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A p53-type response element in the *GDF15* promoter confers high specificity for p53 activation

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Abstract

GDF15 is a transcriptional target gene for p53 and its family members, p63 and p73. Its promoter region contains two p53-type response elements, RE1 and RE2, and RE2 confers p53-specific transactivation. RE2 contains several mismatches from the canonical p53 response element (RRRCWWGYYY). Two mismatches in the RRR span and T base of the RE2 core sequence in the most 3' quarter-site are critical for inhibiting the binding affinity to p63 and p73 and corresponding promoter activity. Our results strongly suggest that differential DNA binding affinities between p53 family member proteins act, at least in part, to confer specific target gene activation.

Keywords

p53 response element; *GDF15*; promoter; p53 transactivation; gene activation

Introduction

p63 and *p73* are members of the *p53* tumor suppressor gene family. Transcriptionally active isoforms of p53, p63 and p73 can activate target gene transcription by binding to response elements such as tandem repeats of RRRCWWGYYY (the canonical p53 response element) [1–6]. Despite structural similarities in their DNA binding domains, the biologic functions of p53 family members are considerably different from each other. Whereas p53 plays a major role in suppressing tumor development, p63 is involved in limb, skin and craniofacial development, and p73 is involved in neuronal and immune system development [7–10]. These differences may be due to differences in target gene regulation.

We have previously demonstrated that *p53*, *TAp63β* and *TAp73β* induce different but overlapping sets of target genes in inducible cell lines using oligonucleotide microarrays [11]. It has also been shown that promoters of several p53 target genes are differentially regulated by p53 family members [12]. To date, a number of genes have been reported by us and others to be targets of p63 and/or p73, such as *JAG1*, *JAG2*, *IL4R*, *Np73*, *AQP3*, *REDD1*, *EVPL*, *SMARCD3*, *BPAG1e* and *WNT4* [11,13–20]. We have also demonstrated that a response element consisting of RRRCATGYYY repeats with mismatched bases in the RRR and YYY stretches and a gap between half-sites was specifically activated by p53 whereas one consisting of RRRCGTGYYY repeats was specifically activated by TAp63γ [17].

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In the present study, we show that the *GDF15* promoter is activated to a greater extent by p53 than by TAp63 β , TAp63 γ and TAp73 β . The most 3' quarter-site of the p53-type response element, RE2, exhibited higher activation by p53 than by other family members due to higher binding affinity for p53. Our results clearly demonstrate the differential binding affinities of p53 family member proteins to a response element as a putative mechanism to confer differential transactivation of a target gene promoter.

Materials and Methods

Response element search, promoter plasmids and luciferase assay

Our computer-based method of searching for putative p53-type response elements was described previously [17]. Promoter plasmids used in Figure 1A and Figure 3B were generated by cloning annealed oligonucleotides into the MluI and XhoI sites of the pGL3Basic plasmid (Promega, Madison, WI) using primer sequences listed in Supplemental Table 1 and Supplemental Table 3, respectively. Luciferase reporter assays were described previously [2]. Briefly, reporter and expression vectors as well as the Renilla luciferase reporter vector were transiently transfected into Saos2 osteosarcoma cells (devoid of p53 and p63 expression) and analyzed after 48 hours for luciferase activity by the Dual Luciferase Reporter Assay (Promega). Data reflect mean fold-change in luciferase activity in experimental cells over cells co-transfected with empty pGL3-Basic and pcDNA3.1-Hygro vectors. Experiments were performed in triplicate.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation assays were performed as previously described using ectopically expressed 3XFLAG-tagged p53 protein. After induction of p53 with 1 μ g/ml of tetracycline, cells were fixed with formaldehyde, and the p53-DNA complex was immunoprecipitated by an anti-FLAG antibody. The PCR primers used for *GDF15* genome amplification were GDF15F, AGCTGTGGTCATTGGAGTGTT and GDF15R, TTCACCGTCCTGAGTTCCTGC.

Immunoblotting and electromobility shift analysis

Immunoblotting and electromobility shift analysis were described previously [11,16,17]. Briefly, *in vitro* translated p53, p63 and p73 proteins were separated on a 9% SDS-polyacrylamide gel. ³²P-labeled oligonucleotides were incubated with p53, p63 or p73 proteins and separated on a 5% native polyacrylamide gel in 0.25X TBE. Probe sequences are listed in Figure 3A and Supplemental Table 2. Two hundred ng of pAb421 (Oncogene Research, San Diego, CA) were used in p53 binding analyses. Probes were separated on a 15% native polyacrylamide gel to confirm equal labeling.

Results

The *GDF15* promoter is activated by p53 family member proteins

We have previously shown that *EVPL*, *SMARCD3*, *BPAG1e*, and *WNT4* promoters are specifically activated by p63 or p63 and p73 but not by p53. These promoters contain p63 or p63 and p73 specific response elements consisting of canonical p53-type response elements or p53-type response elements with an additional response element [11,16,17]. In order to further clarify the mechanism of activation by p53 family members, we examined the activation of various promoters by co-transfection of potent p53 family transactivators such as p53, TAp63 β , TAp63 γ and TAp73 β [1,2,6]. Among the genes activated over 2-fold by either p53, TAp63 β or TAp73 β as detected by our previous microarray analysis, a computational search was performed to identify putative p53-type response elements in the 5' flanking region of

exon 1. Based on this search, 26 promoters were cloned and measured by luciferase assay for transactivation by p53, TAp63 β , TAp63 γ and TAp73 β (Figure 1A). Putative response elements which were included in the cloned fragments are listed in Supplemental Table 4. Four out of 26 promoters, *GDF15*, *GPR56*, *CAPG* and *RIOK3*, were activated over 8-fold to varying levels by p53, TAp63 β , TAp63 γ and TAp73 β .

GDF15, also known as *PDF*, *MIC1*, *PLAB*, *MIC-1*, *NAG-1* and *PTGF β* , has been reported to be a p53 target gene [21–26]. Although the *GDF15* promoter was activated by all p53 family member proteins, it was most strongly activated by p53. The *GDF15* promoter fragment contains two p53-type response elements, one relatively 5' (position -864), and the other relatively 3' (position +15), which we designated as RE1 and RE2, respectively. We made 3 different deletion mutants of the *GDF15* promoter reporter plasmids, an RE1 deletion, an RE2 deletion and an RE1 & 2 deletion, and examined their transactivation by p53 family member proteins (Figure 1B). Consistent with previous reports, deletion of RE1 did not affect transactivation by p53, whereas deletion of RE2 greatly decreased transactivation [21,23,25]. On the contrary, deletion of either RE1 or RE2 decreased transactivation by TAp63 β , TAp63 γ and TAp73 β . Thus, we speculated that RE2 was responsible for conferring activation by p53. In order to determine if RE2 bound to p53 *in vivo*, we performed chromatin immunoprecipitation (ChIP) analysis followed by PCR amplification and clearly showed precipitation of a p53 fragment (Figure 1C) which indicated binding.

p63 and p73 bind less to the RRRCT sequence

The canonical p53-type response element consists of 2 repeats of a RRRCWWGYYY half-site, each half-site consisting of head to head repeats of a quarter-site (RRRCW) [27]. We and another group have shown that RRRCA repeats in which CATG is the core sequence exhibit highest transcriptional activation by p53, and the magnitude of transactivation by p53 varies depending on the nucleotides surrounding the core sequence [16,28]. Thus, we examined the binding affinities of p53, p63 and p73 proteins to different p53-type response elements.

Before examining differential binding affinity to various elements, we first demonstrated the effects of the carboxyl terminus of p53 and p63 on *in vitro* protein-DNA binding because it is known that presence of the carboxyl-terminus of the p53 protein inhibits DNA binding *in vitro* [29]. Consistent with previous reports, deletion of the carboxyl-terminus of p53 or adding the carboxyl-terminus suppressive antibody pAb421 strongly enhanced binding to probe DNA consisting of two tandem repeats of a p53-type RE with perfectly matched RRR and YYY stretches and CATG as its core element (Supplemental Figure 1). In the case of p63, the carboxyl-terminus deletion mutant TAp63-C394, in which the carboxyl-terminus is deleted at amino acid residue 394 (next to the oligomerization domain), exhibited a higher binding signal than all other isoforms of p63. However, the difference in binding affinity between wild-type p63 isoforms and TAp63-C394 was less than that between wild-type p53 and p53-C360 or p53 and pAb421. Supplemental Figure 1 also shows that the binding affinities of the various p63 and p73 isoforms were not significantly different. Taking these results into consideration, we performed *in vitro* DNA-protein binding analyses using p53-type response elements containing different core sequences and various numbers of mismatches and gaps.

We synthesized p53-type response element oligonucleotides which consisted of all possible combinations of core sequences that contained at least one A or T base in the WW of the core sequence with perfectly matched RRR and YYY stretch sequences (Supplemental Table 2). Figure 2A shows *in vitro* DNA-protein binding analysis of probes containing various mutated core sequences with p53 family member proteins. Each p53 family member tested exhibited the strongest binding signal with the CATG-PM probe. A striking difference was found in the binding affinities of p53 to the CTAG-PM probe compared to p63 and TAp73 β . p53-C360 and

p53-pAb421 showed relatively strong binding signals with CTAG-PM, while both p63 isoforms, TAp63-C394, and TAp73 β exhibited none to very weak binding signals.

p53-type response elements consist of combinations of 4 quarter-sites, and a p53 tetramer can bind to each quarter-site on a response element [30]. The results of our binding analysis are summarized according to quarter-site classification in Figures 2C and 2D. p53, p63 and p73 exhibited high affinity to oligonucleotide probes containing RRRCA and low affinity to those containing RRRCS. p53 had moderate binding affinity to probes containing RRRCT while p63 and p73 had low binding affinity to such probes. Wild-type p53 in the absence of antibodies gave a weak binding signal only with the CATG-PM probe (data not shown).

Next we examined the effect of mutations in the RRR stretches and a gap between the two half-sites. We found that CATG sequences consist of 4 high affinity quarter-sites (2 tandem repeats, upper and lower strands), whereas response elements with CGTGs as their core sequences consist of alternately repeated high and low affinity quarter-sites. Both p53-C360 and p53-pAb421 bound to CATG-MM3G1 with higher affinity than CGTG-PM. Whereas the binding of p53 and p63 to CGTG-containing sequences was greatly attenuated by the introduction of mismatches and a gap, only the binding affinity of p63 to CATG-containing sequences was significantly affected by mismatches and a gap. Thus, we conclude that mismatches in the RRR stretches and a gap decreased protein-DNA binding for p63 more efficiently than for p53. That is, despite mismatches in the RRR stretches and a gap between half-sites, p53 still exhibited strong binding affinity to the CATG-containing response element.

Mutating the most 3' quarter-site restores transactivation of RE2 in the *GDF15* promoter by p63 and p73

Based on our *in vitro* binding analysis, we hypothesized that the following two specific features of RE2 in the *GDF15* promoter inhibited its binding affinity to both p63 and p73: 1) The most 5' (upper strand) and 3' (lower strand) RRRCW quarter-sites contain mismatches in the RRR stretches, 2) The W of the most 3' RRRCW is T, not A. Thus, we mutated the most 3' quarter-site as shown in Figure 3A and performed binding analysis with p53, p63 and TAp73. Both p53-C360 and p53-pAb421 bound to Mut1 to Mut7 probes almost similarly, and slightly more intensely than to the wild-type probe. On the contrary, the binding affinities of p63 and TAp73 were greatly enhanced when one or both mismatches were restored (T to G) or the T base in the core of the quarter-site was replaced by A. Binding affinity was greatest when both of these changes were made (Mut7).

In order to examine if these differential binding affinities correlated with transactivation by p53, p63 and p73, we cloned the Mut1 to Mut7 oligonucleotides into a reporter vector and performed luciferase assays. Mutating the most 3' mismatched bases (TT to GG, Mut3 and Mut7) increased luciferase activity in response to all p53 family members, especially p53 and TAp73 β (Figure. 3B). Most remarkably, the mutant containing GGGCA as its most 3' quarter-site (Mut7) increased the transactivation ability of p63s and TAp73 β almost 100-fold compared to wild-type (Figure 3B). By contrast, this quarter-site only enhanced transactivation by p53 less than 2-fold compared to wild-type. Thus, the specific activation of RE2 by p53 was due to the 2 mismatches in the RRR stretches and the T base in the core sequence in the most 3' quarter-site.

Discussion

In this study, we demonstrated that the 3' response element in the *GDF15* promoter, RE2, exhibited higher binding affinity to p53 than to p63 or p73. The most 3' quarter-site of RE2 has 2 mismatches in the RRR stretch and a T as the W in the core sequence which conferred lower affinity to p63 and p73, resulting in p53-specific activation. We also demonstrated that

the binding of p53 family proteins to DNA was theoretically determined by the sequence of each quarter-site which presumably corresponded to binding to each molecule of a p53 tetramer [30]. *In vitro* protein-DNA binding analysis showed that both p63 and p73 had lower binding affinity to the RRRCT quarter-site than p53. Klein *et al.* demonstrated that unlike p53 molecules, p63 molecules do not bind to each other in their DNA binding domain while binding to response elements [31]. This difference may contribute to the differential binding affinities to the RRRCT quarter-site between p53 and p63. p63 and p73 showed similar binding affinity to the RRRCT sequence. This may be due to the higher amino acid sequence similarities between the DNA binding domains of p63 and p73 than those of p53 and p63, or p53 and p73.

Because the carboxyl terminus of p53 has been shown to inhibit its binding to DNA *in vitro*, investigators use the carboxyl terminus neutralizing antibody pAb421 to enhance binding [29]. We also used the carboxyl terminus truncated p53 (p53-C360) in our analysis. However, it is still unclear if *in vitro* binding analyses using pAb421 correctly reflect *in vivo* binding. Our preliminary data demonstrated that p53 binding analysis *in vitro* without using pAb421 underestimated actual *in vivo* binding; on the other hand, using pAb421 may also overestimate *in vivo* binding because addition of pAb421 allowed the binding of p53 to mismatched response elements which were not activated in the luciferase reporter analysis (data not shown). However, *in vitro* binding analyses with GDF15-RE2 and its mutants with the carboxyl terminus-truncated p53 or addition of pAb421 correlated with reporter activation. Thus, our binding analysis using p53-C360 and pAb421 most likely predicts accurate *in vivo* binding of p53 to GDF-RE2.

We and other groups have shown that transactivation of a response element is dependent on its sequence [17,28] p53 forms a tetramer and binds to two repeats of RRRCWWGYYY at its DNA binding domain as well as to several components of the RNA pol II complex, such as TAF_{II}31, TAF_{II}70 and TAF_I, at its transactivation domain to activate target gene transcription [32–34]. The p53 tetramer kinks, bends and twists the DNA while it binds to specific target sites and leads to a change in its 3-dimensional structure allowing the association of p53 with the RNA pol II complex and its recruitment to the transcription start site [35–40]. Thus, various factors, including the binding affinities to response element sequences, can contribute to specific target gene activation for p53 family members.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. *GDF15* promoter activation by p53 family members

A) The left chart shows the fold induction of mRNA in Saos2 inducible cells as detected by microarray analysis. The right chart represents levels of luciferase reporter activation of 26 promoters. Transcriptional activation of the promoters was examined in Saos2 cells transfected with p53, TAp63 β , TAp63 γ or TAp73 β . Numbers in the *GDF15* row are the fold activation of *GDF15* expression and promoter activity. B) *GDF15* promoter deletion mutations were made and examined for transactivation by p53, TAp63 β , TAp63 γ and TAp73 β in Saos2 cells. C) Chromatin immunoprecipitation analysis using ectopically expressed FLAG-tagged p53 protein. After induction of p53 with 1 μ g/ml of tetracycline, cells were fixed with formaldehyde and immunoprecipitated with anti-FLAG antibody. PCR primers were designed to amplify the surrounding RE2 segment of the *GDF15* promoter.

Figure 2. Differential binding of p53, p63 and p73 to p53-type response element quarter-sites

A) *In vitro* translated p53, p63 and p73 proteins were incubated with various oligonucleotide probes and separated on a gel. Probe sequences are listed in Supplemental Table 2. The same core sequences were introduced into both half-sites. CAAG represents GCAGCGGGCAAGCTCGGGCAAGCCCACGGA, PM, perfect match in RRR and YYY stretches, p53-C360, stop codon at amino acid residue 360, p53-pAb421, p53 protein was incubated with an antibody which activates *in vitro* DNA binding of p53 by inhibiting the carboxyl terminus of p53, TAp63-C394, stop codon at amino acid residue 394. Probes were separated on 15% native gels and equal labeling was confirmed. B) The relationship between upper and lower strand sequences. Seven sequences cover all possible core sequences except for 2 Cs and/or Gs sequence in the center of the core sequence. C) Composition of response elements as combinations of quarter-sites. D) Summary of binding affinities of each quarter-site to p53, p63 and p73. E) Effect of 3-nucleotide mismatches in RRR or YYY stretches and a gap between half-sites on binding affinity to p53-C360, p53-pAb421 and TAp63-C394.

Figure 3. Effect of mismatches in RRR stretch and T in the center of the core sequence in the most 3' quarter-site on p53, p63 and p73 binding and activation

A) Mutations were introduced into the most 3' quarter-site and examined for *in vitro* DNA binding with p53, p63 and p73. B) RE2 and its mutant variations were cloned into a luciferase vector and examined for transactivation by p53, p63, and p73 in Saos2 cells.