. Furthermore, transfection of the cells with the plasmid DNA provided resistance to G418, and this facilitated the selection of stable transfectants overexpressing the eIF4E protein (Fig. 3A). The transgenic CHO-K1 cells exhibited significant resistance to the cytotoxicity and cell death induced by CdCl<sub>2</sub>, demonstrating the protective effect of eIF4E overexpression against cadmium-induced cytotoxicity and cell death. Furthermore, the protective effect seen correlated well with the expression level of eIF4E in the transgenic cell lines, and the CHO-K1-14 cell line overexpressing relatively higher levels of eIF4E transgene exhibited a significantly higher protective effect against the CdCl<sub>2</sub>-induced cytotoxicity and cell death compared with the CHO-K1-16 cell line (Fig. 3B).

**Enhanced Ubiquitination and Proteolysis of eIF4E Protein in the Cadmium-exposed Cells**—Results of the studies investigating the potential role of ubiquitination in the inhibition of eIF4E in cells treated with CdCl<sub>2</sub> demonstrated a definite role for the ubiquitination pathway in the CdCl<sub>2</sub>-induced decrease in the cellular level of eIF4E protein. Exposure of cells to CdCl<sub>2</sub> for 24 h resulted in a significant induction of ubiquitin protein as well as increased accumulation of ubiquitinated proteins as evidenced from results of Western blot analysis of the CdCl<sub>2</sub>-treated cells compared with the control cells (Fig. 4A). Exposure of cells to CdCl<sub>2</sub> for 24 h also resulted in the ubiquitination of eIF4E protein (Fig. 4B). Immunoprecipitation of the CdCl<sub>2</sub>-treated cells using an antibody for ubiquitin and further Western blot analysis of the immunoprecipitated proteins using eIF4E antibody demonstrated that eIF4E was pulled down by the ubiquitin antibody in the CdCl<sub>2</sub>-treated cells (Fig. 4C). The absence of eIF4E in the immunoprecipitated proteins of the control cells further supported the observation that exposure of

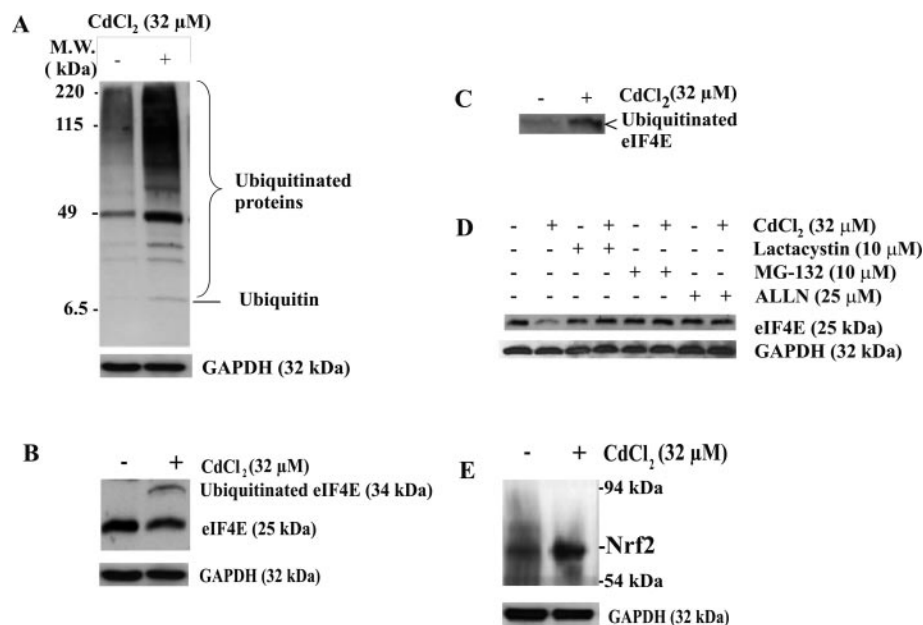
cells to CdCl<sub>2</sub> resulted in the ubiquitination of eIF4E protein. Results of the experiment involving inhibitors of proteasome activity confirmed the role of ubiquitination in the inhibition of eIF4E protein in the CdCl<sub>2</sub>-treated cells. The CdCl<sub>2</sub>-induced decrease in the cellular level of eIF4E protein was reversed by inhibitors of proteasome activity such as lactacystin, MG-132, and ALLN (Fig. 4D). The cellular level of Nrf2, in contrast to that of eIF4E, in the cadmium-treated cells was higher than that of the control cells (Fig. 4E) suggesting that exposure of cells to cadmium may cause effects specific for individual proteins.

**Cyclin D1 Expression Is Inhibited by CdCl<sub>2</sub> and by Silencing of eIF4E Gene Expression**—Results of the Western blot analysis demonstrated that cyclin D1 is a downstream target for both CdCl<sub>2</sub> treatment and eIF4E silencing in cells. Exposure of HeLa cells to CdCl<sub>2</sub> for 24 h resulted in the inhibition of cyclin D1 protein compared with the corresponding control cells. Similarly, silencing the expression of eIF4E by transfecting cells with eIF4E siRNA resulted in a significant inhibition of cyclin D1 (Fig. 5).

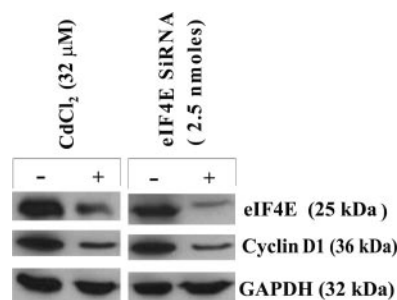
## DISCUSSION

Upon entering the body, toxic chemicals elicit their adverse effect(s) by a variety of cellular and molecular mechanisms. Such mechanisms include, but are not limited to, interactions with cellular machinery that regulate the expression of genes essential for cell survival. Cadmium, like many other toxic chemicals, is recognized for its capacity to deregulate, especially to up-regulate, the expression of genes, including those involved in stress response (23, 24), mitogenesis (25), oncogenesis (26, 27), apoptosis (28, 29) and signal transduction (8). Despite the identification of large numbers of genes that could be potential targets for cadmium toxicity, neither the precise cellular and molecular mechanisms responsible for alterations in the expression of these genes in response to cadmium exposure nor the functional significance of alterations in the expression of these genes with respect to cadmium-induced cytotoxicity and cell death are adequately understood. Results of the present study have identified that eIF4E is a cellular target for cadmium-induced cytotoxicity and cell death. Furthermore, the results have demonstrated that the activation of ubiquitination following cadmium exposure resulted in proteolysis and the subsequent degradation of eIF4E protein. The cadmium-exposed cells, due to their decreased cellular level of eIF4E protein, failed to synthesize/maintain normal cellular level of cyclin D1 protein. Most likely, due to the lack of normal cellular level of cyclin D1, a protein essential for cell cycle progression and growth, the cadmium-exposed cells failed to survive, resulting in cytotoxicity and cell death.

As previously reported (30), we have also found that exposure of cells to cadmium results in toxicity and cell death. The LC<sub>50</sub> value for CdCl<sub>2</sub> determined in all four human cell lines based on the MTT assay results ranged from 19 (PLC/PR/5) to 32  $\mu$ M (HeLa cells). Twenty-four hours of exposure of each of the four human cell lines to the respective LC<sub>50</sub> concentration of CdCl<sub>2</sub> resulted in a significant inhibition of eIF4E protein compared with the corresponding control cells. The fact that the inhibition of eIF4E was noticed in all of the cell lines in response to CdCl<sub>2</sub> exposure suggests that the inhibitory effect noticed was independent of the cell lines employed and is likely to be a response characteristic of exposure to CdCl<sub>2</sub>. The results of our study also suggested that the inhibitory effect of CdCl<sub>2</sub> on eIF4E protein is rather specific. This argument is supported by the observations that (i) eIF4E protein expression was significantly lower in all four cell lines treated with CdCl<sub>2</sub> compared with the corresponding control cells and (ii) the protein level of eIF4G, another member of the eIF4 family of transla-



**FIG. 4. The cadmium-induced decrease in cellular level of eIF4E protein is due to enhanced ubiquitination and the resulting proteolysis.** *A*, exposure of cells to CdCl<sub>2</sub> resulted in the ubiquitination of eIF4E protein. HeLa cells were treated with 32 μM CdCl<sub>2</sub> for 24 h, and cell lysates were prepared from the control (–) and the CdCl<sub>2</sub>-treated (+) cells. The cell lysates were used to determine ubiquitin protein level by Western blot analysis. Subsequently, the blots were stripped to remove the reacted ubiquitin antibody, and the cellular level of the housekeeping gene GAPDH was determined. The experiment was repeated four times, and the results of a representative experiment are presented. *B*, exposure of cells to CdCl<sub>2</sub> resulted in the accumulation of ubiquitinated eIF4E protein. HeLa cells were treated with 32 μM CdCl<sub>2</sub> for 24 h, and cell lysates were prepared from the control (–) and the CdCl<sub>2</sub>-treated (+) cells. The cell lysates were used to determine the cellular level of eIF4E protein by Western blot analysis. Subsequently, the blots were stripped to remove the reacted eIF4E antibody and the cellular level of the housekeeping gene, GAPDH, was determined. The experiment was repeated four times, and the results of a representative experiment are presented. *C*, ubiquitination of eIF4E protein in cells treated with CdCl<sub>2</sub>. HeLa cells were treated with 32 μM CdCl<sub>2</sub> for 24 h, and the cell lysates were prepared from the control (–) and CdCl<sub>2</sub> treated (+) cells. The cell lysates were subjected to immunoprecipitation using a human antibody for ubiquitin by procedures as described under “Experimental Procedures.” The immunoprecipitates were electrophoresed, and the presence of eIF4E protein in the immunoprecipitate was determined by Western blot analysis using a human eIF4E antibody. *D*, cadmium-induced degradation of eIF4E protein is reversed by proteasome inhibitors. HeLa cells were treated with CdCl<sub>2</sub> and various inhibitors of proteasome activity (lactacystin, MG-132, and ALLN) at final concentrations as indicated in the figure. The details regarding the concentration and duration of the various treatments are as described in the text. Lysates prepared from the cells were analyzed for the cellular level of eIF4E and GAPDH proteins by Western blot analysis. The experiment was repeated four times, and the results of a representative experiment are presented. *E*, exposure of cells to CdCl<sub>2</sub> resulted in enhanced cellular level of Nrf2 protein. HeLa cells were treated with 32 μM CdCl<sub>2</sub> for 24 h, and cell lysates were prepared from the control (–) and the CdCl<sub>2</sub>-treated (+) cells. The cell lysates were used to determine cellular level of Nrf2 protein by Western blot analysis. Subsequently, the blots were stripped to remove the reacted Nrf2 antibody, and the expression of the house-keeping gene, GAPDH, was determined. The experiment was repeated four times, and the results of a representative experiment are presented.



**FIG. 5. Exposure of cells to cadmium chloride as well as transfection with eIF4E-siRNA resulted in a decrease in cellular cyclin D1 protein level.** Exponentially growing HeLa cells were treated with 32 μM CdCl<sub>2</sub> for 24 h or transfected with the eIF4E-siRNA (2.5 nmol) as described under “Experimental Procedures.” Cell lysates were prepared, and the cellular levels of eIF4E, cyclin D1, and GAPDH proteins were determined by Western blot analysis. The experiment was repeated four times, and the results of a representative experiment are presented.

tion initiation factors, and that of the house-keeping gene GAPDH, were not inhibited in the cadmium-treated cells compared with the control cells.

Cadmium is a highly toxic chemical, and the proposed mechanisms responsible for cadmium toxicity include its interaction with cellular macromolecules, generation of reactive oxygen species, impairment of cellular anti-oxidant defense systems,

interference with signal transduction, activation of cellular proto-oncogenes, and the inhibition of cellular DNA damage repair process (reviewed in Ref. 9). Cadmium has also been characterized as a potent inducer of gene expression. Significant alterations in the expression of genes involved in oxidative stress response, DNA damage repair, apoptosis, signal transduction, oncogenesis, etc. have been noticed in cells and in animals exposed to cadmium (reviewed in Ref. 9). Results of the present *in vitro* study have demonstrated for the first time that exposure to cadmium down-regulated the expression of eIF4E, the mRNA cap-binding protein essential for initiation of peptide chain synthesis. In all four cell lines treated with CdCl<sub>2</sub>, the cellular expression level of eIF4E was significantly inhibited compared with the corresponding control cells. Inhibition of eIF4E in the CdCl<sub>2</sub>-treated cells was also associated with significant cytotoxicity and cell death. Results obtained from the experiments in which the eIF4E expression was specifically silenced using eIF4E siRNA suggest that the cytotoxicity and cell death noticed among the CdCl<sub>2</sub>-treated cells are due, at least in part, to the chemical-induced inhibition of eIF4E gene expression. Transfection of the cells with eIF4E siRNA resulted in significant silencing of eIF4E gene expression, and this was accompanied by significant cytotoxicity and cell death. The silencing of eIF4E gene expression as well as the resulting cytotoxicity and cell death of the eIF4E siRNA-transfected cells were dependent on the amount of the siRNA that was used in



the transfection. Furthermore, a strong correlation (correlation coefficient,  $r = 0.70$ ) between the siRNA-mediated silencing of eIF4E gene expression and the cytotoxicity and death of the transfected cells was observed illustrating the requirement for normal cellular levels of eIF4E for cell survival. The potential involvement of eIF4E in the cadmium-induced cytotoxicity and cell death was further supported by the results of the cytotoxicity experiment involving the transgenic CHO-K1 cell lines overexpressing the eIF4E gene. Results of earlier studies carried out in our laboratory have validated the usefulness of CHO-K1 cell line for studying gene function following overexpression of cDNA-encoded proteins (19, 20). Compared with the control cells transfected with vector alone, both the transgenic cell lines CHO-K1-14 and CHO-K1-16 overexpressing the eIF4E protein exhibited significantly higher resistance to the cytotoxicity and cell death induced by  $\text{CdCl}_2$ . Furthermore, between the two CHO-K1 transgenic cell lines overexpressing the eIF4E gene, the resistance to  $\text{CdCl}_2$ -induced cytotoxicity and cell death correlated well with the cellular expression level of eIF4E protein. Thus the CHO-K1-14 cell line overexpressing a higher level of the eIF4E transgene compared with the CHO-K1-16 cell line also exhibited significantly higher resistance to cadmium-induced cytotoxicity and cell death. These results, therefore, further support the argument that the cadmium-induced inhibition of eIF4E has a definite role in the ensuing toxicity and cell death.

Cadmium has been demonstrated as a potent inducer of gene expression and at least some of the toxicological properties of cadmium are thought to be mediated through the cadmium-induced alterations in the expression of genes involved in critical cellular functions. The majority of published studies aimed to investigate the cadmium-induced alterations in gene expression have focused on changes taking place at the transcript level. Transcripts for several genes, including those involved in cell proliferation, signal transduction, oncogenesis, apoptosis, oxidative stress response, DNA damage repair, and others have been found altered in response to exposure to cadmium compounds (reviewed in Ref. 9). Cadmium, either because of its effects on transcription factors such as AP1, MTF1, Nrf2, NF- $\kappa$ B, and others or due to the alterations in intracellular levels of reactive oxygen species or calcium ion, is known to result in transcriptional de-regulation of gene expression (27, 31–33). Despite such mounting evidence documenting the potential of cadmium to de-regulate gene expression at the transcriptional level, the cadmium-induced down-regulation of eIF4E does not seem to be mediated through its effects on the transcription of the gene. In any of the cell lines treated with cadmium, the eIF4E transcript level was not significantly different from the corresponding control cells, whereas the transcripts for two of the cadmium-responsive genes, HO-1 and MT-2, were significantly overexpressed in the same samples. The results may thus indicate that the regulatory mechanisms of eIF4E gene expression are different from some or all of the other genes that are known to be de-regulated by cadmium at the transcriptional level. Cloning the gene promoter and further analysis of the regulatory mechanisms may provide insights for the lack of cadmium-mediated de-regulation of eIF4E at the transcriptional level.

Ubiquitination, a post-translational process, has long been considered to be an adaptive response whereby cells eliminate damaged proteins (34). However, recent evidence indicates a major role for ubiquitination in the regulation of specific proteins involved in vital cell functions such as signaling, cell cycle, and differentiation (34). Results of the present study suggest that the reduced cellular level of eIF4E observed among the cadmium-treated cells is not due to transcriptional

repression of the gene but rather due to enhanced ubiquitination and the subsequent proteasome-mediated degradation of eIF4E protein. As reported previously (35), exposure of cells to cadmium resulted in a significant increase in the cellular level of ubiquitin protein. Even though the precise cellular mechanism(s) responsible for the cadmium-induced up-regulation of expression of ubiquitin has not been investigated in the present study, it is possible that oxidative stress potentially resulting from cadmium exposure, among other factors, was responsible for its induction (36). It is also possible that oxidative stress or any other mechanism of toxicity elicited by cadmium might have caused damage to the eIF4E protein leading to its enhanced ubiquitination and the subsequent degradation by proteasome activity resulting in reduction in the cellular level of eIF4E. Results of the Western blot analysis (Fig. 4B) and immunoprecipitation experiment (Fig. 4C) demonstrated ubiquitination of eIF4E in the cadmium-treated cells. Furthermore, the fact that pre-exposure of the cells to three different inhibitors of the proteasome pathway resulted in reversal of the cadmium-induced inhibition of eIF4E further confirmed that the reduced cellular level of eIF4E in the cadmium-treated cells was due to its enhanced ubiquitination and the subsequent proteolysis of the ubiquitinated eIF4E. Similar to the effect of  $\text{CdCl}_2$  on eIF4E, proteasome-mediated degradation of  $\text{Na}^+/\text{K}^+$ -ATPase has previously been reported in rat kidney proximal tubule cells treated with  $\text{CdCl}_2$  (36). However, in contrast to the effect of  $\text{CdCl}_2$  on eIF4E (present study) and on  $\text{Na}^+/\text{K}^+$ -ATPase (36), Stewart *et al.* (37) have reported that exposure of mouse hepatoma (Hepa) cells to  $\text{CdCl}_2$  resulted in the stabilization and activation of Nrf2, which was mediated through the ubiquitin-proteasome pathway. Similar to the findings of Stewart *et al.* (37), we have noticed increased cellular level of Nrf2 protein in HeLa cells treated with  $\text{CdCl}_2$  under the conditions that resulted in the inhibition of eIF4E. Thus our findings as well as those reported by other investigators (36, 37) may suggest that the exposure of cells to cadmium results in protein-specific effects and further studies are required for a better understanding of the reasons for such protein-specific effects of cadmium.

The precise cellular and molecular mechanisms responsible for the cytotoxicity and cell death following exposure of cells to cadmium are not clearly understood. Results obtained from the present study suggest that inhibition of eIF4E is a novel mechanism responsible for cadmium-induced cytotoxicity and cell death. This assumption is based on the observations that (i) cadmium-induced cytotoxicity and cell death are associated with significant inhibition of eIF4E, (ii) the eIF4E siRNA-mediated specific silencing of the eIF4E gene expression resulted in cytotoxicity and cell death, and (iii) ectopic overexpression of eIF4E was protective against the cadmium-induced cytotoxicity and cell death.

The cellular expression level of eIF4E is known to be a critical determinant regulating the translation of mRNAs, especially those possessing a complex 5'-untranslated region. The 5'-cap binding function of eIF4E is essential for the initiation of peptide chain synthesis. In addition, those mRNAs possessing a complex 5'-untranslated region are preferentially translated and in turn overexpressed in several tumor samples and cancer cell lines that exhibit overexpression of the eIF4E gene (38, 39). Those mRNAs possessing a complex 5'-untranslated region and, therefore, translationally more regulated by eIF4E include cyclin D1 (40), *c-myc* (41), and ornithine decarboxylase (42) among other genes (43). Our results demonstrated that the synthesis of cyclin D1, a protein essential for cell growth and cell cycle control, was down-regulated in cells by the cadmium-induced inhibition of eIF4E as well as by the

eIF4E-siRNA-mediated silencing of eIF4E gene expression. Thus, it appears that the exposure of cells to CdCl<sub>2</sub> resulted in a diminished cellular level of eIF4E protein. This, in turn, compromised the ability of the cells to synthesize and maintain proper levels of essential proteins, such as cyclin D1, resulting in cytotoxicity and cell death. Induction of apoptosis has been demonstrated as a major response of cells to exposure to cadmium (reviewed in Ref. 9). eIF4E has been demonstrated as an inhibitor of apoptosis induced by multiple agents (44, 45). Therefore, a potential increase in the incidence of apoptosis taking place in the cadmium-treated cells owing to reduced cellular level of eIF4E may be another mechanism responsible for cadmium-induced cytotoxicity and cell death. Currently, we are investigating the role of eIF4E in apoptosis induced by cadmium.

Cadmium is an established human carcinogen (5). Several previous studies have characterized eIF4E as a potential proto-oncogene (reviewed in Ref. 43). Ectopic expression of eIF4E results in cell transformation and tumorigenesis (14). In addition, marked overexpression of eIF4E has been found to be associated with the malignant transformation of multiple human tissues/organs (46). At this time, however, it is not understood what role, if any, eIF4E has in cadmium carcinogenesis, aside from its role in cytotoxicity and cell death as demonstrated in the present study. The cadmium-induced inhibition of eIF4E as has been demonstrated from results of the *in vitro* experiments described in this communication as well as the previously known roles of eIF4E in apoptosis (44, 45) and in cancer (43, 46) may suggest that eIF4E is less likely to play any significant role in cadmium carcinogenesis. Further validation of the present *in vitro* study using appropriate *in vivo* animal models and additional functional studies are required to understand the potential implication(s) of eIF4E inhibition in human carcinogenesis brought about by cadmium, a common environmental pollutant and a highly useful industrial metal.

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# **Eukaryotic Translation Initiation Factor 4E Is a Cellular Target for Toxicity and Death Due to Exposure to Cadmium Chloride**

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