Overproduced p73α activates a minimal promoter through a mechanism independent of its transcriptional activity

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Abstract p73, the gene for a protein related to the tumor suppressor p53, encodes several variants which bear distinct carboxy-terminal structures as a result of alternative splicing. We and others showed that these splicing variants have different transcriptional effects on promoters with a p53-binding consensus sequence (p53BCS). Here we show that when transiently overexpressed, p73α but not p73β activated several minimal promoters without the p53BCS, while p73γ and p73ε activated them to a much lesser extent than p73α, and p53 suppressed the promoters without p53BCS as reported previously. Moreover, the results of RNase protection and RNA transfection assays suggested that this activation occurred at the transcriptional level. Deletion analysis of p73α revealed that the transactivation domain of p73 was not involved in this activity and the C-terminal region of p73α which is a specific structure of this variant was essential, suggesting that this phenomenon occurs independent of the transactivation activity of p73α and that the C-terminal extension of p73α may affect the basal level of transcription. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: p73; Splicing variant; Transcription; p53

1. Introduction

The p73 gene was identified as a gene encoding a protein homologous to the tumor suppressor p53 [1]. mRNAs for p73 variant proteins, α, β, γ, δ, and ε, which bear distinct carboxy-terminal structures, have been shown to be produced from a single gene as a result of alternative splicing [2,3]. We and others revealed that these splicing variants differ in potential for the transcriptional activation of p53-responsive promoters [2,3]. Extensive homology between all the p73 variants and p53 was found within conserved p53 domains for transactivation, DNA-binding, and oligomerization [1]. Functional similarities were also observed between p73 and p53. When overproduced, just like p53, p73α and β can activate transcription from p53-responsive promoters of several genes including the gene for p21/WAF1, an inhibitor of cyclin-dependent protein kinases [4]. Overproduction of p73α was also observed to promote apoptosis, as in the case of p53 [4,5]. For the first time, p73 was suggested to be a tumor suppressor because of the similarities to p53 mentioned above. This idea seemed to be supported by the fact that the p73 gene was mapped to the chromosomal region at 1p36.33, frequently deleted in a variety of cancers including neuroblastomas, breast carcinoma and colon carcinoma [1,6,7]. However, some functional diversity does exist between p73 and p53. In many tumor cells, a mutation in p73 is seldom found [8]. p53 protein, which is degraded rapidly by the proteasome through the ubiquitination pathway under non-stressed conditions, is known to be accumulated and activated in response to genotoxic stress, inducing cell growth arrest or apoptosis [9,10]. However, p73 was not accumulated in response to genotoxic stress such as UV irradiation or administration of actinomycin D [1]. p73 was recently found to be stabilized and activated on treatment with another genotoxic reagent, cisplatin, through the activation of tyrosine kinase c-Abl [11,12]. And the viral proteins adenovirus E1B 55K, simian virus 40 (SV40) T antigen, and human papillomavirus E6, which are known to bind with and inactivate p53, did not have any effect on p73 [13,14]. Moreover, p73 knock-out mice did not show an increased susceptibility to spontaneous tumorigenesis in contrast to p53-deficient mice [15]. We recently reported that p73β but not p53 activates the Wnt/β-catenin signaling pathway in Saos-2 cells [16].

During a previous study on the transcriptional activities of p73 splicing variants, we found that p73α, γ and ε but not β enhanced the luciferase activity from the reporter plasmid, p55Bluc, which contains an interferon β basal promoter element without apparent homology to the p53-binding sequence. This seemed to be another difference between p73 and p53, because when overexpressed, p53 has been shown to suppress transcription from several viral and cellular promoters without the p53-responsive element, such as SV40 early promoter, cytomegalovirus immediate-early promoter, human immunodeficiency virus long terminal repeat, and promoters of the gene for multiple drug resistance and heat shock protein 70 [17–19]. Recently, Deb et al. showed that p73α but not p73β activated the promoter of the insulin-like growth factor receptor I (IGF-I-R) gene, which was shown to be suppressed by p53 [20]. Given our previous finding, we speculated that the expression of p73α affects the activity of the minimal transcriptional promoter. Therefore, we analyzed several minimal promoters without p53-binding consensus sequence (p53BCS) and determined whether their activation occurred at the transcriptional level. Furthermore, we also show the structural requirement of p73α for this activity using the other

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Abbreviations: p53BCS, p53-binding consensus sequence; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SV40, simian virus 40; MDM2, murine double minute clone 2 oncoprotein; HBV, hepatitis B virus; IGF-I-R, insulin-like growth factor receptor I

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p73 splicing variants and deletion mutants of p73α and discuss the possible mechanisms and functional implications.

2. Materials and methods

2.1. Cell culture

Saos-2 cells were cultured in Dulbecco's modified Eagle's medium (Nissui) supplemented with 10% fetal bovine serum and L-glutamine (584 μg/ml) at 37°C in a 5% CO_2 atmosphere.

2.2. Plasmids and transient transfections

Expression plasmids, pcDNA3-p73α, β, γ, ε, and pcDNA3-p53 as well as pcDNA3-Myc-p73α, β, γ, ε, and pcDNA3-Myc-p53 were described previously [3,21]. Expression plasmids for the deletion mutants of p73s, pcDNA3-p73ΔN56,ΔN130,ΔN315,ΔN423,ΔC505,ΔC424 and p73βΔOD, were constructed by a polymerase chain reaction (PCR)-based cloning technique using pcDNA3-p73α and pcDNA3-p73β as templates. All these constructs were verified by sequencing. pGL3-Promoter was purchased from Promega. Reporter plasmids, p55Bluc and pCAST2Bluc, were kindly provided by Dr. T. Kiyono (Aichi Cancer Center Research Institute, Nagoya, Japan). For plasmid transfection, we used FuGENE6 transfection reagent (Roche) according to the manufacturer's protocol.

2.3. Synthesis of luciferase RNA and RNA transfection

pGEM-luc vector (Promega) digested with *Xho*I was transcribed using a MEGAscript SP6 kit (Ambion) in the presence of m7(5′)ppp(5′)G (cap analog, Ambion). The transfection of synthetic RNA into Saos-2 cells was done using DMRIE-C reagent (Invitrogen) for 3 h, after which the transfection medium was changed to fresh culture medium. After being cultured for 24 h, the cells were harvested and used as samples for the reporter assay.

2.4. Reporter assay

Cell extracts were prepared in Reporter Lysis Buffer (Promega) 24 h after transfection. After the removal of cell debris, the luciferase activity in the extracts was measured with a luciferase assay kit (Promega) using a luminometer (Berthold, Lumat LB 9507) as recommended by the manufacturers.

2.5. Immunoblot analysis

Whole cell extracts were prepared 24 h after transfection. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting analysis using a polyvinylidene difluoride membrane were performed as described previously [22]. The antibody used in these experiments was anti-c-Myc (9E10) (Santa Cruz Biotechnology).

2.6. Ribonuclease protection assay

Total RNA was isolated from the cells 24 h after transfection of the expression and reporter plasmids by Sepasol-RNA I (Nacalai tesque). To obtain the expression plasmids for the antisense RNA probes, pcDNA3-AS-luc and pcDNA3-AS-hGAPDH (human glyceraldehyde-3-phosphate dehydrogenase) were generated as follows; pcDNA3-AS-luc was constructed by inserting the *Eco*RI–*Xho*I fragment of a PCR product, which had been amplified by using the pGL3-Promoter as a template and oligonucleotides 5′-GGGCTCGAGATGGAAGACGCCAAAAACA-3′ and 5′-CCGGAATTCCCTCGATATGTGCATCTGT-3′ as primers, into the *Eco*RI–*Xho*I site of pcDNA3. pcDNA3-AS-hGAPDH was constructed by inserting the *Eco*RI–*Xho*I fragment of a reverse transcription-PCR product, which was amplified using a One Step RNA PCR kit (TaKaRa) with primers 5′-TTTCTCGAGATGGGGAAGGTGAAGGTCG-3′ and 5′-CCGGAATTCTGGAGGGATCTCGCTCCTG-3′. The probe for GAPDH mRNA was used as an internal control of mRNA. ^{32}P-labeled antisense RNA probes for detection of luciferase and GAPDH mRNAs were prepared with a MEGAscript T7 in vitro transcription kit (Ambion) and [α-^{32}P]UTP (Amersham Pharmacia Biotech Inc.) using pcDNA3-AS-luc and pcDNA3-AS-hGAPDH after linearization by digestion with *Xho*I. These probes were hybridized with the total RNA at 37°C overnight. After digestion with RNase Cocktail (Direct Protect, Ambion) for 30 min at 37°C, these samples were separated in 5% acrylamide/8 M urea gel and then by autoradiography. The autoradiogram on the imaging plate was analyzed with a BAS 5000 (Fuji Film).

3. Results

3.1. Enhanced luciferase activity from reporter plasmids without a p53-binding site in cells overproducing p73α

To investigate the effects of p73 splicing variants on the promoters without the p53BCS, p55Bluc reporter plasmid having the interferon β gene basal promoter was co-transfected with an expression plasmid for p73 splicing variant into Saos-2 cells, which do not produce p53 protein (Fig. 1A). We observed that the production of p73α induced a 6-fold increase in luciferase activity compared to mock transfection (Fig. 1B, closed bars). p73γ and ε also raised the level of luciferase activity 2-fold and 1.4-fold, respectively, although these effects may be marginal (Fig. 1B, closed bars). However, no significant effect on reporter activity was seen in the cells producing exogenous p73β. On the other hand, expression of p53 resulted in the suppression of reporter luciferase activity compared with the control (Fig. 1B). This effect of p53 has been reported to be due to repression of transcription under the control of the promoters with a TATA sequence [23]. Similar results were obtained using the reporter plasmids pGL3-Promoter and pGL3-TK containing SV40 early promoter and herpes simplex virus thymidine kinase gene promoter, respectively, upstream of a luciferase gene (data not shown), indicating that this result was not specific for the reporter plasmid p55Bluc.

To rule out the possibility that these results reflect the different production levels of each protein, we examined the correlation between reporter expression from p55Bluc and production of the protein by titrating the amounts of plasmids for p53, p73α, and p73β in transfection experiments. Increased production of each protein was confirmed by immunoblot analysis as shown in Fig. 1C. Under the conditions, reporter expression affected by p73α or p53 was observed to be dependent on production levels (Fig. 1C), while the augmented production of p73β caused no change in the reporter expression. These results indicated that the difference observed between the effect of p73α and p73β on p55Bluc was not due to a quantitative difference but to the potential of each protein.

As the major structural difference between p73α and β is the extra C-terminal region of p73α, it would appear that this region contributes to the augmentation by p73α of the reporter expression from p55Bluc.

To see whether the above phenomenon occurs in other cell lines, a reporter assay using p73α expression plasmid and p55Bluc was performed in Jurkat cells lacking wild-type p53 and HepG2 cells and MCF-7 cells which are known to produce wild-type p53. The activation of reporter expression by exogenous p73α was observed in all these three cell lines (data not shown). This result showed that the effect of p73α was reproduced in several different cell lines and was independent of the production of endogenous wild-type p53.

We previously reported that p73 splicing variants have different potentials to activate p53-binding element-dependent transcription by using the reporter plasmid pCAST2Bluc containing two copies of p53BCS upstream of the interferon β basal promoter ([3] and Fig. 1B, open bars). The expression of p73β induced markedly high luciferase activity as did p53. p73γ activated the protein more than p73α. And p73ε showed no significant effect on the activity of the promoter with p53BCS. This implies that the effects of p73 splicing variants

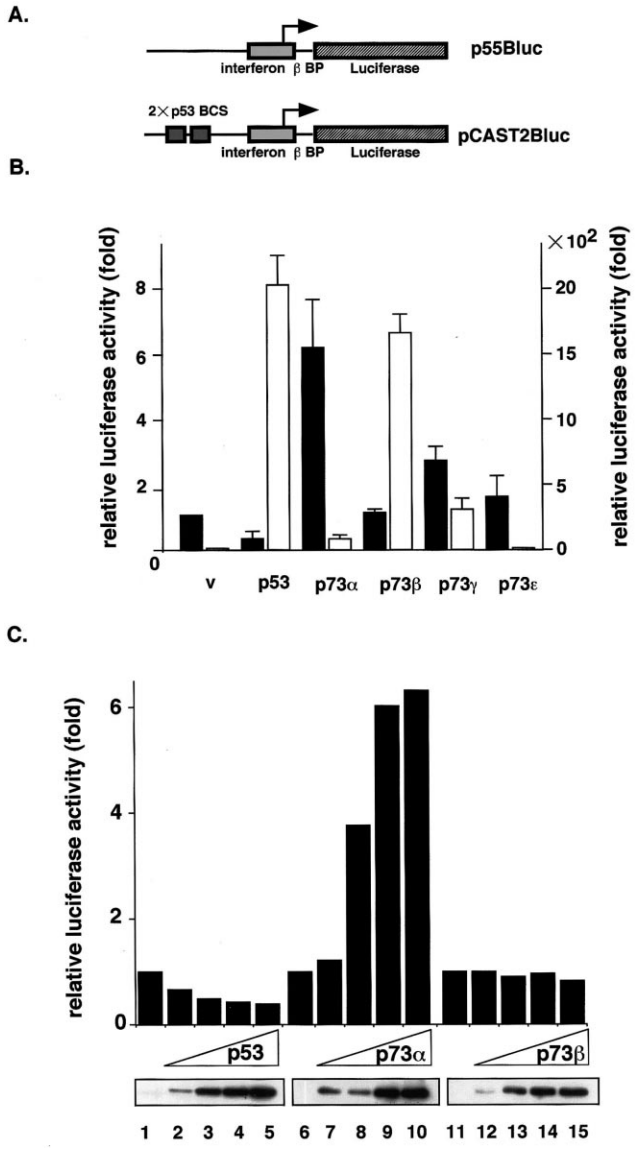

Fig. 1. Overproduced p73α but not p73β enhanced luciferase activity from a reporter plasmid without p53BCS. A: Schematic representation of reporter plasmids. p55Bluc and pCAST2Bluc containing the firefly luciferase gene as a reporter gene were used as reporter plasmids. Interferon β BP and 2×p53BCS indicate the basal promoter of the interferon β gene and a tandem repeat of the p53BCS, respectively. B: Transactivation capacities of p73 variants in the reporter plasmids, p55Bluc and pCAST2Bluc. Luciferase activity in Saos-2 cells transfected with 1 μg of expression plasmid for p73 splicing variants (pcDNA3-p73α, β, γ, and ε), pcDNA3-p53 or pcDNA3 vector only (shown as v) together with 0.3 μg of p55Bluc (closed bars) or pCAST2Bluc (open bars) was measured as described in Section 2. Fold activation of the luciferase activity relative to that from the cells transfected with each reporter plasmid and pcDNA3 vector was calculated and shown with the scale on the left for p55Bluc and right for pCAST2Bluc. The data represent the average of three independent experiments and are shown with the standard error. C: Dose effects of p73α on the reporter expression from p55Bluc in Saos-2 cells. Increasing amounts of pcDNA3-Myc-p53, p73α or β (0 μg: lanes 1, 6 and 11; 0.1 μg: lanes 2, 7 and 12; 0.5 μg: lanes 3, 8 and 13; 1.0 μg: lanes 4, 9 and 14; 1.5 μg: lanes 5, 10 and 15) were transfected into Saos-2 cells together with 0.5 μg of p55Bluc. The total amount of plasmid DNA used for transfection was adjusted to 2 μg by adding an appropriate amount of empty vector pcDNA3. Luciferase activity was measured and the data are presented. Expression levels of Myc-tagged proteins were determined by immunoblotting using anti-c-Myc antibody as shown in the lower panel.
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on the expression of the reporter gene driven by the promoter without p53BCS are independent of their potential for activation of the promoter with p53BCS.

3.2. Quantitative elevation of the reporter mRNA level promoted by production of p73α

To assess whether the induction of luciferase activity by p73α was caused by an increase in its mRNA level, a quantitative analysis was performed using the RNase protection assay to compare the luciferase mRNA levels in Saos-2 cells transfected with pGL3-Promoter together with an expression vector for p73α or p53, or an empty vector. As shown in Fig. 2A, the amount of luciferase mRNA in exogenous p73α-producing cells was larger than that in mock-transfected cells (lanes 4 and 5), while the expression of luciferase mRNA was downregulated in the cells ectopically producing p53 (lane 6). These results suggested that the induction of luciferase activity by p73α was due to the elevated levels of this reporter mRNA. Possible mechanisms for the accumulation of mRNA would be transcriptional activation and/or promotion of mRNA stability. To see whether the stability of luciferase mRNA is changed by p73α or p53, we examined the luciferase activity originating from transfected luciferase mRNA transcribed in vitro in Saos-2 cells expressing p73α or p53. However, no difference in activity was observed between the cells (Fig. 2B, closed bars). These results indicated that the increase in luciferase mRNA caused by p73α was not due to the prolonged life span of the mRNA, suggesting that this phenomenon occurred at the transcriptional level.

3.3. Structural requirement of p73α for activation of promoter without p53BCS

To determine the domain(s) involved in the p73α-mediated transcriptional activation of the promoter without p53BCS, a deletion analysis of p73α coupled with the reporter assay was performed as described in Section 2. Production of each truncated protein in Saos-2 cells was detected by immunoblot analysis (data not shown). Among the amino-terminal deletion mutants, ΔN56 lacking the putative transactivation domain was found to induce the luciferase activity as effectively as wild-type p73α. ΔN315 in which the large N-terminal portion of p73α is absent was also observed to induce the luciferase activity more than mock transfection. However, the other N-terminal and all C-terminal deletion mutants did not show any enhancement of promoter activity (Fig. 3A,B). Although some differences in the production levels were found among the exogenous p73α derivatives, no correlation between the magnitude of reporter gene induction and the production level of each protein was observed (data not shown), indicating that the activities of the deletion mutants on the reporter gene expression were dependent on the properties of those proteins. These results suggested that a putative transactivation domain is not crucial for the induction of the reporter expression and almost all structures except the N-terminal end of p73α are necessary for complete activity. It was also likely that the C-terminal region of p73α plays an essential role in this activation. To confirm that a large part of

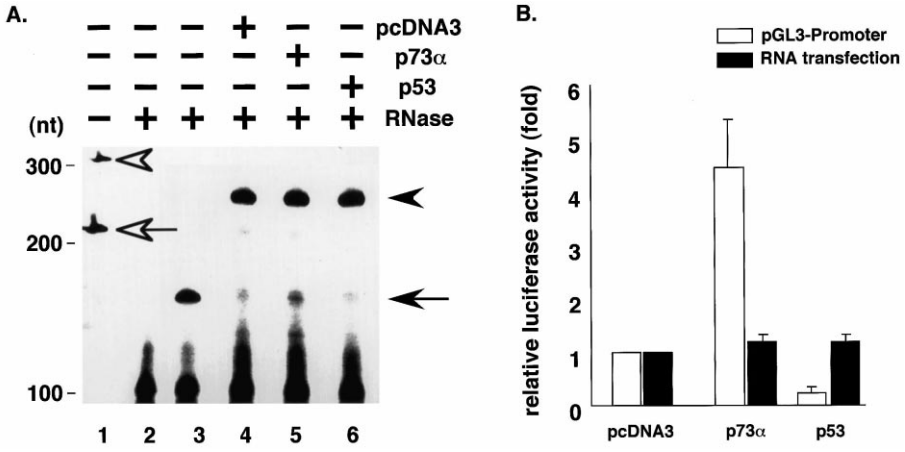

Fig. 2. p73α affected the production level of the reporter mRNA. A: An increase in the amounts of reporter mRNA caused by p73α was detected by RNase protection assay. Total RNA of Saos-2 cells transfected with pGL3-Promoter and pcDNA3, pcDNA3-p73α, or pcDNA3-p53 (lanes 4–6) was isolated 24 h after transfection and used for RNase protection assay. ^{32}P-labeled RNA probes for luciferase and GAPDH mRNAs were prepared as described in Section 2. Detection of GAPDH mRNA was used for a loading control. The open arrowhead and open arrow indicate ^{32}P-labeled anti-GAPDH probe and ^{32}P-labeled anti-luciferase probe, respectively, without RNase treatment (lane 1). After RNase treatment without addition of total RNA, these probes were completely digested (lane 2). The closed arrowhead and closed arrow indicate protected anti-GAPDH and anti-luciferase probes, respectively, after RNase treatment. Luciferase RNA transcribed in vitro was used as a positive control (lane 3). B: p73α did not affect the luciferase activity from the luciferase RNA transfected into Saos-2 cells. Saos-2 cells were transfected with 1 μg of pcDNA3, pcDNA3-Myc-p73α or pcDNA3-Myc-p53. At 24 h after plasmid transfection, cells were transfected with luciferase RNA which was transcribed in vitro as described in Section 2. Then, 24 h after RNA transfection, luciferase activity was measured (closed bars). Plasmid-based reporter assay was performed essentially as described in Fig. 1B. The values shown for the luciferase assays represent the average of three independent experiments. Error bars represent the standard error.

p73α is required for the activity observed in this paper, the promoter activation potentials of several C-terminal polypeptides were examined in the presence of p73β, which did not activate the basal promoter by itself. In this experiment, C-terminal peptide was expected to form a complex with p73β in an oligomerization domain-dependent manner for the following reasons. First, analysis of the amino acid sequence suggested that there exists an oligomerization domain in the primary structure of p73 as well as p53 [1]. Second, interaction between p73 splicing variants through the oligomerization domain has been shown [21,24].

When the C-terminal polypeptide of p73ΔN315 containing the oligomerization domain was produced together with p73β, the basal promoter activity of pGL3-Promoter was apparently increased (Fig. 3C, lane 6). Notably, the reduced activity of p73Δ315 was seen to be completely recovered by coproduction of p73β to the level equivalent to the potential of p73α. On the other hand, when the C-terminal polypeptide of p73ΔN423 lacking the oligomerization domain was coproduced with p73β, basal promoter activity was not seen to be affected (Fig. 3C, lane 7). Furthermore, when p73βΔOD, in which the oligomerization domain was deleted, was produced with the above C-terminal polypeptides of p73α, no augmentation of basal promoter activity was detected (Fig. 3C, lanes 8–10). As p73β has almost an identical structure to the C-terminal deletion mutant of p73α, these results further supported the idea that the C-terminal region of p73α is essential for the activation of pGL3-Promoter, and the region including p73β is required for the complete activity.

4. Discussion

In this report, we have shown that p73α, when overproduced in cultured cells, activates certain transcriptional promoters without p53BCS. The mechanism of the activation by p73α is likely to be different from that of the p73α-dependent activation of the promoter with p53BCS for the following reasons. First, p73ΔN56, lacking the transactivation domain, still had the capacity to enhance the expression of reporter genes from p55Bluc and pGL3-Promoter containing interferon β and SV40 early promoters, respectively, similar to wild-type p73α, although this truncated p73α did not show any activation of the promoter with p53BCS of pCAST2Bluc (Fig. 3B and data not shown). Second, despite the transcriptional activity of the promoter with p53BCS, p73ΔC424, which lacks the C-terminal 213 amino acids of p73α, did not show any activation of promoters without p53BCS ([3] and Fig. 3C). This also seems to imply that the product(s) of the gene(s) which is induced by p73α through p53BCS in its promoter is not involved in this phenomenon.

The idea that the molecular interaction between p73 and a particular transcriptional regulatory factor affects the basal transcriptional activity is one likely explanation for this phenomenon. Transcriptional factors including p73 and p53 have been known to function through interactions with other transcriptional factors or with transcriptional regulatory factors such as coactivators and corepressors [25–30]. Concerning p53-dependent inhibition of promoters without p53BCS, it has been suggested that this transcriptional factor binds with the TATA-binding protein and sequesters it from other transcriptional factors [31]. Recently, Murphy et al. also showed that transcriptional repression in MAP4 and Stathmin gene promoters was mediated through interaction with a corepressor, mSin3a [28]. These reports suggested that the repression of promoters without p53BCS by p53 is mediated by interactions between p53 and cellular proteins, especially transcriptional factors or transcriptional regulatory factors. Because p73α has significant structural homology with p53, it seems reasonable that p73α shares interacting counterparts with p53. In fact, p73α was reported to utilize CREB-binding

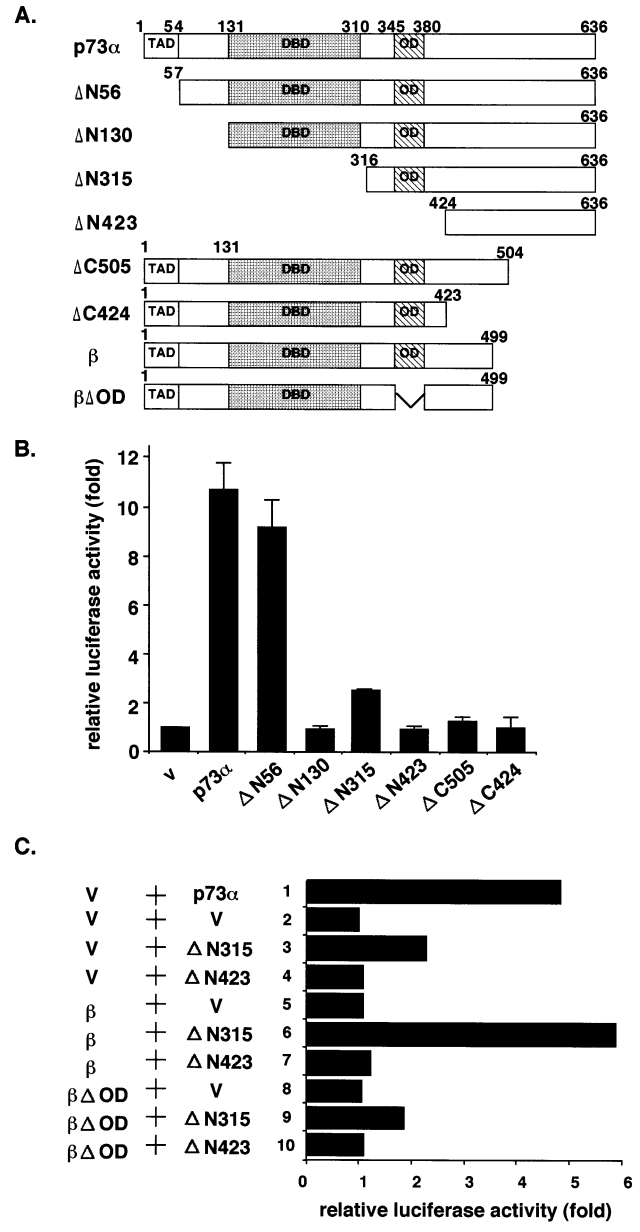

Fig. 3. Structure of p73α required for the activation of reporter gene expression from pGL3-Promoter. A: Schematic representation of the deletion constructs of p73α and β. TAD (open box), DBD (shaded box) and OD (hatched box) indicate the putative transactivation, DNA-binding and oligomerization domains, respectively. The number above the open boxes denotes the amino acid position in the wild-type p73α and β corresponding to the terminal ends of the p73α and β derivatives. B: Effects of deletion mutants of p73α on pGL3-Promoter activity. pGL3-Promoter was co-transfected into Saos-2 cells with an expression plasmid for a p73α derivative or pcDNA3 empty vector and luciferase assays were performed 24 h after transfection. The data for the luciferase assays are shown as in Fig. 1B. C: Coproduction of the C-terminal region of p73α containing the oligomerization domain and p73β activated the reporter expression from pGL3-Promoter. pGL3-Promoter (0.2 μg) was co-transfected with p73β or p73βΔOD (0.5 μg) and pcDNA3, p73αΔN315 or ΔN423 (0.5 μg) into Saos-2 cells. Experimental procedures were as for Fig. 1B. The data represent the average of three independent experiments.

protein/p300 as a coactivator just like p53 for the activation of promoters with p53BCS [25]. However, some differences exist between p73α and p53 in terms of protein–protein interactions. For example, adenovirus E1B 55K, human papillomavirus E6 and SV40 T antigen, which are well known to bind with and inactivate p53, have not been reported to associate with p73α [13]. While it has been shown that human murine double minute clone 2 oncoprotein (MDM2) binds to p53 and promotes its degradation [32], it was also revealed that MDM2 does not play a role in the degradation of p73α in spite of molecular interaction [33]. In this report, the C-terminal region of p73α was suggested to be important for activation of promoters without p53BCS. That region, which was not found in the other splicing variants of p73 and p53, has striking structural similarity with the SAM (sterile α motif) domain [34], which is proposed to function in protein–protein interaction [35]. Moreover, this C-terminal extension of p73α was proposed to contribute to the interaction with several proteins through small ubiquitin-related modifier 1 modification [36]. These findings suggested that p73α has the potential to affect certain cellular events through its molecular interaction with cellular proteins that do not associate with p53 or the other p73 splicing variants. It has been reported that hepatitis B virus (HBV) X protein (HBx) activates transcription through protein–protein interaction. In that case, HBx was considered to interact with RNA polymerase II subunit 5 (RPB5) and inhibit binding of RPB5 with RPB5-mediating protein, a transcriptional corepressor. As a result, the transcriptional promoter was suggested to be activated by a reduction in the amount of the corepressor from the transcriptional machinery [37]. Therefore, it may be possible that p73α modulated the function of the transcriptional corepressor(s) through protein–protein interaction, just like HBx. Identification of the factor interacting with the C-terminal region of p73α should provide important information on the molecular mechanism of this phenomenon.

During the preparation of this paper, Doish et al. reported that p73α enhanced HBV enhancer/promoter activity in contrast to p53 [38]. Deb et al. also reported that p73α but not p73β activated the promoter of the IGF-I-R gene, which was previously reported to be repressed by p53, in a transient transfection reporter assay [20]. These observations may be related with the findings of the present study. Further analysis of the mechanism for the activation of promoters without p53BCS by p73α will provide information on the physiological function of this protein and the transcriptional mechanism of p73 which is different from that of p53.

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