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Redox state of p63 and p73 core domains regulates sequence-specific DNA binding

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ABSTRACT

Cysteine oxidation and covalent modification of redox sensitive transcription factors including p53 are known, among others, as important events in cell response to oxidative stress. All p53 family proteins p53, p63 and p73 act as stress-responsive transcription factors. Oxidation of p53 central DNA binding domain destroys its structure and abolishes its sequence-specific binding by affecting zinc ion coordination at the protein-DNA interface. Proteins p63 and p73 can bind the same response elements as p53 but exhibit distinct functions. Moreover, all three proteins contain highly conserved cysteines in central DNA binding domain suitable for possible redox modulation.

In this work we report for the first time the redox sensitivity of p63 and p73 core domains to a thiol oxidizing agent azodicarboxylic acid bis[dimethylamide] (diamide). Oxidation of both p63 and p73 abolished sequence-specific binding to p53 consensus sequence, depending on the agent concentration. In the presence of specific DNA all p53 family core domains were partially protected against loss of DNA binding activity due to diamide treatment. Furthermore, we detected conditional reversibility of core domain oxidation for all p53 family members and a role of zinc ions in this process. We showed that p63 and p73 proteins had greater ability to resist the diamide oxidation in comparison with p53. Our results show p63 and p73 as redox sensitive proteins with possible functionality in response of p53 family proteins to oxidative stress.

1. Introduction

The p53 protein family (proteins encoded by three genes *TP53*, *TP63* and *TP73*) has important functions in many cellular processes such as DNA repair, cell cycle control, cellular senescence or apoptosis in response to DNA damage [1,2]. The p53 family members are known mainly to be important for the prevention of cancer, but p63 and p73 are crucial also in controlling of development and differentiation [3,4]. All p53 family genes express several protein isoforms because of usage of alternative promoters, multiple splicing sites and alternative initiation of translation. Synergic or antagonistic activities of protein isoforms depend on mechanism of production and structure [5]. Nevertheless, the evolutionary conserved domain structure of these proteins shares a central sequence-specific DNA binding domain (DBD) with a N-terminal transactivation domain and a C-terminal tetramerization domain. As expected, the highest homology between the p53 family members is found in the DBD performing a sequence-specific DNA binding (60% identity between p63 and p53 and 63% between p73 and p53) [6]. Both (p63 and p73) can bind to the same p53 responsive elements (p53CON, containing two half-site decamers 5′PuPuPuCA/TT/AGPyPyPy3′ in direct orientation), but the activated pathways are different [7]. Even though high homology in their core domains p63 and p73 exhibit promoter selectivity and transactivate also a number of unique target genes.

All three DBD contain conserved cysteine residues, which are necessary structure features of all redox-sensitive transcription factors [8]. Number and positions of cysteine residues in central domains are, to some extent, characteristic for the given protein (Fig. S1). Nevertheless, seven of them are highly conserved in all p53 family members [9,10]. Three of these cysteines with one histidine bind one ion of zinc holding together two loops of a conservative structural motif, making it ready for binding to response DNA elements (Fig. S1) as recently showed also for p63 and p73 DNA complexes [7,11,12]. Zinc incorporation within the all core domains is necessary for proper folding and disruption of this interaction implicates conformation changes [13]. Comparison of tagging of thiol groups with maleimide–polyethylene glycol (MAL–PEG) of p53 and p73β revealed that modification of p73β cysteines by this agent is facilitated in the presence of EDTA. Comparison of p53 and p73β proteins showed that zinc chelation by EDTA was more detrimental in the ability of p73β than p53 to bind p53CON [14]. Similar observation in behavior was described for

Abbreviations: p53CON, p53 consensus sequence; diamide, azodicarboxylic acid bis[dimethylamide]; 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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p63CD in the case of a chelating agents 1,10-phenanthroline (phen): p63CD lost DNA binding activity at lower concentrations of phen than p53CD [10].

Redox changes of cysteines can modulate zinc ion coordination near the DNA binding surface as well as a sequence-specific DNA binding and subsequent transactivation of target genes, as was shown for p53 [15] and it is expected for p63 and p73. In the case of p53 it was shown that cysteine residues located on the protein surface are more susceptible to redox modification and therefore are primary candidates for redox modulation. Previously we reported on a mechanism of reversible oxidation of p53. If the p53 oxidation by diamide and subsequent reduction of disulphide groups by DTT takes place in the presence of abundant zinc ions then reduced cysteines can immediately bind the zinc ion in their vicinity and the protein assumes its native conformation. An excess of EDTA removes free and loosely bound zinc ions, thereby making the p53 molecule fully competent for sequence-specific DNA binding. This mechanism does not occur if a stronger oxidation agent (such as permanganate) causes further changes in the protein molecule (including deeper oxidation of sulphur moieties) that cannot be restored by the DTT reduction [16].

In this study we used diamide for study of the oxidation effect of cysteine thiol groups in p53 family core domains on their sequence-specific DNA–protein interaction. Our data show that the sequence-specific DNA binding properties of all three core domains were inhibited by this agent and the presence of zinc ions influenced reversibility of redox modulation of all studied proteins. We also found that binding to the recognition DNA element strongly protected the core domains from a loss of DNA binding due to diamide treatment.

2. Materials and methods

2.1. DNA samples

Synthetic 50-mer oligonucleotides containing p53CON (5′-GACGGTATCGATAAGAGACATGCCTAGACATGCCTCTTGATATCGAATTC-3′ and complementary strand, p53CON sequence underlined) were supplied by VBC Biotech (Vienna, Austria), ^{32}P-labeled oligo was prepared as previously described in [17].

P53CON fragment long 474 bp was prepared by *PvuII* (TAKARA) digestion of supercoiled plasmid pPGM1 (pBSK containing a p53CON sequence AGACATGCCTAGACATGCCT) as described in [19], second fragment long 2513 bp was used as nonspecific competitor. Plasmids encoding human full length wild-type p53 (p53, aa 1–393, pT7-7p53 [18]), p53 core domain (p53DBD, aa 94–312, pET3dp53, [19]) and GST fusion core domains of p53, p63 (p53CD, aa 94–312; p63CD, aa 114–349; both in pGEX-4T [10], Roche Diagnostics GmbH) and p73 (p73CD, aa 104–339, cloned to pGEX-4T similarly as in [10] using cDNA from [20]) were used for protein expression and purification. All vector constructs were confirmed by sequencing.

2.2. Protein expression and purification

Full length protein p53, p53 core domain, and GST fusion core domains of p53, p63 and p73 were purified by protocol described in [21]. Final purification was achieved by preparative size-exclusion chromatography on a 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.

The purity and appropriate size of each protein was analyzed by Coomassie blue staining of 12.5% SDS–PAGE gels (Fig. S2) and by Western blotting with appropriate antibodies: DO11 for p53DBD; DO1 for p53; anti-GST (G1160, Sigma) for GST fusion core domains. Protein concentration was determined densitometrically from Coomasie Blue R-250 stained gels using bovine serum albumin as a standard.

2.3. DNA binding assays

P53 family proteins were mixed with ^{32}P-radiolabeled 50-mer with p53CON (1 pmol) in molar ratio 1/1 (protein tetramer/DNA) and incubated in binding buffer (5 mM Tris–HCl, pH 7.6, 0.01% Triton X-100, 50 mM KCl and 0.2 mM DTT) in presence of 50 ng pBluescript/*SmaI* competitor for 15 min on ice to reach equilibrium. Before or after addition of DNA, proteins were treated with oxidative reagent azodicarboxylic acid bis[dimethylamide] (diamide, Sigma) in increasing concentration from 0 to 2 mM in reaction for 15 min on ice. After incubation protein–DNA complexes were analysed by electrophoretic shift assay (EMSA). Samples were loaded onto 5% native polyacrylamide gel containing 0.5× TBE buffer. After 1 h electrophoresis (at 4–6 V/cm), gels were dried and DNA–protein complexes were detected by autoradiography (Typhoon FLA 9000, GE Healthcare).

Interaction of p53 family proteins with 474 bp long DNA fragment was studied by EMSA in agarose gels. Usually proteins were incubated in binding buffer (5 mM Tris–HCl, pH 7.6, 0.01% Triton X-100 and 50 mM KCl with or without 0.2 mM DTT) and mixed with pPGM1/*PvuII* fragments (200 ng; the 474 bp with p53CON, the 2513 bp fragment as nonspecific competitor) at molar ratio 4/1 (protein tetramer/DNA) for 15 min on ice to reach equilibrium. Before or after addition of DNA the proteins were treated with diamide (0–2 mM in reactions) for 15 min on ice.

Samples were loaded onto a 1% agarose gel containing 0.33× TBE buffer. After 1.5 h of electrophoresis (at 4–6 V/cm), DNA was stained with Ethidium Bromide and photographed using Herolab documentation system (Herolab).

2.4. Influence of zinc ions on proteins oxidation

In study of condition of oxidation reversibility the proteins in DNA binding buffer (with 50 μM DTT) were firstly mixed with zinc ions (100 μM $ZnSO_4$ in final concentration) and then with other reagents (combination and concentrations described in Fig. 4). At last pPGM1/*PvuII* (200 ng) was added to proteins. Each component was left for 15 min on ice.

3. Results

3.1. Sequence-specific binding of p53 family members is inhibited by diamide oxidation

Several studies showed that sequence-specific binding of p53 is inhibited by oxidation of its core domain by diamide [15,16] or hydrogen peroxide [22] *in vitro* as well as in cells [23]. To investigate effects of oxidation agent on other p53 family members, we isolated GST fused p63 and p73 core domains (p63CD, aa 114–349; p73CD, aa 104–339) and their sequence-specific binding to p53CON inserted in 474 bp long DNA fragment (shorter pPGM1/*PvuII* fragment, Fig. 1A) or in 50-mer oligo (Fig. 2A) was studied by EMSA. Full length p53 (p53), p63CD and p73CD proteins were bound to p53CON in 474 bp long fragment at molar ratio 4/1 (protein tetramer/DNA). Under these conditions the majority of 474 bp fragment was bound by the protein (Fig. 1A, lane 2, 8, 14). At first, protein samples were oxidized by increasing amount of diamide (0.125–2 mM) before DNA addition in presence of 0.25 mM DTT. Partial inhibition of binding to p53CON was observed at 0.125 mM diamide concentration which was detected by increasing amount of free 474 bp long fragment (Fig. 1A, lanes 3, 9, 15).

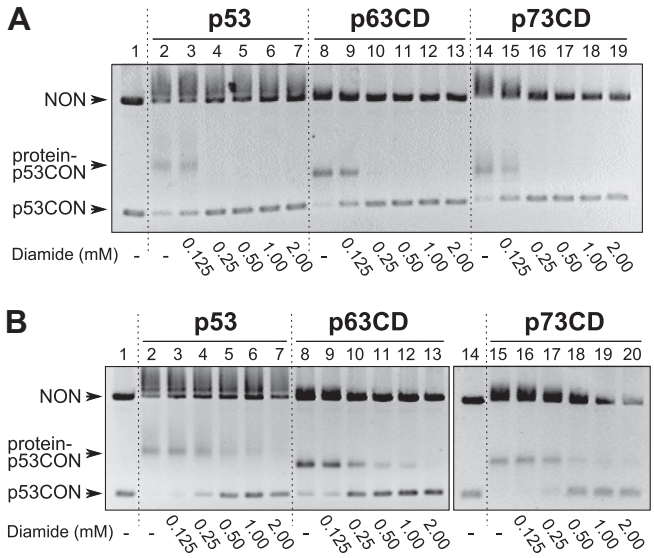

Fig. 1. Influence of diamide on p53 family proteins binding to 474bp fragment with p53CON sequence on agarose gels. Proteins p53 (lanes 2–7), p63CD (8–13) and p73CD (14–19; molar ratio of protein tetramer/DNA was 4/1) in binding buffer with 0.25 mM DTT were treated before (A) or after (B) binding to pPGM1/*PvuII* fragments (200 ng) with increasing concentration of diamide (0; 0.125; 0.25; 0.5; 1 and 2 mM). Lane 1 and lane 14 (in Fig. 1B) represented DNA without protein. DNA was detected by EtBr staining. 474 bp fragment contains p53CON sequence (p53CON); 2513 bp long fragment is nonspecific competitor (NON). For more experimental data see Section 2.

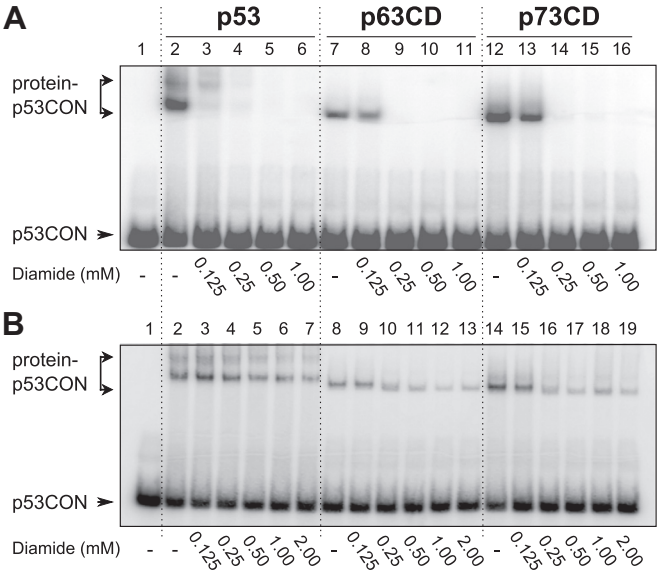

Fig. 2. Influence of diamide oxidation on p53, p63CD and p73CD binding to 50-mer with p53CON sequence on polyacrylamide gels. P53, p63CD and p73CD proteins (molar ratio of protein tetramer/DNA was 1/1) in binding buffer with 0.25 mM DTT were treated with increasing concentration of diamide (0; 0.125; 0.25; 0.5; 1 and 2 mM) before (A) or after (B) binding to ^{32}P-radiolabeled 50-mer oligo (p53CON, 1 pmol) in the presence of nonspecific competitor DNA (50 ng pBSK/*SmaI*). Lane 1 represented DNA without protein. DNA was detected by autoradiography.

Formation of protein–DNA complexes was totally inhibited by 0.25 mM diamide concentration as observed for all p53 family members (Fig. 1A, lanes 4, 10, 16). Similar results were observed in experiments with 50-mer oligo, where a sequence-specific DNA binding of all tested proteins p53, p63CD and p73CD (p53DBD and p53CD not shown) were inhibited in a similar way (Fig. 2A, lanes 3–4, 8–9, 13–14). These results indicate that, similarly as known for p53, also sequence-specific DNA binding of p63 and p73 core domains is strongly affected by diamide oxidation.

3.2. Interaction of p53 family proteins with p53CON partially protects the proteins against diamide oxidation

The p53DBD cysteine residues are involved in zinc coordination and redox regulation [9,23]. We have previously shown that p53 binding to supercoiled DNA partially protected protein against binding inhibition due to oxidation [16]. Here we compared effect of diamide exposition to free proteins with exposition to proteins bound to p53CON sequences (in 474 bp – Fig. 1A and B; in 50-mer – Fig. 2A and B). There were remarkable differences between bound and free proteins. Bound proteins were about 5-times more stable. About 90% of free proteins treated with 0.25 mM diamide (in presence of 0.25 mM DTT) were not able to bind to DNA, while in case of bound proteins a diamide concentration as high as 2 mM was required to reach comparable (80–90%) level of inhibition. In the presence of 2 mM diamide, formation of all protein complexes with 474 bp fragment was inhibited in more than 90% (Fig. 1B, lanes 7, 13, 20), while the amount of analogous complexes with a 50-mer oligo was decreased to 20%, compared to reduced protein (Fig. 2B, lanes 7, 13, 19). Only small differences between individual proteins were observed.

It is well known that the presence of a reducing agent (DTT, 2-mercaptoethanol, tris-(2-carboxyethyl)phosphine (TCEP)) stabilizes all p53 family members [10,14,15,24]. Influence of DTT on effective diamide concentration necessary for full inhibition of p63CD interaction was studied in detail. Effect of diamide was tested in presence of 50 μM and 250 μM DTT (shown for p63CD, Fig. 3). Full inhibition of DNA binding of free protein p63CD was observed at 0.25 mM diamide (in 50 μM DTT) and at 0.5 mM diamide (in 0.25 mM DTT). At least 2-fold molar excess of oxidizing agent was necessary for full inhibition. Similar effect of DTT was

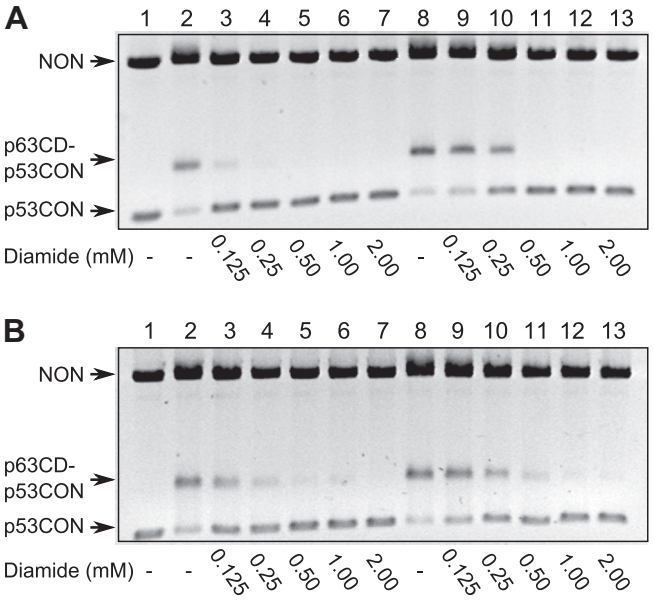

Fig. 3. DNA binding properties of p63CD protein were protected against diamide oxidation by DTT and interaction with p53CON. Protein p63CD (molar ratio of p63CD/DNA was 4/1) was treated with increasing concentration of diamide (0; 0.125; 0.25; 0.5; 1 and 2 mM) before (A) or after (B) addition of pPGM1/*PvuII* fragments (200 ng; p53CON, short 474 bp fragment; NON, long 2513 bp fragment). Reactions in lanes 2–7 were with 50 μM DTT, lanes 8–13 were with 0.25 mM DTT. Lane 1 represented DNA without protein. DNA was detected by EtBr staining.

observed in the case of diamide treatment of protein bound to DNA (Fig. 3B).

3.3. Oxidation of p63 and p73 core domains is reversible process

We have previously shown that in the presence of excess zinc ions, loss of p53 DNA binding due to diamide treatment was reversible even in absence of DNA during oxidation [16]. This phenomenon was discussed in terms of zinc coordination in p53. Also inhibition of p63 and p73 DNA complexes by chelating agents (EDTA or phen) was reversible in the presence zinc ions. The following experiments were focused on reversibility of p63CD and p73CD DNA binding after diamide oxidation in excess of zinc ions by DTT (Fig. 4A). All DBDs exposed to 1 mM diamide in the presence of abundant zinc ions (100 μM $ZnSO_4$) in solution showed full restoration of their p53CON binding capacity after reduction by 5 mM DTT followed by treatment with EDTA (to remove excess of zinc ions). Such behavior was observed for p53DBD, p63CD, p73CD (Fig. 4A), p53CD and p53 (not shown).

The following experiments with p53, p63CD and p73CD were focused on combined effects of zinc, EDTA, DTT and diamide with respect to reversibility of p63 and p73 inhibition due to oxidation (Fig. 4B). If, after diamide oxidation, a chelating agent EDTA was applied followed by DTT, p63CD binding to DNA was partially restored in the case of higher excess of DTT (lane 11). DNA binding of other proteins (p53 and p73CD) was not recovered under this condition (lanes 3–4 for p53 and lanes 17–18 for p73CD). Interestingly, in the case of immediate DTT application after diamide treatment followed by EDTA, both proteins p63CD (lanes 12–13) and p73CD (lanes 19–20) were able to bind p53CON. Also in the case of p53 we observed partial restoration of DNA binding by 1.5 molar excess of DTT over diamide (lane 6). Finally, if the proteins were oxidized in the presence of zinc ions, followed by EDTA and DTT addition (in this order), oxidation of all proteins p53, p63CD and p73CD was fully reversible in this case (lanes 7–8 for p53, lanes 14–15 for p63CD and lanes 21–22 for p73CD). Taken together, our results indicate protective effects of available (unchelated by EDTA) excess zinc ions on the p53 family core domain structures under oxidative conditions. Moreover, our observations suggest a higher resistance of p63CD and p73CD towards irreversible inhibition due to oxidation, compared to p53.

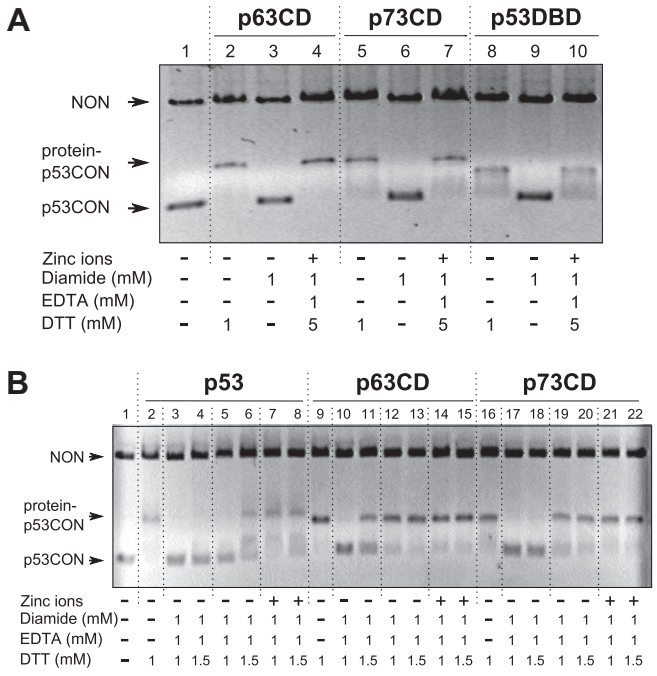

Fig. 4. Diamide oxidation of p63 and p73 core domains is reversible. Influence of zinc ions on p53 family core domains redox behavior. (A) Proteins p63CD (lanes 2–4), p73CD (5–7) and p53DBD (8–10) in binding buffer with 50 μM DTT were treated with 1 mM DTT (lanes 2, 5 and 8) or 1 mM Diamide (lanes 3, 6 and 9) or 100 μM $ZnSO_4$ followed by reagents in order of addition 1 mM Diamide, 5 mM DTT and 1 mM EDTA (lanes 4, 7 and 10). In the end pPGM1/*PvuII* fragments (200 ng; p53CON, short 474 bp fragment; NON, long 2513) were added. Lane 1 represented DNA without protein. (B) Proteins p53 (lanes 2–8), p63CD (9–15) and p73CD (16–22) in binding buffer with 50 μM DTT were treated with 1 mM Diamide and then with combination of reagents in order of addition EDTA–DTT (lanes 3–4, 7–8, 10–11, 14–15, 17–18 and 21–22) or DTT–EDTA (lanes 5–6, 12–13 and 19–20). The first lane in each couple enclosed within spotted lines contains 1 mM DTT and second lane 1.5 mM DTT, amount of EDTA was 1 mM in each reaction. The last couple of each protein (lanes 7–8, 14–15 and 21–22) was enriched with 100 μM $ZnSO_4$ added before oxidative reagent. In the end pPGM1/*PvuII* fragments (200 ng; p53CON, short 474 bp fragment; NON, long 2513 bp fragment) were added. Lane 1 represented DNA without protein and lanes 2, 9 and 16 contained only protein, DNA and 1 mM DTT.

4. Discussion

Although p53, p63 and p73 share considerable homology in their DNA binding domains and transactivate target genes from the same responsive elements, there have been only few detailed studies examining their interactions at the level of purified core domains or full length proteins with DNA. Recently, it was shown that binding affinity of p63 and p73 core domains (DBDs) to the same response elements was about 20–100 times smaller than DBD of p53 [7]. Authors proposed that these differences may be due to diversity in the oligomerisation interfaces which is in p73DBD and p63DBD smaller than in p53DBD [12]. Using fluorescence anisotropy assay conservation of DNA-binding specificity and oligomerisation within p53 family (ΔNp63α,β,γ, ΔNp73β and p73DBD) was shown. All studied proteins had similar affinities to response element DNA with only exception of p73DBD (more than 4–5 times weaker binding than the inherently tetrameric ΔNp73β) [25]. Using EMSA assay similar affinity and equal specificity were detected in binding of purified full length proteins (p63γ, p73β to GADD45 response sequence [26,27]. Interestingly, gel shift assays and nitrocellulose filter DNA binding assay did not detect p63DBD binding to response elements [10,12] while SPR revealed approximately three order of magnitude lower p63DBD affinity to a 20-bp response element, compared to p53DBD [12]. On the other hand, it was observed that fusion of p63DBD with GST which forms dimers in solution dramatically enhanced its binding to response elements [10].

In this work we therefore used GST fusion constructs of p63DBD and p73DBD to confer the DBDs reasonable DNA binding affinity, allowing us to compare redox modulation of the DNA binding domains within the p53 family. We observed similar DNA binding affinities of reduced untagged p53DBD and GST fused p63DBD and p73DBD to a synthetic p53 consensus sequence [19]. GST fused p53 core domain exhibited even higher affinity to this sequence (not shown); we thus selected the former three construct for the comparative study.

Redox regulation of p53-DNA binding activity has been well documented and shown to affect zinc coordination and correct p53 folding [15,16,22]. Recent structure analysis of core domains p63 and p73 in complexes with sequence-specific DNA [7] as well as studies of effects of metal chelating agents (1,10-phenantroline or EDTA) [10,14] led us to a hypothesis that DNA binding of p63 and p73 may be redox-modulated similarly to p53. Indeed, our present study has revealed that sequence-specific DNA binding of p53, p63 and p73 core domains is sensitive to treatments with a thiol oxidizing agent diamide. At first, all investigated proteins were inhibited in their sequence-specific binding to p53CON in

50-mer oligonucleotide as well as in a 474 bp long DNA fragment. We observed protection of all proteins from oxidation by interaction with the p53CON. Our results further show the p53-family core domains differ from each other in condition necessary for maintaining of reversibility of the redox modulation. In general p63 and p73 core domains showed a higher apparent DNA stability under oxidizing conditions than p53.

Diamide, hydrogen peroxide, glutathione, and metals were endogenous and exogenous molecules which have been shown to alter the redox status of p53 (reviewed in [9]) and influence its function as transcription factor. So also redox control of p63 and p73 might represent simple biochemical mechanism to explain how activity of these proteins can be regulated at physiological processes associated with cell signaling, response to stress, DNA damage and chemotherapy.

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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.2013.02.097.

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