Cap43, a Novel Gene Specifically Induced by Ni^{2+} Compounds

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ABSTRACT

To better understand the molecular mechanism(s) involved in the essentiability, toxicity, and/or carcinogenicity of nickel compounds, a RNA differential display technique was used to identify gene(s) that were specifically induced by these carcinogens. Differential expression of several genes was observed in human lung A549 cells exposed to nickel sulfide. One gene, Cap43, which expressed a 3.0-kb mRNA encoding a M, 43,000 protein, was found to be induced within 4–6 h by either NiS_{2} or NiCl_{2} in A549 cells and attained a level as high as 30-fold within 24–36 h of treatment. Twelve other tested metal compounds failed to induce Cap43 expression, leading to the conclusion that, with regard to metals, the induction of this gene was nickel-specific. Oxidative stress that is often caused by metals and heat shock did not induce Cap43 further, suggesting a specific nature in the signaling pathway involved in Cap43 induction. Activation of signaling pathways with vanadate did not induce Cap43 nor did trifluoperazine block its induction by nickel; however, okadaic acid, a serine/threonine phosphatase inhibitor, induced Cap43 to a greater extent than any nickel compound tested. Homocysteine did not induce Cap43 in a number of cell lines, with the exception of human endothelial cells. The Cap43 gene was found to be induced by nickel not only in all tested human and rodent cell lines in vitro but also in several rat organs after oral exposure to NiCl_{2}. We have found that the primary signal for Cap43 induction was an elevation of free intracellular Ca^{2+} caused by Ni^{2+} exposure because Cap43 was induced by calcium ionophores and its induction was attenuated by bis-(O-aminophenyl)-ethane-N,N,N',N'-tetraacetic acid (tetraacetoxyethyl-ester), a chelator of intracellular Ca^{2+}. We found that the Cap43 gene was evolutionarily conserved and similarly regulated in humans, mice, and rats. Recent studies have shown that Cap43 is expressed at lower levels in colon cancer. Further studies of Cap43 regulation by Ca^{2+} should enhance our understanding of the role of Cap43 in cell function and cancer pathogenesis.

INTRODUCTION

Nickel compounds are widely used in modern industries (1). Several industrial processes, e.g., nickel refining, electroplating, the production of long-lasting nickel-cadmium batteries, the combustion of fossil fuels, and the incineration of nickel-containing solid waste, are responsible for the production of nickel-containing aerosols in the workplace and in the surrounding environments (1). Because of the widespread use of these agents, workers in these facilities are at risk of occupational exposure (1). In addition, the release of nickel into the environment represents a potential for nonoccupational exposure (1). The average daily exposure to nickel by inhalation has been estimated to be 0.2 and 0.4 μg for rural and urban dwellers, respectively (1). Inhalation is the main route for human exposure to nickel compounds, and epidemiological studies have demonstrated a correlation between the incidence of respiratory (lung and nasal) cancer and worksite exposure to nickel (2–4). Tumors have also been induced in several animal models after inhalation, ingestion, or injection of various nickel compounds (5–7).

The molecular mechanisms of nickel toxicity and carcinogenicity have been the focus of numerous studies (8–10). It was found that nickel compounds, via direct or indirect mechanisms, can cause multiple types of cellular/nuclear damage (8, 11, 12). Based on these findings, the majority of cellular damage caused by nickel compounds may ultimately result in altered gene expression, rather than in direct DNA damage. It has been reported that the expression of transcription factors, MT, heat shock proteins, and three subgroups of acute phase reactants, such as α1-acid glycoprotein, serum amyloid A, and C-reactive protein can be induced by heavy metals, e.g., mercury, cadmium, lead, copper, zinc, and nickel (13–18). However, induction of these proteins is a common effect shared by several heavy metals, including mercury, cadmium, lead, copper, zinc, and nickel (13, 17–20). There are no reports of gene expression specifically induced by a nickel compound.

Ni^{2+} has been shown to block Ca^{2+} channels and release stores of free intracellular Ca^{2+} in a variety of systems, and some studies have reported that longer exposure to nickel compounds can elevate intracellular Ca^{2+} (21, 22). Water-soluble nickel salts are considered less carcinogenic than water-insoluble compounds, such as NiS_{2} and NiO, because the uptake of soluble Ni^{2+} is poor (9). Phagocytosis of NiS_{2} causes buildup of very high levels of nickel inside the cell after its intracellular dissolution catalyzed by the acidic pH of endocytic vacuoles (9). The carcinogenic potency of nickel compounds is related to the ability of Ni^{2+} to access chromatin, where it produces an increased chromatin condensation, enhanced DNA methylation, and turning off of the transcription of tumor suppressor and senescence genes (12).

In the present study, we have used the differential display technique (23) to isolate genes that might be induced by nickel compounds in human bronchoalveolar epithelial type II A549 cells. We report the identification and characterization of a novel gene (Cap43) that is induced in a dose- and time-dependent manner by exposure to non-toxic levels of both water-soluble and -insoluble Ni^{2+} compounds. No other metal compound significantly induced expression of this gene in A549 cells. Furthermore, this Cap43 gene is induced by nickel, not only in all other human cell lines tested, but also in several rat organs after a single in vivo oral exposure to nickel. The Cap43 gene encoded a 3.0-kb mRNA that can be translated into a M, 43,000 protein. The induction of Cap43 is mediated by a rise of free intracellular Ca^{2+} caused by exposure to nickel compounds (22), and because Cap43 has recently been found to be down-regulated in colon cancer (24), additional studies of Cap43 will enhance our understanding of the molecular mechanisms of carcinogenesis.

MATERIALS AND METHODS

Cell Culture. A549 (CCL 185), HUV-EC-C (CRL 1730), HOS (CRL 1543), Calu-1 (HTB 54), and WI-38 (CCL 75) cells were purchased from the American Type Culture Collection (Rockville, MD). HTE cells were a gift from Dr. L-C. Chen from New York University Medical School. The A549 cell line, derived from explant culture of lung carcinomatous tissue of a 58-year-old

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The abbreviations used are: MT, metallothionein; FBS, fetal bovine serum; PKA, cAMP-dependent protein kinase; APR, acute phase reactant.
Caucasian male, has epithelial-like morphology and a likely type II origin/ function. HUV-EC-C is an endothelial cell line derived from a normal umbilical cord vein. HOS is a human osteogenic sarcoma cell line derived from a 13-year-old Caucasian female. Calu-1 is an epidermoid carcinoma from a Caucasian male that originated in the lung and metastasized to the pleura. The WI-38 cell line was derived from normal embryonic lung tissue of a Caucasian female and has fibroblast-like morphology. HTE is a human tracheal epithelial cell transformed by an origin-defective SV40. All cells were maintained at 37°C as monolayers in a humidified atmosphere containing 5% CO₂. Cells were passaged at 70–80% confluence by trypsinization. The culture medium for each cell line was as follows: A549 cells were grown in Ham’s F-12K medium supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin; HOS and HTE cells in α-MEM supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin; WI-38 cells in Eagle’s basal medium supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin; Calu-1 cells in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin; and HUV-EC-C cells in Ham’s F-12K medium supplemented with 10% FBS, 100 μg heparin/ml, 30 μg of endothelial cell growth supplement/ml, 100 units/ml penicillin, and 100 μg/ml streptomycin.

The insoluble nickel compounds were suspended by sonication for 10 min in serum-free F-12K medium. Soluble compounds were either dissolved in sterile water or DMSO. Monolayers (75% confluent in 60- or 100-mm dishes) were incubated for 24 h with varying doses of each compound or with a fixed dose of each compound for varying lengths of time (time course study).

mRNA Differential Display and Cloning of the Cap43 Gene. We used the differential display technique to identify genes induced by nickel compounds (23). A549 cells were exposed to Ni₃S₂ at either 0.0, 0.1, or 0.3 μg per cm² for 24 h. mRNA differential display was performed using MessageClean and RNAimage kits (GenHunter Corp.) according to the manufacturer's instructions. Total RNA was extracted from the A549 cells using an ULTRASPEC RNA isolation system (Biotect) and treated with DNase I from MessageClean kit (GenHunter Corp.) to eliminate chromosomal DNA contamination. Forty-eight different combinations of primer pairs (3 anchor primers and 16 arbitrary primers) were divided into kits 1 and 2. Oligo dT primers (23) were used for mRNA differential displays. After isolation of the Cap43 cDNA fragment (295 bp) from the differential display gel, the fragment was cloned into pCR-TRAP vector (GenHunter Corp.). The insert was then PCR amplified and used as a probe for Northern blot analysis and cDNA library screening.

Northern Blot Analysis. Total RNA was extracted from cells immediately after exposure using an ULTRASPEC RNA isolation system (Biotect) and electrophoresed (20 μg total RNA/lane) in 1.2% agarose/formaldehyde gels. The probe amplified from the differential display gel was labeled with [α-³²P]dCTP using a Random Primed DNA labeling kit (Boehringer Mannheim).

cDNA Library Screening and Sequencing. Human lung and brain cDNA libraries were purchased from Stratagene. A human lung cDNA library, constructed using oligo dT primers, had inserts (>0.5 kb) ligated to EcoRI and XhoI cloning sites in Uni-Zap XR vectors. The human brain cDNA library, constructed using oligo dT and random primers, had inserts (>0.5 kb) ligated to the EcoRI cloning site in lambda Zap II vectors. XLI-Blue MR++ strain is the recommended host strain for amplification and screening of both Uni-Zap XR and lambda Zap II cDNA libraries. Host cells and phage preparation, screening, and in vivo excision were performed according to the instruction manual from Stratagene. The human lung cDNA library was first screened using the Cap43 cDNA fragment isolated by differential display, and inserts from the positive colonies were sequenced by automated sequence analysis and aligned using MacVector software (Dr. Bernard Goldschmidt, New York University Medical Center, Kaplan Comprehensive Cancer Center).

In Vivo Exposure of Rats to Nickel. The rats used in this study were pathogen-free female and male Sprague Dawley rats (200–250 g) obtained from Taconic Farms (Germantown, NY). All rats were quarantined for 2 weeks prior to exposures. Rats were housed in individual nickel-free cages in a temperature (20°C)- and humidity (50% relative humidity)-controlled rooms and provided Purina Rodent Chow and water ad libitum. A solution of 20 mg of NiCl₂/ml in normal saline was prepared and administered by gavage at a dose of 50 mg of nickel/kg body weight; control rats were gavaged with vehicle only. At 36 h after NiCl₂ administration, rats were euthanized by i.p. injection of Nembutal (125 mg/kg, i.p.), and the lungs, liver, kidneys, heart, brain, and skeletal muscle were removed and immediately processed in an ULTRASPEC RNA isolation solution. Total RNA extraction and Northern analysis were performed as described earlier.

RESULTS

Identification and Cloning of a Cap43 Gene Induced by Nickel Compounds in Human Lung A549 Cells by mRNA Differential Display Analysis. A mRNA differential display assay was performed to compare gene expression in A549 cells treated with either 0.0, 0.1, or 0.3 μg Ni₃S₂/cm² for 24 h. The doses of Ni₃S₂ used here were weakly toxic, as assessed by the trypan blue exclusion assay and colony proliferation assays (data not shown). A total of 48 different combinations of primer pairs (3 anchor primers and 16 arbitrary primers) from RNAimage kits 1 and 2 were used for the differential display. To assess assay reproducibility, duplicate RNA samples were used for RT-PCR amplification. A representative differential display autoradiograph with four different primer combinations and PCR products (Cap43 cDNA fragments) is shown in Fig. 1. The band intensities that were reproducibly increased by nickel treatment, are highlighted (Fig. 1). The Cap43 cDNA fragment was isolated from the differential display gel, reamplified by PCR using the same primer pair, and used as a probe for Northern blotting. A single band corresponding to approximately 3.0 kilobase pairs was found to be induced by nickel in A549 cells. As shown in Fig. 2A and B, both water-soluble nickel chloride (NiCl₂) and water-insoluble nickel subsulfide (Ni₃S₂) were able to induce Cap43 gene expression in a time-dependent manner. Equitoxic doses of NiCl₂ and Ni₃S₂ were equally potent in inducing Cap43 expression (Fig. 2C).

The Cap43 Gene Belongs to a Novel Gene Family. The Cap43 cDNA fragments were cloned from a human lung cDNA library and sequenced. Fig. 3A represents the cDNA sequence of the Cap43 gene obtained in our laboratory. The Cap43 mRNA had a 1759-bp 5′ untranslated region, and its predicted open reading frame encoded 394 amino acid residues with a deduced molecular weight of 43,400 and an isoelectric point of 5.3. The high content of serines and threonines

Ni₃S₂ (μg/cm²)
The blot was first hybridized with the Cap43 fragment (top) isolated in the differential probe was performed to show the loading (bottom panel). C. effects of soluble (NiCl₂) and insoluble (Ni₃S₂) nickel compounds on Cap43 expression. A549 monolayers (75% confluent) were exposed to 2.0 μg of NitS₂/cm² or 1.0 mM NiCl₂ for the time periods indicated in the figure. RNA was isolated, and Northern blot analysis was performed as described in “Materials and Methods.” Hybridization with an actin gene, the cytoplasmic protein Ndrl (U60593), 4 and TDD5 (U52073; Ref. 24) were also found in GenBank. Multiple gene alignment of these four sequences is shown in Fig. 3B. The Cap43 and RTP genes have the same predicted amino acid sequences, although there are a few differences in the 3′-untranslated region, and there was a single amino acid difference from Drg1. In Drg1, isoleucine was changed to threonine because of a T-to-C transition, but all other cloned genes, including two mouse genes, have isoleucine in this position (Fig. 3B). In spite of one amino acid difference in Cap43, RTP and Drg1 represented the same human gene, and Ndrl was their mouse homologue. The TDD5 had the same NH₂-terminal part of the protein, but significant differences in the COOH-terminal part existed, suggesting that it probably represented another member of this gene family with an as yet to be described human homologue. The efficient hybridization of rat mRNA with the human probe under stringent conditions indicated the existence of a rat homologue for Cap43-RTP-Drg1 gene.

The cellular functions of Cap43 are not known. Although a number of computer programs available for protein analysis including GCG, PROSITE pattern search, and MacVector were used to search for the possible functions of the Cap43 gene, little additional information was obtained. Apart from a putative domain containing a phosphopentathione attachment site, the Cap43 gene had no transmembrane domain or zinc finger motif or metal-binding domains, but it had a new motif consisting of 10 amino acids repeated three times in the COOH terminus of the protein (Fig. 3B). This repeated motif was found in all members of Cap43 family except for TDD5, providing additional support to the suggestion that Ndrl and TDD5 were products of different genes.

Cap43 Induction in Various Cell Lines by NiCl₂ or Homocysteine. Because this gene was first found to be induced by homocysteine, we tested this agent (25). The following human cell lines were analyzed to determine whether nickel or homocysteine could induce Cap43 expression in other cell lines derived from various human tissues; (a) pulmonary epithelial cells (A549, HTE, and Calu-1); (b) lung fibroblasts (WI-38); (c) osteosarcoma (HOS); and (d) endothelial cells (HUV-EC-C). Fig. 4 shows the data for HTE, Calu-1, WI 38, and HOS cells. Data for HUV-EC-C cells were similar to that previously published for homocysteine, and the nickel response was similar to that obtained for HOS cells (Fig. 4). All cultures were treated for 24 h with 1 mM NiCl₂ or with 6 mM of homocysteine, or with both agents, but results showed that NiCl₂ significantly induced Cap43 expression in all of the human cell lines tested including HUV-EC-C cells (data not shown), whereas homocysteine only induced Cap43 in HUV-EC-C cells (25). When nickel and homocysteine were added together, the level of expression was identical to that attributed to nickel alone (Fig. 4).

Basal Expression and Lung Induction of the Cap43 Gene in Vivo. To determine the basal level of Cap43 mRNA expression in human tissues, a blot containing poly(A) mRNA from different human organs was hybridized with the Cap43 probe. The results in Fig. 5A indicated that the Cap43 gene was ubiquitously expressed in human heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas. To assess the Cap43 induction by nickel in vivo, rats were exposed to 110 mg of NiCl₂/kg (equivalent to 50 mg of Ni/kg) body weight by gavage for 36 h. RNA was then extracted from the lungs, liver, kidneys, heart, brain, and skeletal muscle, and Northern blot analyses were performed with a human probe from the coding region. The results in Fig. 5B indicated that NiCl₂ induced Cap43 expression in the liver and other organs, but its induction in the lung was variable in multiple experiments and is not shown here.

Specificity of Cap43 Induction by Nickel in A549 Cells and the Search for Signal Transduction Pathway Involved in Nickel-induced Cap43 Expression. To investigate whether other metal compounds were able to induce Cap43 gene, A549 cells were treated with various metal compounds, and total RNA from exposed cells was assessed for Cap43 expression. Apart from Ni₃S₂ and NiCl₂, the metal compounds tested were water-soluble chloride salts of zinc, cobalt, copper (II), cadmium, magnesium, and mercury; water-soluble lead acetate, cisplatin, sodium vanadate, sodium arsenite, and potassium chromate (data not shown); and water-insoluble ferrous sulfide. None of these metal compounds significantly induced the expression of...
The proteins homologous to Cap43 were identified by searching the Entrez database using a BLAST program and aligned using the GCG program. Sequences identical to Cap43 are highlighted. Ten-amino acid repeats on the COOH-terminal of the proteins are underlined. Potential phosphorylation sites for protein kinase C (solid boxes), casein kinase 2 (dotted boxes), and tyrosine kinase (dashed boxes) were identified using a PROSITE pattern search.

Fig. 3. A, nucleotide sequence of Cap43. GenBank Accession No. AF004162. The coding part of the gene is shown in bold. B, multiple gene alignment of the novel gene family.
Cap43 gene (Table 1) at doses that ranged from nontoxic to lethal levels.

To study whether oxidative stress was involved in the induction of Cap43 expression, paraquat and hydrogen peroxide were used in a wide range of concentrations. Both failed to induce Cap43 expression (Table 2), indicating that the observed increased expression of Cap43 after nickel exposure was not likely to be due to oxidative stress induced by the nickel compounds themselves. Heat shock at 42°C for 15 min also failed to induce Cap43 (data not shown).

Sodium vanadate, a tyrosine phosphatase inhibitor alone or in combination with nickel, did not affect Cap43 expression (Fig. 6A), suggesting that tyrosine phosphorylation was not involved; however, okadaic acid (Fig. 6B) alone was found to be a good inducer, suggesting that serine/threonine phosphorylation was a component of the signaling pathway. To narrow down the possible serine/threonine phosphorylation pathways, we used dibutyryl cyclic AMP, an activator of PKA, in concentrations up to 2 mM and found that PKA was not involved. Trifluoperazine, an inhibitor of calmodulin-dependent phosphorylation, also had no effect on nickel-induced Cap43 expression (data not shown), and it alone did not induce Cap43 (Table 2).

Enhanced Cap43 mRNA Expression Involves Transcriptional Activation. To investigate whether Cap43 induction was due to an increase in the rate of Cap43 mRNA synthesis or an enhancement of its stability, the transcriptional inhibitor actinomycin D was used. Pretreatment with 4 µM actinomycin-D for 30 min completely abolished Cap43 induction by 1 mM NiCl₂ (Fig. 7A). In Fig. 7B, cotreatment of cells with 4 µM actinomycin-D and 2.0 µg Ni₅S₄/cm² also completely inhibited Cap43 induction by Ni₅S₄. This suggested that nickel caused an increase in Cap43 expression by stimulating the rate of its transcription. In addition, cycloheximide did not abolish Cap43 induction, and alone it produced some induction of Cap43 (Fig. 7B).

DISCUSSION

Nickel carcinogenesis involves multiple molecular events such as oxidative stress (27), enhanced DNA methylation, and altered gene expression (28, 29). We have identified a signaling pathway affected by nickel by cloning Cap43, a gene induced by this metal. Cap43 encoded for a 3.0-kb mRNA and a predicted Mr 43,000 protein. Expression of the Cap43 gene was induced in human lung carcinoma A549 cells by both water-soluble and -insoluble nickel compounds in a dose- and time-dependent manner. Twelve metal compounds tested failed to induce this gene in A549 cells. Therefore, to date the Cap43 gene is the only gene known to be induced by nickel in mammalian cells, and its level of induction (~30-fold) was remarkably large for a mammalian gene. Because there are many metals that share some common chemical characteristics (i.e., valance and atomic radius)
with nickel and nickel is not considered an essential element for humans, it is unusual for a mammalian gene to respond specifically to nickel. Recent studies have shown that nickel exposure elevated free intracellular Ca\(^{2+}\) levels, and this Ca\(^{2+}\) elevation was the signal for Cap43 induction by 1 mM of NiCl\(_2\).

Table 1 Cap43 induction by metal compounds in A549 cells\(^a\)

<table>
<thead>
<tr>
<th>Metals</th>
<th>Dose range tested (µg/cm(^2))</th>
<th>Maximal induction (fold over basal levels)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ni(_2)S(_2)</td>
<td>0.16-1.5</td>
<td>1</td>
</tr>
<tr>
<td>NiCl(_2)</td>
<td>2.0-20</td>
<td>1</td>
</tr>
<tr>
<td>ZnCl(_2)</td>
<td>1.0-10</td>
<td>1</td>
</tr>
<tr>
<td>CoCl(_2)</td>
<td>5.0-1000</td>
<td>2</td>
</tr>
<tr>
<td>CuCl(_2)</td>
<td>5.0-1000</td>
<td>1</td>
</tr>
<tr>
<td>PbCl(_2)</td>
<td>5.0-1000</td>
<td>1</td>
</tr>
<tr>
<td>CdCl(_2)</td>
<td>10.0-50</td>
<td>1</td>
</tr>
<tr>
<td>MgCl(_2)</td>
<td>5.0-50</td>
<td>1</td>
</tr>
<tr>
<td>Na(_2)VO(_4)</td>
<td>10.0-1000</td>
<td>1</td>
</tr>
<tr>
<td>Cisplatin(^b)</td>
<td>10.0-1000</td>
<td>1</td>
</tr>
<tr>
<td>Fe(_2)SO(_4)</td>
<td>2.0-2000</td>
<td>1</td>
</tr>
</tbody>
</table>

\(^a\) A549 cells were exposed to the above agents for 24 h.
\(^b\) These agents can neither induce Cap43 expression nor change the extent of Cap43 induction by 1 mM of NiCl\(_2\).

Table 2 Cap43 induction by various compounds in A549 cells\(^a\)

<table>
<thead>
<tr>
<th>Agent</th>
<th>Dose range tested (µM)</th>
<th>Maximal induction (folds of basal levels)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H(_2)O(_2)</td>
<td>0.04-500</td>
<td>1</td>
</tr>
<tr>
<td>Paraquat</td>
<td>20-200</td>
<td>1</td>
</tr>
<tr>
<td>Dibutyryl cAMP(^b)</td>
<td>250-2000</td>
<td>1</td>
</tr>
<tr>
<td>Trifluoperazine</td>
<td>2.5-20</td>
<td>1</td>
</tr>
<tr>
<td>Homocysteine(^b)</td>
<td>74-60000</td>
<td>1</td>
</tr>
<tr>
<td>Homocysteine thiolactone</td>
<td>6.0</td>
<td>1</td>
</tr>
<tr>
<td>S-Adenosyl-homocysteine</td>
<td>5.0</td>
<td>1</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td>50-20000</td>
<td>1</td>
</tr>
<tr>
<td>Amethopterin</td>
<td>0.4-50</td>
<td>1</td>
</tr>
</tbody>
</table>

\(^a\) A549 cells were exposed to the above agents for 24 h.
\(^b\) These agents can neither induce Cap43 expression nor change the extent of Cap43 induction by 1 mM of NiCl\(_2\).

Fig. 6. A. effect of sodium vanadate on Cap43 expression. Cells were treated with various doses of sodium vanadate for 20 h. Fifteen µg of total RNA were subjected to Northern blot analysis as described in "Materials and Methods." B. effect of okadaic acid on Cap43 expression. Cells were treated with 250 nM okadaic acid for different periods of time or treated with nickel for 8 h. Fifteen µg of total RNA were subjected to Northern blot analysis as described in "Materials and Methods."

Fig. 7. A. effect of actinomycin-D on Cap43 expression. A549 monolayers (75% confluent) were treated for 24 h. Fifteen µg of total RNA were subjected to Northern blot analysis as described in "Materials and Methods." B. effect of actinomycin-D or cycloheximide on Cap43 expression. A549 cells (75% confluent) were treated for 24 h with the indicated combinations of Ni\(_2\)S\(_2\) (2.0 µg/cm\(^2\)), actinomycin-D (4 µM), or cycloheximide (35 µM). Total RNA was then extracted, and Northern analysis was performed as described in "Materials and Methods."
organs from rats treated in vivo with nickel, suggesting that its induction by nickel is a general phenomenon and not tissue or cell specific. De novo mRNA and protein synthesis were essential for the induction of the Cap43 gene; Cap43 mRNA was elevated about 6–8 h after nickel exposure. Cap43 induction by nickel was completely inhibited by pretreatment with actinomycin D and partially inhibited by cycloheximide. The protein synthesis inhibitor cycloheximide alone, however, slightly induced Cap43 expression. There are two possible explanations for the effects of cycloheximide. Inhibition of cellular protein synthesis may block the synthesis of a repressor for this gene, or it may just be a nonspecific response of cells to cycloheximide treatment; similar examples of mRNA induction by cycloheximide have been observed for other genes, such as the insulin receptor or the inducible isofrom of nitric oxide synthase (35, 36).

It has been suggested that metals are important factors in gene expression and may be significant in signal transduction and gene activation or as components of regulatory proteins. The possible cellular pathways of nickel interactions that could lead to Cap43 expression were explored. The involvement of the following major signal transduction pathways was examined: (a) the adenylate cyclase cascade that leads to an increased level of cyclic AMP and activation of protein kinase A (37); (b) the calmodulin-dependent pathway (38); and (c) the two major oncogenic pathways (the Jak-Stat pathway and the mitogen-activated protein kinase cascade), which are controlled by tyrosine phosphorylation (39, 40). Surprisingly, none of these pathways was found to be involved in Cap43 induction. The lack of Cap43 induction by dbcAMP indicated that Cap43 induction by Ni was not likely mediated by elevation of cAMP levels and PKA activation. Similarly, the trifluoperazine data indicated that Cap43 induction by nickel was not mediated by calmodulin activation. To assess the involvement of tyrosine phosphorylation, two different approaches were used. A tyrosine phosphatase inhibitor, sodium vanadate, was used and found to have no effect on Cap43 expression, and Western blots of total cellular proteins were probed with an anti-phosphotyrosine antibody. The Western blot results indicated that there were no significant differences between untreated and nickel-exposed A549 cells in levels of tyrosine phosphorylated proteins (data not shown). Okadaic acid, a serine/threonine phosphatase inhibitor, induced Cap43 expression more rapidly and more efficiently than nickel, suggesting that it acted more directly in a signaling pathway. These data indicated that serine/threonine phosphorylation may be a signal transduction pathway controlling the Cap43 gene; however, the protein kinase involved in Cap43 expression has not been identified. These results are consistent with studies showing that nickel-elevated free intracellular Ca2+ levels and Cap43 induction by nickel was blocked with the Ca2+ chelator bis-(O-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid tetra(acetoxymethyl)-ester (22, 30), and that a Ca2+ ionophore also strikingly induced Cap43 (22).

A Cap43 homologue, the human RTP gene, was recently cloned by another research group based upon its homocysteine inducibility in human umbilical cord vein cells (HUV-EC-C; Ref. 25). The Cap43 gene was also induced by homocysteine in HUV-EC-C cells but not in any other human cell line tested. In contrast, nickel induced Cap43 expression in all cell lines tested. The agents that presumably increase intracellular homocysteine levels, such as homocysteine itself, and homocysteine-thioibactole were unable to induce Cap43 expression in A549 cells. In addition, the antifolate drug amethopterin, which prevented remethylation of homocysteine to methionine by methionine synthase, also was found to have no effect on Cap43 expression. To examine whether uptake of homocysteine was normal in A549 cells, the intracellular concentration of homocysteine was measured in nickel- or homocysteine-treated A549 cells by high-performance liquid chromatography (data not shown). The high-performance liquid chromatography data indicated that both nickel and homocysteine treatments increased intracellular homocysteine levels, and thus the lack of Cap43 induction by homocysteine was not due to a defect in homocysteine uptake by A549 cells.

We found that the Cap43 gene was conserved in different species including mouse, rat, hamster, and human. It was ubiquitously expressed in all of the human organs tested, including brain, heart, liver, lung, kidney, placenta, skeletal muscle, and spleen. In the rat, the Cap43 gene was also constitutively expressed in all of the organs tested. The observed high basal levels of the Cap43 gene in human organs compared with that in rat organs was due to the high sensitivity of the poly(A) RNA used in the human Northern assay. Because the Cap43 gene was highly conserved between humans and rodents, it was likely to have important cellular functions. Recently, when this gene was cloned independently by a group from the Netherlands (24), it was found to be a differentiation marker for colon epithelium, and its expression was lost or decreased in 17 colon adenocarcinomas. These findings indicated that the Cap43 gene was relevant in studying the molecular mechanisms of carcinogenesis, and it was possible that Cap43 was lost or underexpressed in other tumors.

The role of the Cap43 gene in whether nickel is essential, as well as the toxicity of nickel, is not clear. Kumar et al. (41) presented evidence for a new biological role of nickel in anaerobic bacteria through the formation of a methylnickel intermediate in carbon monoxide dehydrogenase, which is a metalloenzyme. It is still unclear whether the same mechanism is operative in mammalian cells, because in mammalian systems similar metalloenzymes have not yet been identified, and whether nickel is essential has not been established. Therefore, at present is difficult to investigate whether the Cap43 gene plays a role in the nickel as an essential element.

In conclusion, the Cap43 gene was cloned using an acute exposure of A549 cells to nickel. In vitro human cells originating from the lung showed the highest levels of Cap43 expression in response to nickel compounds. Serine/threonine phosphorylation was shown to be involved in Cap43 expression. Other studies from our laboratory have shown that nickel exposure elevated free intracellular Ca2+, and this elevation was the direct signal for Cap43 induction (22). It is also of interest that both water-soluble and relatively insoluble nickel compounds induced Cap43 equivalently because water-insoluble compounds produced much higher levels of intracellular soluble Ni2+ after 24 h of exposure (9). These results suggest that Ni2+ ions may be acting extracellularly at a Ca2+ sensing site on the membrane to affect an increase in free intracellular Ca2+. The finding that Cap43 is a differentiation marker in colon epithelium and is down-regulated in colon cancer makes it an interesting candidate for studies of molecular mechanisms of nickel carcinogenesis (24).

REFERENCES

Cap43, a Novel Gene Specifically Induced by Ni²⁺ Compounds

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