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Calcyclin (S100A6) expression is stimulated by agents evoking oxidative stress via the antioxidant response element

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Abstract

Calcyclin (S100A6) is a cell-specific, calcium binding protein of the S100 family whose expression is augmented in many types of cancer. By means of luciferase activity assays, RT-PCR and Northern blot hybridization, we established that transcription of S100A6 gene is increased by agents known to evoke oxidative stress. Mutation of the antioxidant response element (ARE) located at position −290/−281 of the calcyclin gene promoter, and overlapping the E-box sequence recognized by the upstream stimulatory factor (USF), led to inhibition of calcyclin gene promoter activity stimulated by cadmium ions. Electrophoretic mobility shift assays (EMSA) with the −302/−260 calcyclin gene promoter fragment revealed, apart from USF binding, the presence of another protein complex (N) shown by competitive EMSA to be bound to ARE. DNA affinity chromatography followed by Western blot showed the binding of Nrf2 transcription factor to the immobilized calcyclin gene promoter fragment and concomitant appearance of complex N in EMSA of the eluted fractions. The results indicate that agents evoking oxidative stress activate calcyclin gene via the ARE sequence in its promoter.
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Keywords: Calcyclin (S100A6); Cadmium; Oxidative stress

1. Introduction

Calcyclin (S100A6) is an EF-hand calcium binding protein of the S100 protein family. Most of the genes coding for the S100 proteins are clustered on human chromosome 1q21 [1]. Calcyclin is found in epithelial cells and fibroblasts [2] as well as in some neurons [3], Schwann cells and subpopulations of astrocytes [4]. So far no defined physiological role has been attributed to the S100A6 protein although it was postulated to participate in and modulate numerous processes such as cell cycle progression [5], exocytosis [6], cytoskeleton rearrangement [7] and others. It has been shown recently that calyclin interacts with CacyBP/SIP [8], a component of the β-catenin degradation complex [9]. A characteristic feature of calcyclin gene expression is its marked up-regulation in many types of tumor cells including melanoma [10], adenocarcinoma [11] and neuroblastoma [12]. These data, mostly obtained by biochemical and histological methods, are now being confirmed by microarray studies [13]. In spite of the well-established correlation between the level of calcyclin and tumor progression, our knowledge of the mechanism involved in the regulation of calcyclin gene expression is rather scarce and the potential regulatory features of the calcyclin gene promoter have not been extensively explored. We have previously found that the transcriptional activity of the promoter is stimulated by the binding of upstream stimulatory factor (USF) to the E-box sequence located at position −283/−278 [14]. USF was reported to be involved in the activation of transcription of

Abbreviations: ARE, antioxidant response element; HO-1, heme oxygenase-1; CacyBP/SIP, calcyclin binding protein/Siah interacting protein; EpRE, electrophile response element; USF, upstream stimulatory factor; EMSA, electrophoretic mobility shift assay

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many genes including those engaged in glucose and lipid metabolism [15] and cell cycle progression [16]. Recent data provide evidence that USF may be another stress responsive transcription factor activated by UV and osmotic shock [17], heat-shock [18] and cadmium [19,20]. NF-kB binding was also reported and mapped to position −460/−451 of the calcyclin gene promoter [21] implicating the role of inflammatory processes in calcyclin gene activation. In this work, we describe calcyclin gene regulation by agents evoking oxidative stress and present data on the role of the antioxidant response element (ARE) in this process. Since cadmium and many other agents acting via the ARE sequence are carcinogenic and ultimately cause DNA damage, they may constitute an important environmental factor contributing both to the increase in calcyclin level and to cell transformation. Epithelial cells, like Hep-2 cells used in this work, constitute an excellent material to perform such studies since epithelium lines the surface of the body at the border of the internal and external environment and, being continually exposed to environmental hazards, is thus the primary site of adaptive response.

2. Materials and methods

2.1. Cell culture and transfection

Hep-2 (human epithelial) cells were maintained in DMEM with 10% FCS, 100 IU/ml penicillin and 100 μg/ml streptomycin. Cells were seeded in 12-well plates and after 24 h transfected for 6 h in DMEM without FCS or antibiotics using Lipofectamine Reagent (Gibco) with 2 μg of the calcyclin gene promoter-luciferase construct and 0.25 μg of the pRL-CMV vector to assess transfection efficiency. A complete medium was then added and cells were grown for 18 h before treatment. Cells were treated either with freshly prepared 50 μM $CdCl_2$ for 3 h or with 0.8 mM H_2O_2 for 1 h or with 10 μM curcumin (in alcohol) for 1 h. Cells were subsequently lysed and luciferase activities were measured on a TD-20/20 luminometer (Turner designs) using Dual-Luciferase Reporter Assay System (Promega).

2.2. Plasmids

pGL2 vector containing the luciferase gene and the −361/+134 fragment of the human calcyclin gene promoter was described earlier [10]. Plasmid bearing the −361/+134 calcyclin gene promoter fragment with the E-box sequence, CACGTG, at position −283/−278 mutated to CAGCTT was obtained as described [14]. Plasmids with mutations in the ARE sequence (AREmut) and in both the E-box and ARE sequences (AREmut/E-boxmut) were constructed according to the QuikChange Site-Directed Mutagenesis method (Stratagene) using the following sense and complementary antisense oligonucleotides: 5′-GCTCCCCTTttCGAtT**CACGTG**TCACGAA-3′ (AREmut) and 5′-GCTCCCCTTttCGAtT**CAgcTt**TCACGAA-3′ (AREmut/E-boxmut) where the ARE sequence is underlined, the E-box is in bold and mutated bases are indicated by small letters. The PCR consisted of one cycle of 1 min at 95 °C and 12 cycles of 30 s at 95 °C, 45 s at 55 °C, and 12 min at 68 °C followed by 5-min incubation at 68 °C. Wild-type −361/+134 plasmid was used as a template to obtain the AREmut plasmid and the AREmut plasmid served as a template to obtain the AREmut/E-boxmut plasmid.

2.3. Northern blot

Total RNA was isolated from control Hep-2 cells and cells treated with 50 μM cadmium for 3 h using RNeasy kit (Qiagen), applied on a 1.2% agarose gel in the presence of formaldehyde and blotted to Hybond+ nitrocellulose membrane in 20× SSC buffer. Methylene blue staining was used to visualize equal sample loading. For detection of calcyclin mRNA a specific, RT-PCR-derived, 274-bp-long DNA probe, complementary to the sequence of the second and third exons of the calcyclin gene, was labeled with [^{32}P]-deoxycytidine 5′ triphosphate using the Rediprime random primer labeling kit (Amersham Pharmacia Biotech). Prehybridization was performed for 2 h at 42 °C and hybridization, with 3×10^7 cpm of the probe, was performed overnight at 65 °C. Membranes were washed three times for 5 min in 2× SSC and 0.2% SDS at room temperature and twice in 0.25× SSC and 0.2% SDS at 65 °C and subjected to autoradiography at −70 °C for 48–72 h.

2.4. RT-PCR

cDNAs were obtained using 3 μg of RNA isolated as described above, random nonamers and M-MuLV reverse transcriptase (Stratagene) according to the manufacturer's protocol. Heme oxygenase-1 (HO-1) and GAPDH cDNAs were amplified using the following PCR program: 1 cycle of 95 °C for 1 min, 34 cycles consisting of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s followed by 1 cycle of 72 °C for 5 min. Calcyclin cDNA was amplified with PCR program consisting of 1 cycle of 95 °C for 1 min, 34 cycles of 95 °C for 30 s, 55 °C for 1 min and 72 °C for 1 min followed by 1 cycle of 72 °C for 5 min. The following forward and backward PCR primers were used: GAPDH, 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′; HO-1, 5′-CCAGCGGGCCCAGCAACAAAGTGC-3′ and 5′-AAGCCTTCAGTGCCCACGGTAAGG-3′; and calcyclin (mouse), 5′-ATGGCATGCCCTCTGGAT-3′ and 5′-TTATTTCAGAGCTTCATT-3′.

2.5. Electrophoretic mobility shift assays (EMSA)

Nuclear extracts, radioactive probe preparation, incubation and electrophoresis were performed as previously

described [14]. The oligonucleotides used as probes or competitors in EMSA were as follows: wt, 5′-CCTG CTCCCCTTGCCGAGT**CACGTG**TCACGAAGAG-CAAACTGA-3′; E-boxmut, 5′-CCTGCTCCCCTT GCCGAGT**CAgcTt**TCACGAAGAGCAAACTGA-3′; AREmut, 5′-CCTGCTCCCCTTttCGAtT**CACGTG**TCAC-GAAGAGCAAACTGA-3′; Ferritin ARE, 5′-GCA-GAATGCTGAGTCACGGTGGAA [22]; only the sequence of the sense strand is given. The E-box sentence is in bold, the ARE sequence is underlined, mutations are indicated by small letters. Antibodies used for EMSA and Western blot assays were from Santa Cruz Biotechnology: USF1, sc-229X, and Nrf2, sc-722X.

2.6. Affinity chromatography

Two-hundred micrograms of double-stranded oligonucleotide equivalent to the −302/−260 fragment of the calcyclin gene promoter (Fig. 1), but containing a four-base extension, TGAC, at the 5′ end of the antisense strand, was coupled to 0.25-g CNBr-Sepharose 4B (Sigma). Nuclear extract (0.2 mg) from cadmium-treated Hep-2 cells was incubated with the resin for 1 h at 4 °C in EMSA buffer containing 0.2 M NaCl and 50 μg herring sperm DNA/ml. After extensive washing, proteins bound to the column were eluted with 1.2-ml portions of EMSA buffer containing 0.4, 0.6 and 0.8 M NaCl. The fractions eluted with 0.6 and 0.8 M NaCl were diluted with EMSA buffer to achieve a 0.4 M final concentration of NaCl and then concentrated to about 250-μl final volume using Centricon YM-10 filter, Milipore. Twenty microliters of these fractions was used in EMSA and 100 μl was examined for Western blot.

2.7. Western blot analysis

Proteins were resolved on 12% polyacrylamide gels and electroblotted onto nitrocellulose membrane. The detection of Nrf2 protein was performed using the ECL Western blotting analysis system (Amersham Pharmacia Biotech) as described by the manufacturer.

2.8. Statistical analysis and densitometry

The results of luciferase assays are presented as means ± S.E. Densitometric analysis of RT-PCR and Northern blot results was performed with the Scion Imaging program, version Beta 4.0.2. The mean pixel intensity of the band representing control cells was subtracted from that representing cadmium-treated cells and the difference was expressed as percent increase. Mean values ± S.D. obtained from at least three independent experiments are given in the text, while the results of a representative experiment are mounted on Fig. 3. All band measurements were background-subtracted.

3. Results

3.1. Presence of the ARE in the proximal calcyclin gene promoter

Promoter sequence analysis revealed that the E-box sequence at −283/−278 of the human calcyclin gene promoter is overlapped by the antioxidant response element (ARE) on the noncoding strand or by its counterpart, the so-

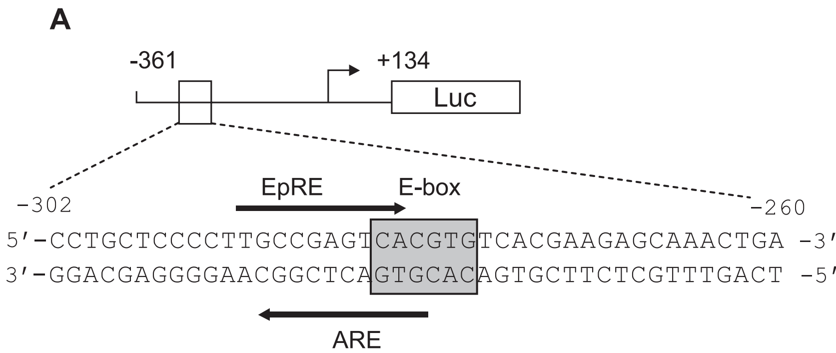

Fig. 1. Schematic representation of the −302/−260 fragment of calcyclin gene promoter. Panel A. Schematic representation of the vector used in transfection experiments and of the *cis* elements identified in the −302/−260 fragment of calcyclin gene promoter. The E-box sequence is boxed, the ARE and EpRE sequences are indicated by arrows. Panel B. Representation of the E-box and ARE sequences found in the calcyclin gene promoter, of the respective consensuses and of the mutations introduced in vectors used in transfection experiments and in oligonucleotides used for EMSA. The ARE sequence of the ferritin gene promoter is also shown for comparison.

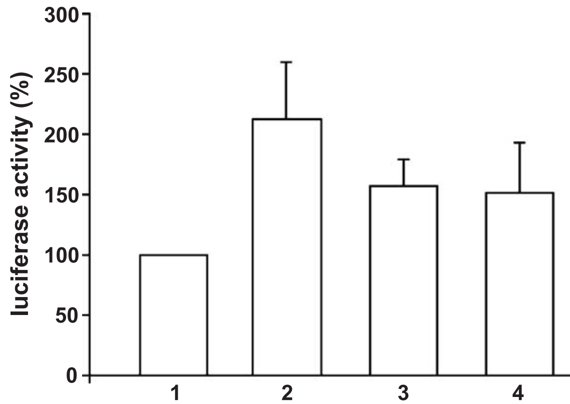

Fig. 2. Effect of agents evoking oxidative stress on the calcyclin gene promoter activity. Cells were transfected with the luciferase vector containing the −361/+134 fragment of the calcyclin gene promoter and were either left untreated (1) or treated with 50 μM $CdCl_2$ (2), 10 μM curcumin (3) or 800 μM H_2O_2 (4) for an appropriate time as described in Materials and methods. Cells were then lysed and luciferase activity was assayed in the lysates. All data are mean±S.E. of five independent determinations; $P<0.01$ for data in 2 and 3 and $P<0.1$ for data in 4, compared with control.

called electrophile response element (EpRE), on the coding strand (Fig. 1). Both these elements were reported to mediate cell response to agents evoking oxidative stress, many of which are carcinogenic substances [23]. The GTGACTCGGC sequence found on the noncoding strand of the calcyclin gene promoter perfectly matches the consensus ARE sequence, G/ATGACnnnGC [24], while the TGCCGAGTCA sequence on the coding strand differs in only one nucleotide from the consensus EpRE, TGCTGAGTCA (Fig. 1). The ARE sequence in calcyclin gene promoter does not contain a consensus AP1 binding site, TGACTCA, which is often contained within the ARE; however, several AP1-like sequences, i.e., TGACTCG, TGACACG and CGAGTCA, can be found on both strands within the −302/−260 sequence.

3.2. Effect of agents evoking oxidative stress on calcyclin gene expression

Prompted by the structural evidence, we have examined whether calcyclin gene expression can be stimulated by agents known to act via the ARE sequence, namely cadmium, H_2O_2 [25] and curcumin [26]. Fig. 2 shows that luciferase activity of a reporter construct containing the −361/+134 fragment of calcyclin gene promoter was increased in Hep-2 cells treated with these agents, with more than twofold activation observed for 50 μM $CdCl_2$. The effect of cadmium was also observed when a longer calcyclin gene promoter construct (−1371/+134) was used in transfection but was much less pronounced with the shorter (−167/+134) one (not shown), suggesting that, although some other *cis*-elements may be also involved, the −361/−167 promoter region encompassing the ARE is sufficient to generate the change in gene expression.

To verify the results obtained using promoter constructs in another experimental system, we investigated the effect of cadmium ions on calcyclin mRNA level in Hep-2 cells by means of RT-PCR and Northern blot. The RT-PCR assay was performed for calcyclin mRNA as well as for HO-1 mRNA to confirm, on a well-established cadmium-inducible protein [25], that the conditions applied in our experiments indeed resulted in oxidative stress. Fig. 3A presents the RT-PCR results of a typical experiment showing that the level of calcyclin and heme oxygenase mRNA derived from Hep-2 cells grown at 50 μM $CdCl_2$ concentration is higher than in untreated cells. Densitometric data of three independent experiments indicated no changes in GAPDH mRNA (Fig. 3A, 3), above threefold increase in HO-1 mRNA expression and 56±12% increase in calcyclin mRNA (Fig. 3A, 1–2). The corresponding value obtained from three independent Northern blot experiments was 106±26% increase in calcyclin mRNA in cells treated with cadmium (Fig. 3B). These data indicate that cadmium up-regulates calcyclin mRNA level.

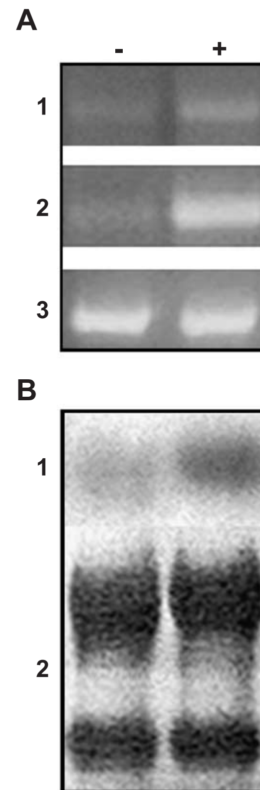

Fig. 3. RT-PCR and Northern blot analysis of calcyclin mRNA level in control and 50 μM $CdCl_2$-treated Hep-2 cells. Total RNA was isolated from untreated Hep-2 cells or cells treated with 50 μM $CdCl_2$ for 3 h and processed for Northern blot or RT-PCR analyses as described in Materials and methods. Panel A: RT-PCR analysis of calcyclin (1), HO-1 (2) and GAPDH mRNA (3); panel B: Northern blot of calcyclin mRNA, autoradiogram (1) and methylene blue staining of 28S and 18S rRNA bands (2). Results from untreated (−) and cadmium-treated cells (+) from a representative experiment are presented.

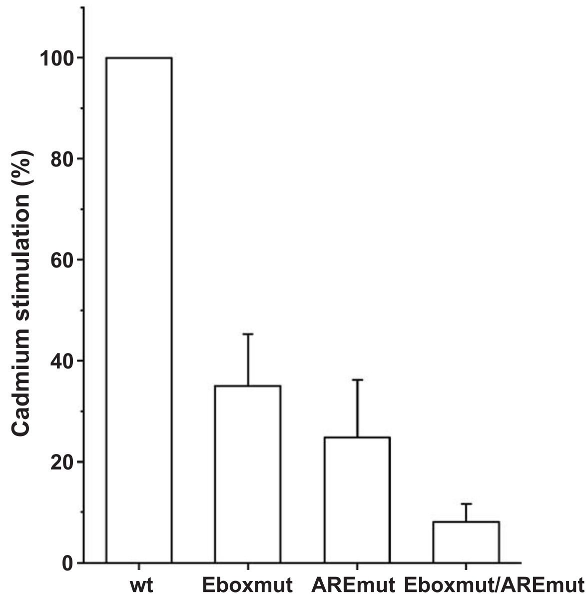

Fig. 4. Effect of mutations in the E-box and ARE sequences on cadmium ion-dependent stimulation of calcyclin gene expression. Hep-2 cells were transfected with pRL-CMV reporter vector and with pGL2 vector containing luciferase gene under control of the −361/+134 fragment of calcyclin gene promoter with no mutation (wt), carrying a mutation in the E-box (Eboxmut), in the ARE sequence (AREmut) or in both E-box and ARE sequences (Eboxmut/AREmut). Incubation with $CdCl_2$ and luciferase activity assays were performed as described in Materials and methods. Results are presented as percentage of cadmium stimulation measured for cells transfected with a mutated vector when compared to cadmium stimulation seen for the non-mutated vector (taken as 100%). All data are mean±S.E. of five independent determinations.

3.3. Mutation in ARE inhibits cadmium-dependent stimulation of the calcyclin gene promoter

To assess the role of the ARE sequence in the activity of the calcyclin gene promoter, we transfected Hep-2 cells either with a vector containing the −361/+134 wild-type promoter fragment, a vector with mutation within the ARE (Fig. 1) or a vector with a mutation within the USF binding site, which was shown to prevent USF binding [14]. Fig. 4 shows that mutation in the ARE sequence, which preserved an intact E-box, but changed three bases within the ARE consensus including the GC dinucleotide essential for the sequence to be functional, diminished cadmium ion-evoked stimulation of the calcyclin gene promoter activity to about 25% of its original value. Mutation in the E-box sequence resulted in about 60% decrease in calcyclin gene promoter activity, indicating that this site may also be important for cadmium activation [19,20]. It should be noted, however, that mutation in the E-box introduces a change in the first base of the ARE sequence (G to C), so it no longer complies with the ARE consensus (Fig. 1), which suggests that the effect may be partly due to the change in the ARE sequence. Double mutation results in almost a complete loss of cadmium-dependent stimulation of promoter activity (Fig. 4).

3.4. Protein binding to the ARE sequence within the −302/−260 calcyclin gene promoter fragment

We then proceeded to examine protein binding to the ARE sequence in the calcyclin gene promoter. For EMSA we used a probe comprising residues −302/−260 which accommodates both the ARE and the E-box sequences (Fig. 1). Two prominent DNA binding protein complexes were formed upon incubation of the probe with nuclear extracts from Hep-2 cells (Fig. 5A, lane 2). Both complexes were effectively competed by a 50-fold excess of non-radioactive probe, indicating that all interactions were specific (Fig. 5A, lanes 2 versus 3). As we have already shown for nuclear extract from Ehrlich ascites tumor cells [14], the slower migrating complex is formed by the USF1/USF2 dimer and could be suppressed upon addition of the anti-USF1 antibody (Fig. 5B, lane 5). The band containing USF was competed by a 50-fold excess of an oligonucleotide with mutation in the ARE but not by a 50-fold excess of an oligonucleotide containing mutation in the E-box or by an oligonucleotide corresponding to the EpRE/ARE sequence from the ferritin H gene promoter (Fig. 5A, lanes 4–6). The lower band assigned as complex N, on the other hand, had different binding characteristics, i.e., it was competed by an oligonucleotide carrying a mutation within the E-box, but not by an oligonucleotide with mutation in the ARE (Fig. 5A, lanes 4–5). Furthermore, addition of a 50-fold excess of an oligonucleotide corresponding to the EpRE/ARE sequence from the ferritin H gene promoter effectively competed with the formation of complex N while USF binding was not perturbed (Fig. 5A, lane 6; Fig. 5B, lane 4). Consequently, when the fragment of ferritin gene promoter was used as a probe, we observed a single protein–DNA complex with mobility equal to that of complex N (Fig. 5B, lane 7). As expected, this binding could be competed by both the ferritin and calcyclin promoter derived probes (Fig. 5B, lanes 8–9). As the ARE sequence is the only motif shared by both oligonucleotide probes, it is justified to consider that the binding of complex N occurs at the ARE motif in the −302/−260 fragment of calcyclin gene promoter. To further test the hypothesis that complex N may represent protein(s) bound to the ARE sequence, we compared the binding pattern of control and cadmium-treated cells. As can be seen in Fig. 5A, lanes 7–8, the intensity of the faster migrating band was increased in nuclear extracts from cadmium-treated cells in agreement with its putative role in ARE activation.

3.5. DNA affinity chromatography to identify proteins bound to the ARE

We examined the protein content of complex N by means of affinity chromatography, often used in identification of transcription factors [27] and proved to be successful in identification of USF [14]. Nuclear extract (200 μg) derived from cadmium-treated Hep-2 cells was applied on a column,

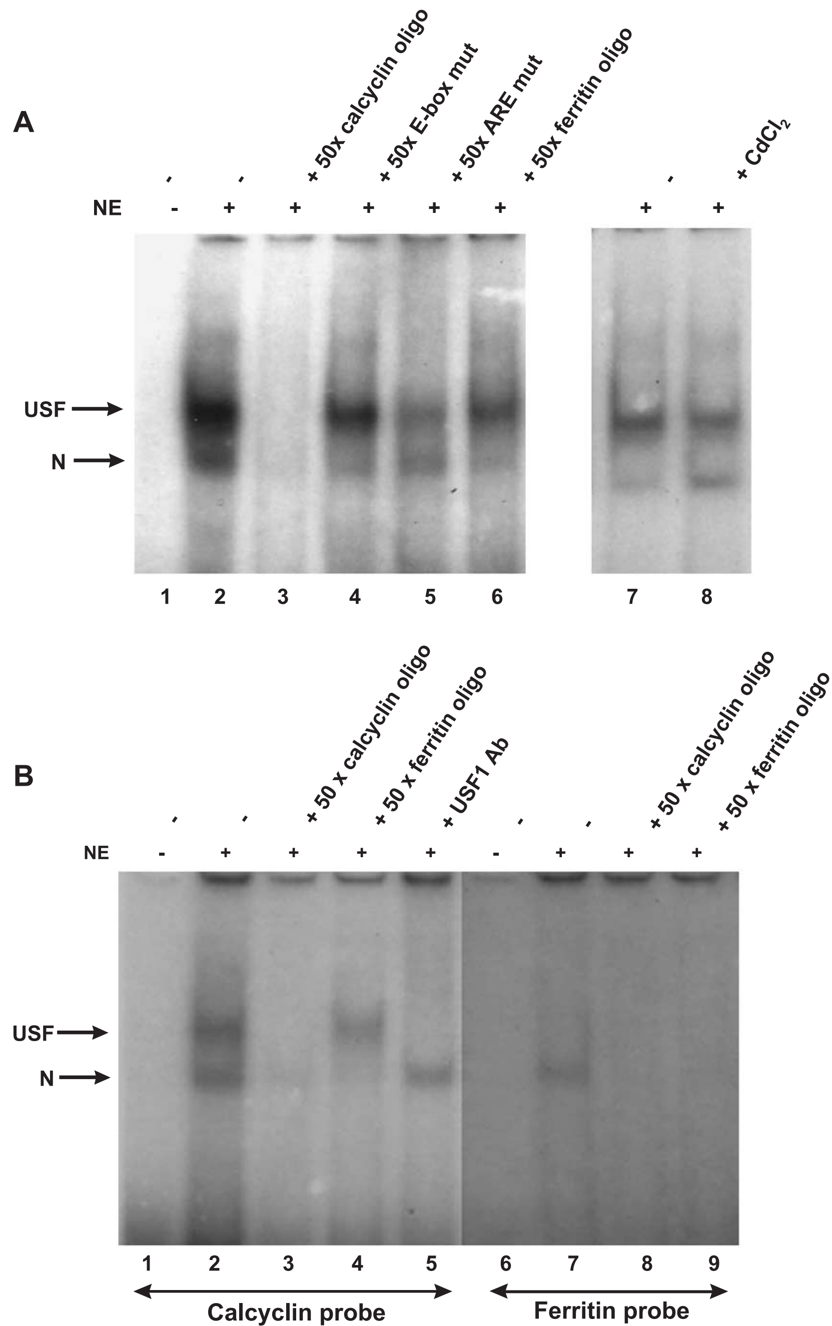

Fig. 5. Analysis of protein binding to the −302/−260 fragment of calcyclin gene promoter. Nuclear extracts (10 μg) obtained from control (panel A, lanes 2–7 and panel B, lanes 2–5, 7–9) or cadmium-treated (panel A, lane 8) Hep-2 cells were incubated with radioactive double-stranded oligonucleotide corresponding to the −302/−260 fragment of the calcyclin gene promoter (panel A, lanes 2–8; panel B, lanes 2–5) or with an oligonucleotide representing the EpRE/ARE sequence of ferritin H promoter (panel B, lanes 7–9). Panel A. No extract (lane 1); control extract (lanes 2–7); extract from Hep-2 cells treated with cadmium (lane 8); addition of a 50-fold excess of nonradioactive oligonucleotide corresponding to the −302/−260 fragment of the calcyclin gene promoter: wild type (lane 3), E-boxmut (lane 4) ARE mut (lane 5) or corresponding to the EpRE/ARE sequence of ferritin H gene promoter (lane 6). Panel B. No extract (lanes 1 and 6); control extract (lanes 2–5 and 7–9); addition of a 50-fold excess of nonradioactive oligonucleotide corresponding to the −302/−260 fragment of the calcyclin gene promoter (lanes 3 and 8) or corresponding to the EpRE/ARE sequence of ferritin H gene promoter (lanes 4 and 8); addition of anti USF1 antibody (lane 5). Antibodies (1 μl) and competitors (50-fold excess over the probe) were added 30 min prior to the addition of the radioactive probe. Samples were run on a 5% non-denaturating polyacrylamide gel, the gel was then dried and exposed at −70 for 20 h.

filled with CNBr Sepharose coupled to the −302/−260 fragment of calcyclin gene promoter, in buffer used for EMSA containing 0.2 M NaCl. Bound proteins were eluted after extensive washing by a stepwise NaCl concentration gradient and all fractions were examined by Western blot and EMSA. Western blot analysis revealed the presence of both USF1 (not shown) and Nrf2 (Fig. 6) proteins in fractions eluted from the column, indicating that the latter

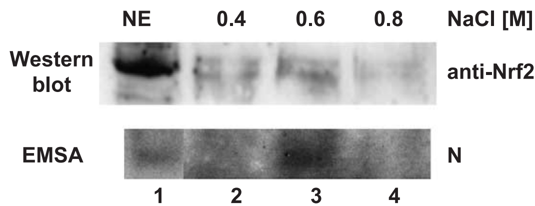

Fig. 6. EMSA and Western blot analysis of fractions obtained from the affinity column. Nuclear extract (NE) (lane 1), fractions eluted with: 0.4 M NaCl (lane 2), 0.6 M NaCl (lane 3) and 0.8 M NaCl (lane 4). Fractions eluted from the column were diluted to obtain a final NaCl concentration equal to 0.4 M NaCl and then concentrated. Fifty or ten micrograms of nuclear extract and 100- or 20-μl portions of the concentrated fractions were used for Western blot and EMSA, respectively.

transcription factor is also effectively bound to the calcyclin gene promoter fragment. The presence of Nrf2 protein in the 0.4 and 0.6 M NaCl fractions coincides with the EMSA results showing that complex N is formed when these fractions are incubated with radioactive probe (Fig 6). These results indicate that Nrf2 binds to the ARE sequence within the calcyclin gene promoter and that the binding results in the formation of complex N in EMSA.

4. Discussion

The non-genotoxic carcinogens cause DNA damage, mutations and neoplasia through various mechanisms that include oxidative stress and glutathione depletion [28]. An adaptive reaction of cells, the so-called stress response, involves activation of intracellular signaling pathways leading to increased transcription of phase II detoxification enzymes and enzymes controlling the cellular redox state. Evidence has accumulated that the stress response, including that evoked by cadmium, may be mediated by the antioxidant response element (ARE), also called the electrophile response element (EpRE), which are found in promoters of many genes up-regulated by such stress [29]. This regulatory sequence can be recognized by heterodimers of Nrf1 or Nrf2 transcription factors with the Maf family proteins [30].

Analysis of the promoter of the calcyclin gene, coding a small calcium binding protein often up-regulated in cancers, revealed the presence of a sequence strictly matching the ARE consensus [24] and only slightly different from the extended core sequence 5′-TMANNRT-GAYNNNGCR-3′ [29] in its 5′ end. This sequence overlaps an E-box sequence that interacts with USF and is important for calcyclin gene expression [14]. We have shown by luciferase assay that cadmium, H_2O_2 and curcumin, which were earlier shown to stimulate HO-1 expression via ARE, evoked higher reporter gene activity in treated Hep-2 cells and confirmed the effect of cadmium by means of RT-PCR and Northern blot assay, both showing an increase in calcyclin mRNA level. Furthermore, mutation of the ARE resulted in a severe inhibition of the cadmium effect on reporter gene expression. Mutation of the E-box sequence had a less pronounced inhibitory effect, which may be due to the involvement of E-box and USF in response to cadmium, as found earlier for methallotionein-I [19] and heme oxygenase [20], or to a change of the first base of the ARE consensus.

EMSA studies identified USF as the component of the lower mobility complex by supershift assay with anti-USF1 antibody, in agreement with our earlier results performed on extracts from Ehrlich ascites tumor cells [14]. Competitive EMSA confirmed that the higher mobility complex involved the ARE sequence of the calcyclin gene promoter. However, we could not observe a supershift upon addition of the antibody against Nrf2 (not shown), a transcription factor interacting with ARE. Therefore, we used affinity column to compare the appearance of the lower band in EMSA with the presence of Nrf2 protein in Western blot in fractions bound to the resin. Using the anti-Nrf2 antibody, we observed a positive immunochemical reaction, most pronounced in the fraction eluted with 0.6 M NaCl, revealing a double protein band just below the 91-kDa molecular weight standard, and matching the appearance of complex N in EMSA. This finding indicates that the Nrf2 transcription factor binds to the ARE sequence of the calcyclin gene promoter and its binding can be attributed to the formation of complex N. A very similar situation, i.e., overlapping of E-box and ARE sequences, was found in the promoter of metallothionein I gene [31]. Methallothionein is a small, cystein-rich protein implicated in scavenging toxic metals, like cadmium or mercurium, and in the maintenance of cellular redox balance [32]. EMSA studies of the methallothionein I gene promoter also revealed the presence of two protein complexes—the upper one was identified as USF–DNA complex, while the lower one, shown by competition studies to bind to the ARE, was not identified [31]. All our findings suggest that the ARE within the calcyclin gene promoter, whether independently or in cooperation with E-box, contributes to the stimulatory effect exerted by cadmium, and possibly other agents employed in this study, on promoter activity most probably via the Nrf2 transcription factor.

It is difficult to assess the physiological importance of the increase in calcyclin expression by oxidative stress since its function has not been unequivocally clarified. Nonetheless, some analogies can be evoked based on the structural similarity among the S100 protein family members. The obvious possibility seems to be a direct antioxidant effect since most of the S100 proteins, including calcyclin, contain cystein residues and can form covalent S–S bonds; such covalent dimerisation as a consequence of exposure to H_2O_2 was observed for S100A2 protein [33]. Calcyclin is also a zinc binding protein and this binding was shown to be competed by cadmium [34]. It can thus be presumed that calcyclin has the ability to bind cadmium and other heavy metals and participate in their neutralization. For example,

S100B was shown to act as a copper sink and to protect cells from oxidative stress and copper overload [35]. It is worth to mention that the expression of S100B increased in cells overexpressing Nrf2 in mixed glial-neuronal culture [36]. Metal binding and formation of S–S bridges is also a feature of metallothionein, whose role in protecting cells from oxidative stress is well established [32]. Furthermore, calcyclin has been shown to interact with CacyBP/SIP, a component of ubiquitination complex [3]. Since the process of ubiquitination is activated by cadmium and other agents causing oxidative stress [37], regulation of calcyclin expression may be a part of this process.

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