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Impact of cadmium, cobalt and nickel on sequence-specific DNA binding of p63 and p73 *in vitro* and in cells

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ABSTRACT

Site-specific DNA recognition and binding activity belong to common attributes of all three members of tumor suppressor p53 family proteins: p53, p63 and p73. It was previously shown that heavy metals can affect p53 conformation, sequence-specific binding and suppress p53 response to DNA damage. Here we report for the first time that cadmium, nickel and cobalt, which have already been shown to disturb various DNA repair mechanisms, can also influence p63 and p73 sequence-specific DNA binding activity and transactivation of p53 family target genes. Based on results of electrophoretic mobility shift assay and luciferase reporter assay, we conclude that cadmium inhibits sequence-specific binding of all three core domains to p53 consensus sequences and abolishes transactivation of several promoters (e.g. BAX and MDM2) by 50 μM concentrations. In the presence of specific DNA, all p53 family core domains were partially protected against loss of DNA binding activity due to cadmium treatment. Effective cadmium concentration to abolish DNA–protein interactions was about two times higher for p63 and p73 proteins than for p53. Furthermore, we detected partial reversibility of cadmium inhibition for all p53 family members by EDTA. DTT was able to reverse cadmium inhibition only for p53 and p73. Nickel and cobalt abolished DNA–p53 interaction at sub-millimolar concentrations while inhibition of p63 and p73 DNA binding was observed at millimolar concentrations. In summary, cadmium strongly inhibits p53, p63 and p73 DNA binding *in vitro* and in cells in comparison to nickel and cobalt. The role of cadmium inhibition of p53 tumor suppressor family in carcinogenesis is discussed.

1. Introduction

Members of p53 protein family (p53, p63 and p73) control transcription of many genes involved in cell cycle control, DNA repair, cellular senescence or apoptosis in response to DNA damage [1,2]. Evolutionarily older p63 and p73 display strong homology to p53 in three major domains: N-terminal transactivation domain, central DNA binding domain (DBD) and C-terminal oligomerisation domain. Even though subtle structural differences were observed, a generally conserved conformation of DBDs and DNA–protein contact sites were revealed from structure analysis of co-crystals of p53, p63 and p73 DBDs with DNA target sequences. Their central DBDs are highly homologous and share an immunoglobulin-like folded structure [3–5] responsible for binding to sequence-specific response elements (REs), whose overall consensus sequence is similar to that of the canonical p53 consensus sequence (p53CON, containing two half-site decamers 5′PuPuPuC(A/T)(T/A)GPyPyPy3′ in direct orientation, [3]). Mediated by DBD, sequence-specific interactions with REs in regulatory regions of p53 family target genes are crucial for their function as transcription factors.

p53 family belongs to metalloproteins, a zinc ion coordination in DBD by three cysteines and one histidine is necessary for DNA binding of all family members [3–7]. These and other cysteines are involved in already reported p53 redox sensitivity [8]. Recently, we described that p63 and p73 DBDs share redox sensitivity to diamide and hydrogen peroxide with p53 [8,9]. We have shown that oxidation of p63 and p73 DBDs abolished p53CON recognition

Abbreviations: p53CON, p53 consensus sequence; TCEP, tris(2-carboxyethyl)phosphine; DTT, dithiothreitol; p53CD, GSTp53CD (aa 94–312); p63CD, GSTp63CD (aa 114–349); p73CD, GSTp73CD (aa 104–339); p53, full length p53; p53DBD, p53CD (aa 94–312); DBD, central DNA binding domain.

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and that reversibility of DBD oxidation was dependent on zinc accessibility [9]. Similarly, binding of transition metals like cadmium, cobalt, nickel, mercury and copper to the p53 protein abolishes p53 conformation and DNA binding [10–14], likely through very similar set of cysteines and histidines respective to metal ion preferences [10]. Metal sensitivity was currently established only for p53.

Metals can influence p53 on several levels, apart from indirect effects (ROS and NOS induced alterations to DNA bases, altered lipid metabolism and calcium homeostasis) [15–17], p53 is sensitive to local perturbations induced by metal ions themselves [10,12]. In this regard, redox inactive metals, such as cadmium and nickel exhibit their toxicity via binding to sulfhydryl groups of proteins and depletion of glutathione (reviewed in [17]). On the other hand, redox active metals like cobalt are as well potent inducers of oxidative stress, causing generation of free radicals. As environmental pollutants, selected metals pose ever-growing carcinogenic threat. Cadmium (Cd) and nickel (Ni) were classified by IARC as carcinogenic for humans while cobalt (Co) belongs to the group of "possibly carcinogenic to humans" [17].

In this study we used divalent ions of transition metals cadmium, cobalt and nickel to study their effect on sequence-specific binding of p53 family core domains *in vitro* and in cells. Our data show that the sequence-specific DNA binding properties of all three core domains were inhibited at micromolar $CdCl_2$ range and millimolar concentrations of $NiCl_2$ or $CoCl_2$. Also transactivation of MDM2, BAX or p53CON promoter in H1299 cells transfected with p53 family members was inhibited by micromolar concentration of $CdCl_2$ but not yet by $NiCl_2$ or $CoCl_2$. We also found that binding to DNA response element strongly protects core domains from a loss of DNA binding as a response to heavy metal treatment *in vitro*. Additionally, the inhibitory effect of cadmium on p53 family binding *in vitro* was reverted by sub-millimolar concentrations of chelating agent EDTA and only partially by redox agent DTT.

2. Materials and methods

2.1. DNA samples

Fragment p53CON (474 bp) was prepared by *Pvu*II (TAKARA) digestion of supercoiled plasmid pPGM1 (pBSK containing a p53CON sequence AGACATGCCTAGACATGCCT, [18]), second fragment long 2513 bp was used as nonspecific competitor (NON). Plasmids encoding human full length wild-type p53 (p53, aa 1–393, pT7-7p53 [19]), p53 core domain (p53DBD, aa 94–312, pET3dp53, [18]) and GST fusion core domains of p53, p63 (p53CD, aa 94–312; p63CD, aa 114–349; pGEX-4T [6]) and p73 (p73CD, aa 104–339, pGEX-4T [9]) were used for protein expression and purification.

2.2. Protein expression and purification

Full length protein p53 (p53), p53 core domain (p53DBD), and GST fusion p53 family core domains (p53CD, p63CD, p73CD) were purified by protocol described in [9,20]. Proteins after final purification on Superdex 200 HiLoad 26/60 column (Amersham Pharmacia Biotech) in 25 mM Hepes (pH 7.6), 200 mM KCl, 10% glycerol, 1 mM DTT and 1 mM benzamidine were dialyzed against the same buffer with 1 mM TCEP instead of DTT.

2.3. DNA binding assays

Interaction of p53 family proteins with 474 bp long fragment DNA was studied by EMSA in agarose gels. Usually 50–100 ng of proteins were incubated in binding buffer (5 mM Tris-HCl, pH 7.6, 0.01% Triton X-100 and 50 mM KCl with 1 mM TCEP) and mixed with pPGM1/*Pvu*II fragments (200 ng; 474 bp with p53CON, 2513 bp fragment as nonspecific competitor) at molar ratio 3–5/1 (protein tetramer/DNA) for 20 min on ice to reach equilibrium. Before or after addition of DNA the proteins were treated with metal ions ($CdCl_2$, $NiCl_2$ or $CoCl_2$) for 20 min on ice. Samples were loaded onto a 1% agarose gel containing 0.33× TBE buffer, DNA was stained with ethidium bromide.

2.4. Influence of EDTA and DTT on protein-DNA binding inhibited by heavy metals

To study influence of EDTA and DTT on cadmium inhibition the proteins were firstly incubated with cadmium (for 20 min on ice), then 200 ng of pPGM1/*Pvu*II were added and the incubation continued with DTT or EDTA at molar excess (2–200) for 20 min on ice.

2.5. Human cell lines, transfections and luciferase assays

Human non-small cell lung carcinoma line H1299 (p53 null cell line, NCI-H1299, ATCC) was grown in DMEM medium supplemented with 5% FBS (Gibco). The luciferase reporter constructs containing different p53 recognition sites (pGL3-BAX, pGL3-MDM2-APP, pGL3-PGM1 (p53CON sequences were cloned in *Sma*I site in pGL3 promoter vector) [21]), pGL3-promoter (Promega) and pRL-SV40 (Renilla control) were used. H1299 cells seeded in 24-well plates were transfected using Lipofectamine (Invitrogene) according to the manufacturer's instructions. When appropriate, 50–100 ng of the p53 family expression vectors based on pCDNA3.1: pcDNA3-HA-TAp73β, pcDNA3-HA-TAp73γ [22], pcDNA3.1-p53 [21], pcDNA3.1-TAp63α-FLAG (Addgene plasmid 27008, [23]), pCDNA3.1-TAp63γ-(3X)HA (Addgene plasmid 26977, [23]) or empty vector pCDNA3.1 was co-transfected with 200 ng of reporter constructs. About 16 h after transfection cadmium treatment was started, after 12 h treatment extracts were prepared using the Dual Luciferase Assay System kit (Promega) following the manufacturer's protocol (for more details see in [21]). For each construct, relative luciferase activity was defined as the mean value of the Firefly luciferase/Renilla luciferase ratios obtained from at least three independent experiments.

3. Results

3.1. Cadmium inhibits sequence-specific binding of p53 family members

Previously it was shown that cadmium induces modifications of p53 conformation, inhibits p53 sequence-specific binding to DNA and suppresses p53 response to DNA damage [10–14,24]. To characterize the effect of cadmium on sequence-specific binding of other p53 family members, p63 and p73 core domains (p63CD, aa 114–349; p73CD, aa 104–339) were isolated. Proteins p63CD and p73CD recognize p53CON sequences with affinities comparable to p53 core domain also due to fusion of DBD with GST which facilitates dimer formation in solution as was described in [6,9]. We tested the influence of cadmium at the concentration range 10–100 μM on p63CD, p73CD, full length p53 and p53DBD binding to p53CON (inserted in 474 bp DNA fragment, Fig. 1A and B) on agarose gel. Molar ratios of protein to DNA was 3–5/1; at this condition the majority of p53CON fragment was bound by the p53 family proteins (Fig. 1A and B, lanes 2, 6, 10, 14). At first, protein samples were exposed to increasing amount of $CdCl_2$ (10–50 μM) before DNA addition in the presence of 1 mM TCEP in binding buffer (Fig. 1A). Partial inhibition of p63CD and p73CD binding was

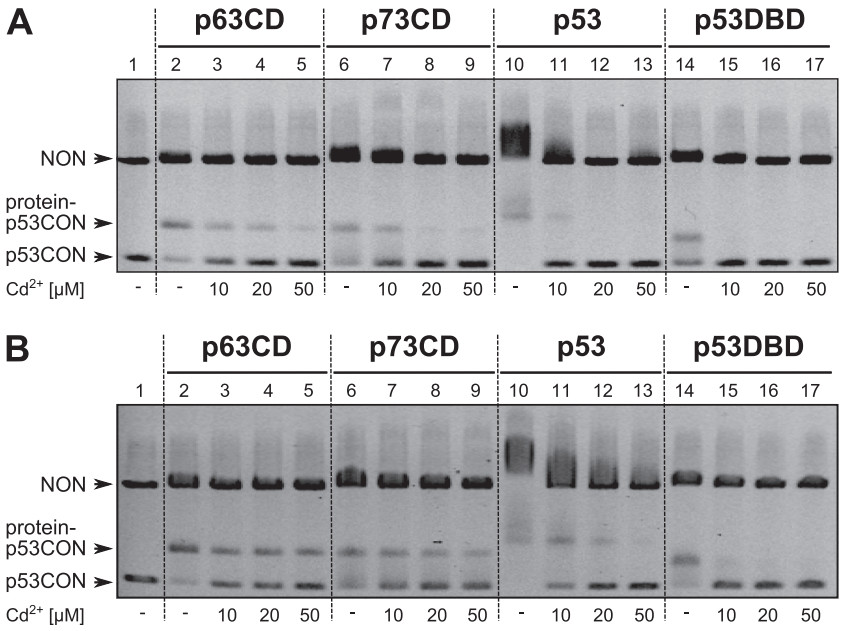

Fig. 1. Influence of Cd^{2+} ions on p53 family proteins binding of p53CON sequence (474 bp long fragment) on agarose gels. Proteins p63CD (lanes 2–5), p73CD (lanes 6–9), p53 (lanes 10–13) and p53DBD (lanes 14–17; molar ratio of protein tetramer/DNA was 4/1) in binding buffer with 1 mM TCEP were treated before (A) or after (B) binding to pPGM1/*Pvu*II fragments (200 ng) with increasing concentration of $CdCl_2$ (0; 10; 20; and 50 μM). Lanes 1 represented DNA without protein. DNA was detected by EtBr staining. 474 bp long fragment contains p53CON sequence; 2513 bp long fragment is nonspecific competitor. For more experimental data see Section 2.

observed at 10 μM $CdCl_2$ (Fig. 1A, lanes 3 and 7). As for p53 and p53DBD, almost full inhibition of DNA interaction was already detected at this concentration (Fig. 1A, lanes 11 and 15). Formation of p63CD-DNA and p73CD-DNA complexes was almost completely inhibited by 50 and 20 μM $CdCl_2$ respectively (Fig. 1A, lanes 5 and 8). Similar results were observed in experiments with p53CON oligonucleotides, where a sequence-specific DNA binding of p63CD and p73CD was inhibited by comparable concentrations (Fig. S1).

The sensitivity to cadmium was stronger for p73CD than for p63CD; even more sensitive to cadmium treatment were p53 constructs. These results indicate that sequence-specific DNA binding of p63 and p73 core domains is strongly affected by cadmium but the concentrations for efficient inhibition of p63CD and p73CD is about two times higher than for p53 proteins.

3.2. Protective effect of DNA–protein interactions against cadmium

We have previously shown that binding to p53CON protects p53 family proteins against cysteine oxidation by diamide [9,11]. In this study, we compared the effect of $CdCl_2$ exposition on free proteins p63CD, p73CD, p53DBD and p53 (Fig. 1A) with exposition on proteins bound to p53CON sequences (Fig. 1B). In the case of p53, the bound protein was about two-three times more stable than its free counterpart (Fig. 1A and B, lanes 11–13). Differences between cadmium effect on bound versus free p63CD and p73CD proteins were similar (Fig. 1A and B, lanes 5, 9). Overall, these data indicate protective role of DNA–protein interaction against inhibitory effect of cadmium.

3.3. Reversibility of $CdCl_2$ inhibition of p53 family DNA binding by EDTA and DTT

In our laboratory, it was previously observed that chelating agents EDTA and DTT reverted inhibition of sequence-specific binding of p53 caused by transition ions (zinc, cobalt, nickel) [14]. Millimolar concentration of EDTA abolished DNA binding of p53 and p73; full inhibition was observed by 10–20 mM EDTA [6,7,10]. The presence of a reducing agent is necessary for stabilization of core domains of all p53 family members [6–9,12] but the often used DTT is also potent chelating agent. For this reason reducing agent tris-(2-carboxyethyl)phosphine (TCEP) was used in all experiments instead of DTT.

To determine whether the cadmium effect on p53 family was reversible, cadmium-exposed proteins were incubated with DNA and then 2–100 molar excess of chelating agents EDTA and DTT (Fig. 2A–C) was added. Core domain of p53 exposed to 10 μM $CdCl_2$ showed full restoration of its p53CON binding capacity in dose dependent manner for both DTT and EDTA (Fig. 2A, lanes 4–9). Similarly p63CD and p73CD proteins treated by 50 μM $CdCl_2$ were able to recover after incubation with 2–100 molar excess of EDTA. Binding to p53CON was fully reversible by at least two times molar excess of EDTA (Fig. 2B and C, lanes 7, 8). Regarding DTT, we observed only partial reversibility of cadmium inhibition for p73CD (Fig. 2C, lanes 4–6) and DTT was not able to markedly revert cadmium inhibition of p63CD (Fig. 2B, lanes 4–6).

3.4. Nickel and cobalt inhibit sequence-specific binding of p53 family members less efficiently than cadmium

To compare cadmium with other transition ions, nickel and cobalt were tested on sequence-specific binding of p63CD and p73CD to p53CON. Previously, we and other groups showed that both ions inhibit binding of p53 protein in sub-millimolar concentration [10,13,14]. In agreement with our previous data with p53, p53DBD binding to p53CON was fully inhibited by 300 μM $NiCl_2$ or $CoCl_2$ when proteins were pre-incubated with ions (Fig. 3A and B). p63CD and p73CD were less sensitive to nickel and cobalt treatment, only partial inhibition of binding to p53CON was observed for 1.2 mM concentration of $NiCl_2$ and $CoCl_2$ (Fig. 3A and B, lanes 6, 12). Full inhibition of p63CD and p73CD binding was observed for >2 mM $CoCl_2$ and 10 mM $NiCl_2$ (Figs. 3A and B, S1). Also in the case of nickel and cobalt, the DNA–protein complexes were more resistant to ion treatment than protein alone (not shown). We conclude that nickel and cobalt are less effective

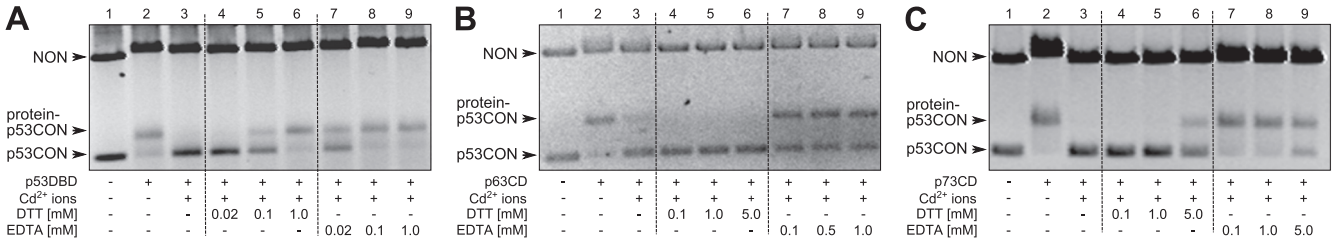

Fig. 2. Cadmium inhibition of p53, p63 and p73 core domains is reversible by EDTA. (A) Protein p53DBD (lanes 2–9) in binding buffer with 1 mM TCEP was treated by 10 μM $CdCl_2$ (lanes 3–9) and then increasing concentrations (0.02; 0.1; 1 mM) of DTT (lanes 4–6) and EDTA (lanes 7–9) were used to reverse the cadmium effect on binding to pPGM1/*Pvu*II fragments (200 ng). (B) p63CD, concentrations of DTT (lanes 4–6) and EDTA (lanes 7–9) were 0.1, 1 and 5 mM; $CdCl_2$ was 50 μM (C) p73CD, concentrations of DTT (lanes 4–6) and EDTA (lanes 7–9) were 0.1, 1 and 5 mM; $CdCl_2$ was 50 μM. Lane 1 represented DNA without protein.

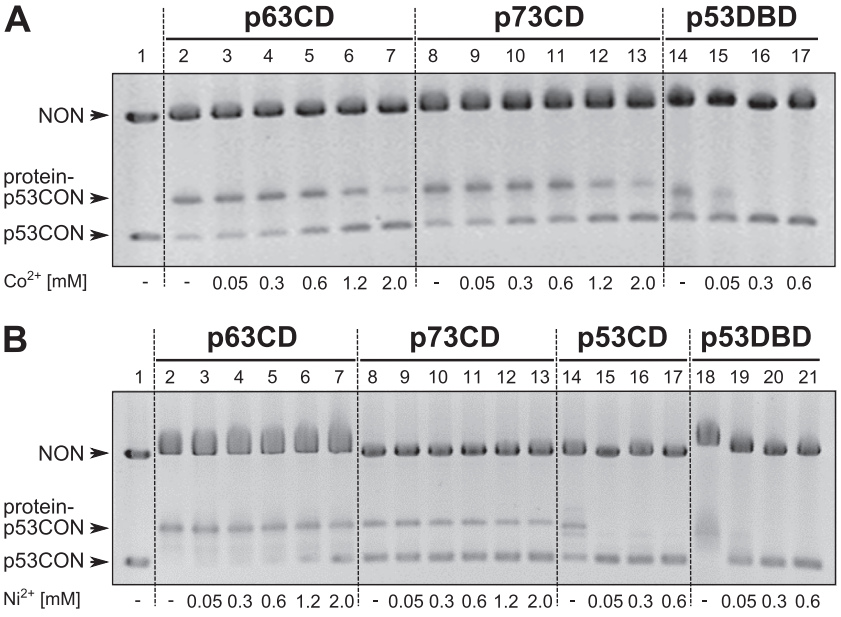

Fig. 3. Influence of Ni^{2+} and Co^{2+} ions on p53 family proteins binding to p53CON sequence (474 bp fragment) is less effective in comparison with cadmium effect. (A) Proteins p63CD (lanes 2–7), p73CD (lanes 8–13) and p53DBD (14–17; molar ratio of protein tetramer/DNA was 4/1) in binding buffer with 1 mM TCEP were treated before binding to pPGM1/*Pvu*II fragments (200 ng) with increasing concentration of $CoCl_2$ (0; 0.05; 0.30; 0.6; 1.2 and 2 mM). (B) Proteins p63CD (lanes 2–7), p73CD (8–13), p53CD (lanes 14–17) and p53DBD (18–21; molar ratio of protein tetramer/DNA about 4/1) were treated in the same way as in (A) by $NiCl_2$ (0; 0.05; 0.30; 0.6; 1.2 and 2 mM). Lanes 1 represented DNA without protein. DNA was detected by EtBr staining. 474 bp fragment contains p53CON sequence; 2513 bp long fragment is nonspecific competitor. For more experimental data see Section 2.

than cadmium in inhibition of p53 family binding to p53CON sequence. Full inhibition of p63CD and p73CD binding to DNA was observed in millimolar concentrations of $NiCl_2$ and $CoCl_2$ in contrast to micromolar concentrations of $CdCl_2$.

3.5. Cadmium impairs p53, p63 and p73 transactivation of target genes

Previously it was shown that cadmium inhibits p53 transactivation of $p21^{waf-1}$ in MCF7 cells and p53-dependent reporter pRGC in p53 null mouse fibroblast cell lines [13]. To determine if $CdCl_2$, $NiCl_2$ and $CoCl_2$ may impair p63 and p73 function as transcription factors, different p53 family isoforms (p63α, p63γ, p73β, p73γ and p53) were co-transfected with p53 family-dependent luciferase reporter vectors containing REs in front of luciferase gene (pGL3-MDM2-APP, pGL3-PGM1 and pGL3-BAX) into p53-null H1299 cells. Luciferase activity of reporters was measured 12 h after metal treatment. The transcription activity of p53, p63γ and p73β on BAX promoter is shown on Fig. 4. The addition of 50 μM $CdCl_2$ reduced p53, p63γ and p73β transcription activity on pGL3-BAX reporter by more than 95% in cells transfected with. In the absence of p53 family proteins, 50 μM $CdCl_2$ had no detectable

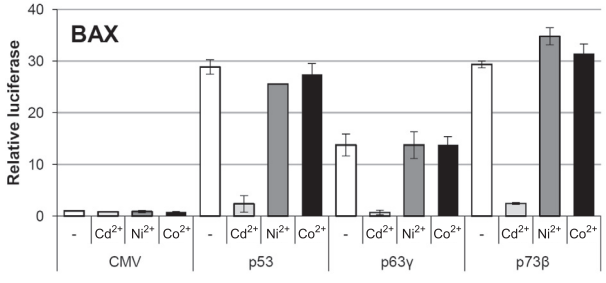

Fig. 4. Inhibition of p53 family transactivation activity by cadmium. H1299 cells were transiently transfected with plasmids expressing the p53, p63 and p73 constructs (based on pCDNA3.1) or pCDNA3.1 vector alone (CMV) together with the reporter plasmids expressing the Firefly luciferase gene under the transcriptional control of the indicated gene promoter (BAX) and a reference plasmid with the Renilla gene under control of the SV40 promoter. Luciferase activity was analyzed 12 h after $CdCl_2$, $CoCl_2$ and $NiCl_2$ treatment, as described in Section 2. Transfections were carried out in triplicates and at least three independent times, standards deviations are indicated.

effects on pGL3-BAX vector alone. Similarly, also p53, p63α, p63γ and p73γ transactivation of pGL3-MDM2 and pGL3-PGM1 reporter was inhibited by more than 90% by 50 μM $CdCl_2$ (Fig. S2). In

contrast to $CdCl_2$; $NiCl_2$ and $CoCl_2$ had no major effect on p53 family transcriptional activity on tested reporter vectors at 50 μM concentration (Figs. 4 and S2). We conclude that 50 μM cadmium strongly inhibits p53, p63 and p73 dependent transactivation of several promoters (BAX, MDM2 and PGM1) in H1299 cells.

4. Discussion

While there is clear separation of p53, p63 and p73 functions in development and differentiation; analysis of trascriptome, genome occupancy and other biochemical approaches indicate high degree of conservation in sequence-specific DNA binding [25]. Recently, contribution of sequence features on p63 and p73 transactivation, cooperativity of DNA binding, recognition of DNA damage as activation stimulus and structure of p63 and p73 complexes with DNA have been intensively investigated (e.g. [3,4,24,25]). Still, on the level of purified core domains there are only few detailed DNA binding studies of p63 and p73 (e.g. [6,7,9,26]). In this work, we have documented for the first time that the DNA binding domains of all p53 family members are metal sensitive. So far all tested metals (cadmium, cobalt and nickel) have been shown to interfere with the structure and DNA interactions of only p53. Metal ions caused inhibition of DNA binding and conformational perturbation of purified p53 protein was observed by biochemical methods (EMSA and ELISA) [10,13]. Due to high homology of DBDs inside p53 family, our results are in good agreement with a premise that also p63 and p73 DBD expose amino acid residues for interactions with metals and behave similarly to p53. As possible binding sites for cadmium, cobalt and nickel in p53 protein were suggested predominantly cysteines and histidines [10]. DBDs of p63 and p73 are less rich in cysteine and histidine residues than p53DBD and we hypothesize that this can be responsible for their lower sensitivity to cadmium, cobalt and nickel.

The situation becomes more complicated in cell systems, where inhibition of p53 dependent transactivation of luciferase reporter vectors was so far observed only for cadmium [13]. Additionally metal inhibition of the p53 dependent stress response was documented, although opposite effects have been also reported in different cell backgrounds [13,15,16]. In this study, we observed that p63 and p73 transactivation of several p53 dependent promoters (e.g. BAX, MDM2) was inhibited by cadmium treatment but not by cobalt and nickel in tested concentration.

Importance of our study can be based on the notion that there is an increasing number of studies describing that diverse repair systems including mismatch, base excision and nucleotide excision are inhibited by low non-cytotoxic concentrations of cadmium, cobalt and nickel; inhibition of DNA binding or enzymatic activity was observed for many repair proteins [27]. Ability of XPA protein, which participates in the first step of nucleotide excision repair, to bind DNA was disrupted by Cd, Co and Ni while Hg and Pb didn't show any alternation. Poly(adenosine diphosphateribose)polymerase 1, a molecular sensor in DNA strand-break responses, is inactivated by Ni, Co, Cd and Cu [27]. Also enzymatic activity of isolated DNA repair proteins hOGG1 and APE1 was impaired by cadmium [27]. Many of the above mentioned proteins are metalloproteins containing zinc in their DNA binding domains similarly to p53 family.

Hence, we propose that the metal sensitivity of p53 family proteins is conserved through whole family and can strongly affect DNA binding affinity not only on purified proteins but also in cell based assays. We suppose that inhibition of p53 family sequence-specific interactions with DNA together with activation of oxidative stress and inhibition of repair systems plays a role in metal carcinogenesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.11.027.

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