Transcriptional activities of p73 splicing variants are regulated by inter-variant association

Yoshihide UEDA*, Makoto HIJIKATA^{†1}, Shinji TAKAGI*, Tsutomu CHIBA† and Kunitada SHIMOTOHNO*

*Department of Viral Oncology, Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto 606-8507, Japan, and †Division of Gastroenterology and Hepatology, Department of Internal Medicine, Kyoto University, Sakyo-ku, Kyoto 606-8507, Japan

p73 has been identified as a gene that encodes a protein with significant identity with the tumour suppressor p53. The main structural difference between p73 and p53 is the additional C-terminal region of p73. Six isoforms of p73 with differing C-terminal structures, α , β , γ , δ , ϵ and ξ , have been reported. These variants differ in transcriptional activity on p53-responsive promoters. Here we report a possible mechanism of transcriptional activation by p73 splicing variants. C-terminal deletion mutants of p73 α showed a significantly higher level of transcriptional activity than wild-type p73 α , suggesting that the C-terminal structure of p73 α functions to repress the transcriptional activity of p73 α . The results of immunoprecipitation assays and two-hybrid assays in mammalian cells showed that the p73

variants interacted with each other, but not with p53. The transcriptional activity of p73 β was reduced by co-expression with either p73 α or p73 ϵ , which bears an identical C-terminal structure to p73 α . Co-expression of the C-terminal portion of p73 α or p73 ϵ with p73 β also resulted in reduced transcriptional activity. Moreover, we observed that the level of endogenous p21 protein induced by p73 β was decreased by co-expression of full-length p73 ϵ or the C-terminal region of p73 α or p73 ϵ . These observations suggest that p73-mediated gene expression is regulated by the interactions of p73 splicing variants in the cell.

Key words: oligomerization, p53, splicing variant, transcription.

INTRODUCTION

p73 has been identified as a gene encoding a protein with significant identity with the tumour suppressor p53 [1]. p73 induces the expression of p53 target genes, such as p21, GADD45 (growth arrest and DNA damage 45) and MDM2 (murine double minute clone 2 oncoprotein), resulting in cell cycle arrest or apoptosis, when it is transiently expressed in cells [1–4].

However, unlike p53, mutations of p73 have not been found frequently in human tumour cells [5]. Some reports have suggested that p73 mRNA is overexpressed in tumour cells of hepatocellular carcinoma [6], lung cancer [7], breast cancer [8], etc. p73-deficient mice have neurological, pheromonal and inflammatory defects, but lack spontaneous tumours, unlike p53-deficient mice [9]. These findings suggest that the physiological functions of p73 are different from those of p53.

The most marked structural difference between p73 and p53 is the additional C-terminal portion of p73. The presence of six isoforms, α , β , γ , δ , ϵ and ξ , with differing C-terminal structures has been reported [10–12]. Among these, only p73 α has been found to have a C-terminal portion containing a region with a striking structural similarity with the SAM (sterile α motif) domain [13,14], which is proposed to be a putative protein–protein interaction domain [15]. The p73 isoforms are produced by alternative splicing. They vary in their transcriptional activity on p53-responsive promoters, and have different abilities to suppress cell growth [10,11]. The pattern of expression of these p73 splicing variants also differs in cell lines and in normal human tissues [10,11]. The different patterns of distribution of each variant in different cells and the differing abilities of each variant to transactivate p53-responsive genes suggest that each splicing variant has a different function.

Furthermore, the ability of each protein to regulate target genes may be achieved through the intermolecular association of

p73 variants, as suggested by the following findings. p53 functions as a tetramer [16,17], and the identity between the oligomerization domains (ODs) of p53 and p73 suggests that each p73 variant can form hetero-oligomers with p53 as well with as the other variants. In the yeast two-hybrid system, interactions between p73 and p53 or between p73 variants α , β , γ and δ have been reported [1,11]. For example, p73 α interacted weakly with p73 β , whereas p73 β showed strong homotypic interaction in the same system. However, in an immunoprecipitation assay of endogenous p73 proteins using a p73 α -specific antibody, p73 β was co-immunoprecipitated with p73 α [18]. This suggested that at least some p73 α forms a complex with p73 β . Moreover, it was also reported that the purified recombinant polypeptide of the putative p73 OD (amino acids 345–383) associated with itself, although it did not interact with the p53 OD [19]. As the p73 OD used in that study is present in all p73 variants, it is likely that p73 variants can interact with each other. All of the above observations suggest that inter-variant association of p73 occurs and modulates transcriptional activity in the cell. Here, to test this possibility, we have examined the correlation between the transcriptional activities and the molecular interaction between the p73 splicing variants α , β , γ and ϵ .

MATERIALS AND METHODS

Plasmid construction

The expression plasmids pcDNA3-Myc-p73 α , -p73 β , -p73 γ and -p73 ϵ , p73 α ΔC424 and p73 α ΔC269 and pcDNA3-Myc-p53 were constructed by inserting the *KpnI*–*Bam*HI fragment of a PCR product amplified by using pCMV-Tag1 (Stratagene) as template with S1 and R1 as primers (see below for primer sequences) into the *KpnI*–*Bam*HI sites of pcDNA3-p73 α , -p73 β , -p73 γ , -p73 ϵ , p73 α ΔC424 and p73 α ΔC269 and pcDNA3-p53, which have been described previously [10], to fuse the c-Myc epitope tag with the

Abbreviations used: p53BCS, p53-binding consensus sequence; DBD, DNA-binding domain; GADD45, growth arrest and DNA damage 45; MDM, murine double minute clone 2 oncoprotein; OD, oligomerization domain.

¹ To whom correspondence should be addressed (e-mail mhijikat@virus.kyoto-u.ac.jp).

Figure 1 Modulation of the transcriptional activity of p73α by its C-terminal structure

(A) Schematic representation of p73 deletion mutants. The c-Myc epitope tags introduced at the N-terminus are shown by grey boxes. TAD1 and TAD2 indicate putative transactivation domains 1 and 2 respectively of p73. The numbers above the boxes indicate the numbers of amino acids from the N-terminus of p73α. The closed box in the p73β C-terminus and the hatched box in the p73γ and p73ε C-termini indicate unique sequences. (B) Detection of exogenous p73 deletion mutants and splicing variants. Saos-2 cells were transiently transfected with 1.0 μg of expression plasmid encoding a p73α deletion mutant (ΔC566, ΔC505, ΔC424 or ΔC269) or a p73 splicing variant (α, β, γ or ε), or with 1.0 μg of pcDNA3 vector (shown as v). Whole-cell extracts prepared 30 h after transfection were analysed by Western blotting using anti-Myc epitope tag antibody. Arrowheads show the detected recombinant proteins of the expected size. The positions of molecular size markers are shown on the left. (C) Activation of the p53BCS promoter by p73 deletion mutants and splicing variants. Saos-2 cells were transfected with 0.2 μg of pCAST2Bluc and 1.0 μg of the indicated expression plasmids encoding p73α deletion mutants, p73 splicing variants or p53. Fold activation was quantified relative to the level of luciferase activity in the cells transfected with the reporter plasmid and the pcDNA3 vector (v). (D) Activation of a promoter with GAL4-binding elements by GAL4 DBD-fused p73 variants. Saos-2 cells were transfected with 0.2 μg of pFR-Luc, which contains five repeats of the GAL4-binding element upstream of the luciferase gene, and 1.0 μg of expression plasmids encoding the indicated GAL4 DBD-fused p73 variants. The lower panel shows the production of exogenous splicing variants of p73 analysed by Western blotting using anti-(GAL4 DBD) antibody. Whole-cell lysates prepared 30 h after transfection were examined. Arrowheads show the positions of GAL4 DBD-fused p73 variants. The values in the luciferase assay represent the means of three independent experiments; error bars represent S.E.M.

protein encoded by the expression plasmid. Plasmids pcDNA3-Myc-p73ΔC566 and pcDNA3-Myc-p73ΔC505 were generated by inserting the *EcoRI*–*XhoI* fragments of PCR products prepared by using pcDNA3-p73α as a template, with S2 and R2 as primers for p73αΔC566 and S2 and R3 as primers for p73αΔC505, into the *EcoRI*–*XhoI* site of pcDNA3-Myc-p73α. pcDNA3-Myc-p73αΔOD, for expression of p73α lacking the putative OD, was constructed by inserting the *EcoRI*–*MluI* fragment of a PCR product amplified with primers S2 and R4, and the *MluI*–*XhoI* fragment of a PCR product amplified with primers S3 and R5 from pcDNA3-p73α as a template, into the *EcoRI*–*XhoI* site of pcDNA3-Myc-p73α.

pcDNA3-FLAG-p73α, -p73β, -p73γ and -p73ε, and pcDNA3-FLAG-p53 (where the FLAG epitope has the sequence DYKDDDDK), were made by inserting the *BamHI*–*XhoI* frag-

ments of pCMV-FLAG-p73α, -p73β, -p73γ and -p73ε, and pCMV-FLAG-p53 respectively (which have been described previously [10]), into the *BamHI*–*XhoI* site of pcDNA3. pcDNA3-FLAG-p73αΔOD was constructed by inserting the *EcoRI*–*XhoI* fragment of pcDNA3-Myc-p73αΔOD into the *EcoRI*–*XhoI* site of pcDNA3-FLAG-p73α.

pcDNA3-αC, -βC, -γC and -εC were generated by inserting the *BamHI*–*XhoI* fragments of PCR products amplified by using pcDNA3-p73α, -p73β, -p73γ and -p73ε respectively as templates with primers S4 and R5 into the *BamHI*–*XhoI* site of pcDNA3. pcDNA3-αCAN was constructed by inserting the *EcoRI*–*XhoI* fragment of a PCR product amplified by using pcDNA3-p73α as template with primers S5 and R5 into the *EcoRI*–*XhoI* site of pcDNA3. pcDNA3-αCAC was generated by inserting the *BamHI*–*XhoI* fragment of a PCR product amplified by using

pcDNA3-p73 α as template with primers S4 and R6 into the *Bam*HI–*Xho*I site of pcDNA3.

pM-p73 α , -p73 β , -p73 γ and -p73 ϵ , and pVP16-p73 α , -p73 β , -p73 γ and -p73 ϵ were obtained by inserting the *Bam*HI–*Sal*I fragments of pcDNA3-p73 α , -p73 β , -p73 γ and -p73 ϵ respectively into the *Bam*HI–*Sal*I sites of pM and pVP16 (Clontech). pM-p53 and pVP16-p53 were generated by inserting the *Bam*HI–*Sal*I fragment of a PCR product amplified by using pcDNA3-p53 as a template with primers S6 and R7 into the *Bam*HI–*Sal*I sites of pM and pVP16 respectively.

The reporter plasmid pCAST2Blue, in which the p53-binding consensus sequence (p53BCS) comprising two copies of 5'-GAGCATGCCCCGGGCATGCTC-3' is located upstream of the luciferase gene [20], was kindly provided by Dr Tohru Kiyono (Division of Virology, Aichi Cancer Center Research Institute, Nagoya, Japan). The reporter plasmid pFR-Luc, in which five tandem repeats of the GAL4 binding element are located upstream of the luciferase gene, was obtained from Stratagene.

The primers used in this experiment were as follows: S1, 5'-GACGGTACCCACCATGGAGCAGAACTCATC-3'; S2, 5'-GGGACGGAATTCACCACCAT-3'; S3, 5'-AATACGCGTCAGCAGCAGCAGCTCCTAC-3'; S4, 5'-AGAGGATCCAGATGAACGAGAGCTCCGCCA-3'; S5, 5'-GTCGAATTCTGCTGCCCTCCGTCAACCAG-3'; S6, 5'-TTCGGATCCTTGGCAGCCAGACTGCCTTC-3'; R1, 5'-CCAGGATCCTCAGATCCTTTCAGA-3'; R2, 5'-CAACCGAGTCAGTTGCTAGAGCGGAGCAG-3'; R3, 5'-CCTCTCGAGTCAGTTTGACACCCCAATCC-3'; R4, 5'-AACACGCGTCTTCACACCGGCACCAAG-3'; R5, 5'-ATTAGGTGACACTATAG-3'; R6, 5'-CCTCTCGAGTCAGTTTGACACCCCAATCC-3'; R7, 5'-AGAGTCGACCAGTGGGGAACAAGAAGTGG-3'.

Cell culture and transfection

Saos-2 and COS-7 cells were cultured in Dulbecco's modified Eagle's medium (Nissui) with 10% (v/v) fetal bovine serum and L-glutamine. For plasmid transfection into the cells, we used the FuGENE6 transfection reagent (Boehringer Mannheim) according to the manufacturer's protocol.

Luciferase assay

Saos-2 cells were transfected with reporter plasmid pCAST2Blue or pFR-Luc, together with an expression vector for the desired p73 derivative. At 30 h post-transfection, cells were lysed and the luciferase activity was quantified with a luciferase assay kit (Promega), as recommended by the manufacturer.

Western blotting analysis

The preparation of cell lysates, SDS/PAGE and immunoblotting analysis with a PVDF membrane were performed as described previously [21]. The antibodies used in these experiments were anti-c-Myc (9E10) (Santa Cruz Biotechnology), anti-[GAL4 DNA-binding domain (DBD)] (Clontech), anti-(FLAG M2) (Sigma), anti-p21 (C-19) (Santa Cruz Biotechnology) and anti- α -tubulin (Ab-1) (Calbiochem). Immunocomplexes on the filters were detected by enhanced chemiluminescence assay (Renaissance; NEN).

Immunoprecipitation

Approx. 1×10^5 COS-7 cells were transfected in 3.5-cm wells with 0.5 μ g of plasmid encoding Myc-tagged protein and 1.0 μ g of plasmid encoding FLAG-tagged protein, and were harvested at 36 h post-transfection. Appropriate amounts of the lysate were

used in this experiment to adjust the protein level for detection, because the level of production of each construct was different, as shown in Figure 1(B). After cells were washed with cold PBS, they were lysed with 0.5 ml of RIPA buffer comprising 50 mM Tris/HCl (pH 7.2), 150 mM NaCl, 1% Triton X-100 and 0.5% sodium deoxycholate. The lysates were pre-adsorbed with 30 μ l of Protein G–Sepharose suspension (Pharmacia) for 30 min. After centrifugation (1930 g, 1 min, 4 °C), the supernatant was incubated with anti-c-Myc antibody (9E10) for 2 h. The immune complexes were recovered by adsorption to Protein G–Sepharose. The complexes were washed with 1 ml of RIPA buffer three times. The immunoprecipitates were dissolved in sample buffer comprising 125 mM Tris/HCl (pH 6.8), 4% SDS, 150 mM 2-mercaptoethanol, 20% glycerol and 0.01% Bromophenol Blue, and boiled for 5 min. The samples were separated by SDS/7% PAGE.

RESULTS

Modulation of the transcriptional activity of p73 α by its C-terminal structure

We reported previously that the ability of p73 α and p73 ϵ to transactivate a transcriptional promoter with the p53BCS promoter was much weaker than that of p73 β [10]. This suggested that the C-terminal structure that is shared by p73 α and p73 ϵ functions to suppress the transcriptional activities of these splicing variants of p73. It was reported that C-terminal deletion mutants of p73 α showed higher transcriptional activity than wild-type p73 α on p21, MDM2 and Bax promoters, and that the transcriptional activity was correlated with the DNA-binding activity [22]. However, the lower transcriptional activity of p73 β , which has a level of DNA-binding activity comparable with that of the C-terminal deletion mutants of p73 α , compared with these mutants suggests the existence of an alternative regulatory mechanism.

In order to avoid the effects of other transcription factors on the promoter, we conducted reporter assays using a reporter plasmid with a p53BCS promoter, in which only the p53BCS is used to analyse the transcriptional activity of the C-terminal deletion mutants of p73 α with a Myc epitope tag, as shown in Figure 1(A). We chose Saos-2 cells for this assay, as they are known to lack production of endogenous p53 and p73 proteins [10,23]. Production of exogenous proteins of the expected size in Saos-2 cells transfected with the expression plasmids for these mutants was confirmed by Western blotting of cell lysates with anti-Myc antibody, although the production levels of these proteins were seen to vary to some extent (Figure 1B). As shown in Figure 1(C), p73 α ΔC566 and p73 α ΔC505, in which the C-terminal 71 and 132 amino acids respectively of p73 α were deleted, showed significantly higher transcriptional activity than wild-type p73 α , as shown previously for the p21, MDM2 and Bax promoters [22]. The 71 amino acids deleted in p73 α ΔC566 are included in the shared region of p73 α and p73 ϵ , suggesting that this region is essential for the suppressive effects of these variants. The magnitude of activation by these deletion mutants of p73 α was as high as that by p53 or p73 β . With further C-terminal deletion (p73 α ΔC424), the transactivation capacity was diminished. There was no positive correlation between the magnitude of reporter luciferase gene activation and the level of production of the respective p73 derivative, although there was an approximate inverse correlation, with the exception of p73 α ΔC505 (see Discussion). The differences in luciferase activity among the deletion mutants of p73 α were not affected by titration of the amount of expression plasmid used for transfection (results not shown), as we have reported previously for p73 variants [10].

Figure 2 Interactions between p73 splicing variants

(A) Immunoprecipitation (IP) coupled with Western blotting analysis (WB). COS-7 cells were transfected with two expression plasmids, one for a Myc-tagged p73 derivative [α , β , γ , ϵ or $\alpha\Delta OD$ (p73 α lacking the putative OD)] or p53, and another for a FLAG-tagged derivative, as indicated. Myc-tagged proteins were immunoprecipitated from cell extracts using anti-c-Myc antibody. Western blotting analysis of the immunoprecipitates was done by using anti-FLAG (top panel) and anti-c-Myc (middle panel) antibodies. The bottom panel shows the results of Western blotting in which the total cell extract from transfected cells was analysed using anti-FLAG antibody. (B) Mammalian two-hybrid assays. Two-hybrid assays were performed by using GAL4 DBD- and VP16 activation domain (AD)-fused p73 α , p73 β , p73 γ , p73 ϵ or p53 as bait and prey respectively in Saos-2 cells (ΔN denotes a construct lacking the N-terminal domain). Luciferase activity relative to that in control cells transfected with empty pM and pVP16 vectors (lane 1) is shown.

These results suggest that the differences in transcriptional activity found among the deletion mutants were not caused by their levels of expression. The lower activity of p73 $\alpha\Delta C424$ compared with that of p73 $\alpha\Delta C505$ may be due to the deletion of the second transcription activation domain, as reported by Takada et al. [24].

To examine the mechanism of transcriptional suppression by the C-terminus of p73 α , we investigated the subcellular localization of each p73 variant and of p73 $\alpha\Delta C566$. As reported previously, p73 α and p73 β were located mainly in the nucleus [2]. We found that p73 γ , p73 ϵ and p73 $\alpha\Delta C566$ were also located in the nucleus (results not shown), indicating that the varied transcriptional activities are not due to differences in subcellular localization.

Next we examined whether the level of transcriptional activity is dependent on the DBD of p73, as follows. We utilized a reporter assay based on the interaction between GAL4-binding elements and the GAL4 DBD. Four p73 splicing variants, which were fused with the GAL4 DBD at the N-terminus, were used as effector proteins, and pFR-Luc, which contains five repeats of

the GAL4-binding elements upstream of the luciferase gene, was used as the reporter plasmid. In this system, transactivation through GAL4 DBD-based DNA binding of these fusion products was expected. We observed that the luciferase activities induced by p73 α , p73 γ and p73 ϵ fused proteins were much weaker than that induced by the p73 β fusion construct in Saos-2 cells (Figure 1D). This pattern of transcriptional activity of fused p73 variants was similar to that shown in Figure 1(C), in which pCAST2Bluc was used as the reporter plasmid. The levels of production of these GAL4 DBD-fused p73 variants also showed a similar pattern to that of the Myc-tagged p73 shown in Figure 1(B), indicating the lack of correlation between luciferase activity and the amount of each product. These results indicate that the difference in transactivation potential of these isoforms is not dependent on the DBD of p73.

Physical association between p73 splicing variants

To examine the homotypic and heterotypic interactions among p73 splicing variants in a co-immunoprecipitation assay, we

Figure 3 Suppression of transcriptional activity of p73β by co-expression of p73α or p73ε

SaOs-2 cells were transfected with 0.2 μg of pCAST2Bluc and 0.5 μg of Myc-tagged p73β or p53 expression plasmid or empty vector (shown as v), together with increasing amounts (0, 0.1, 0.5 or 1.0 μg) of Myc-tagged p73α or p73αΔOD (p73α lacking the putative OD) expression plasmid (**A**), or 1 μg of Myc-tagged p73α, p73β, p73γ or p73ε or empty vector (**B**). The total amount of plasmid DNA used for transfection was adjusted to 1.7 μg by adding an appropriate amount of empty vector. The lower panels show the results of Western blot analysis of whole-cell extracts for detection of Myc-tagged protein production. The data from the luciferase assay were analysed essentially as described in the legend to Figure 1.

constructed mammalian expression plasmids in which the FLAG epitope tag or Myc epitope tag sequence was introduced at the N-terminus of p73α, p73β, p73γ, p73ε, p53 and p73αΔOD, which is a deletion mutant of p73α lacking the putative OD. We confirmed that these FLAG- and Myc-tagged products also induced the transcription of the reporter gene of pCAST2Bluc similarly to the authentic proteins in SaOs-2 cells (results not shown). We then performed co-immunoprecipitation experiments using extracts of COS-7 cells transiently expressing various combinations of FLAG-tagged and Myc-tagged constructs. We found that all FLAG-tagged p73 variants were detected by Western blotting using the anti-FLAG antibody to probe immunoprecipitates, which were pulled down with anti-Myc antibody and contained each Myc-tagged p73 variant (Figure 2A). In contrast, p53 did not co-immunoprecipitate with p73α or p73β. These results indicated that p73 variants interact with each other, but not with p53. Furthermore, we found that p73αΔOD was co-precipitated with p73α, but not with p73β. These results suggested that p73α interacts with p73β through the putative OD of p73, but that homotypic interactions of p73α may occur through another domain as well.

We also investigated the interactions between p73 variants

Figure 4 Transcriptional suppression by the C-terminal structure of p73α and p73ε in trans

(**A**) Schematic representation of p73 C-terminal structures used. The closed box in βC and hatched boxes in γC and εC indicate the amino acid sequences found in those splicing variants that are different from p73α. TAD2 indicates the putative region of transactivation domain 2 of p73. The numbers above the boxes indicate the positions of the amino acid from the N-terminus of p73α. (**B**) Suppression of transcriptional activity of p73β on the p53BCS promoter by the C-terminal structures of p73α and p73ε. SaOs-2 cells were transfected with 0.2 μg of pCAST2Bluc and 0.5 μg of Myc-tagged p73β together with expression plasmids for the C-terminal regions of p73. The negative control experiments were done by using empty vector (v) instead of the expression plasmid. The data from the luciferase assay were analysed essentially as described in the legend to Figure 1.

using the two-hybrid system in mammalian cells. As shown in Figure 2(B), reporter gene expression was activated to some extent with all combinations of a GAL4 DBD-fused p73 variant and a VP16 transactivation domain-fused p73 variant. On the other hand, activation due to interactions between p53 and p73 variants was not detected in this system, in agreement with the results in Figure 2(A). In this experiment, however, we observed that only a low level of reporter gene activation was induced through the interaction with p73α or p73ε derivatives used as either bait or prey (see the scale of the ordinate of Figure 2B; lanes 9–12, 15–18, 27, 30, 33 and 36). As shown in Figure 2(A), p73α and p73ε are relatively better partners for complex-formation with any isoforms, whereas p73β and p73γ appear to

Figure 5 Effects of p73 splicing variants on the level of endogenous p21 protein

All panels show the results of Western blotting analysis for detection of endogenous p21 (top panels), α -tubulin (bottom panels) or exogenous Myc-tagged p73 variants or p53 (middle panels) in Saos-2 cells transiently transfected with the expression plasmids. **(A)** Induction of endogenous p21 protein by p73 splicing variants. The results of Western blotting analysis of cells transfected with 1.5 μ g of expression plasmids for Myc-tagged p73 α , p73 β , p73 γ , p73 ϵ or p53 alone are shown. **(B)** p73 β -induced endogenous p21 protein is reduced by co-expression of p73 ϵ , but not of p73 α . Saos-2 cells were co-transfected with 0.5 μ g of pcDNA3-Myc-p73 β and 1.0 μ g of expression plasmid for p73 α , p73 β , p73 γ , p73 ϵ , α C, β C, γ C, ϵ C or α C Δ N (lanes 3–11; see Figure 4A), and the cell extracts were analysed. The fold increase in the p21 protein level, which was quantified relative to the level in cells transfected with the pcDNA3 vector (v) by densitometry, is shown below the top panels in **(A)** and **(B)**. **(C)** Effects of co-expression of p73 α and p73 ϵ on p53-induced endogenous p21 protein levels. The effects of production of p73 α , p73 ϵ , α C and ϵ C on the induction of p21 protein by Myc-p53 were analysed. Transfection experiments were performed with 0.5 μ g of pcDNA3-Myc-p53 and 1.0 μ g of expression plasmid for p73 α , p73 ϵ , α C or ϵ C (see Figure 4A). The negative control experiments were done by using empty vector (v) instead of the expression plasmid.

be poorer partners, as indicated by the band intensities. Although stronger association (such as that provided by p73 α and p73 ϵ) normally results in more potent transactivation in mammalian two-hybrid systems, the weaker association provided by p73 β and p73 γ resulted in more potent transactivation in the present case. This implies that p73 α and p73 ϵ have repressive activity against the VP16 transactivation domain. Moreover, in Figure 1, we showed that p73 α and p73 ϵ have much lower transactivation activities not only for the p53BCS promoter but also for the promoter that contains GAL4-binding elements. Based on these findings, we conclude that p73 variants α , β , γ and ϵ interact with each other, but not with p53.

Suppression of the transcriptional activity of p73 β by p73 α or p73 ϵ

It was reported recently that the C-terminal region of p73 α (amino acids 327–636) bound to p73 α and p73 β *in vitro* and blocked p73-dependent transcriptional activation and apoptosis in Saos-2 cells [25]. To examine whether wild-type p73 α influences the transcriptional activity of p73 β , which has much stronger activity than p73 α , a reporter gene assay using pCAST2Bluc as a reporter plasmid was performed under conditions whereby p73 α was expressed simultaneously with p73 β . When p73 α was

co-expressed with p73 β , activation of the reporter gene was markedly reduced compared with the level seen in cells producing p73 β alone (Figure 3A, lanes 5–8), suggesting that p73 α suppressed the transcriptional activity of p73 β . The suppression was dependent on the dose of p73 α expression plasmid. As the level of production of p73 β was seen to be increased by co-expression with p73 α , the suppressive effect was not due to a decreased amount of p73 β . No transcription-suppressing effect of p73 α was observed when p73 α was co-expressed with p53 (Figure 3A, lanes 9–12), or p73 α Δ OD with p73 β (lanes 13–16). Since neither p53 nor p73 α Δ OD interacts with p73 β (Figure 2A), these findings suggest that the transcriptional suppression by p73 α occurs through interaction with p73 β . On the other hand, we also observed that p53-dependent reporter gene expression was augmented by co-expression of p73 α (Figure 3A, lanes 10–12). Since no interaction was detected between p73 α and p53 in the immunoprecipitation assay or two-hybrid assay, it seems that p73 α activates transcription by p53 indirectly.

We next investigated whether p73 γ and p73 ϵ also affect the transcriptional activity of p73 β . p73 γ had little suppressive effect on the transcriptional activity of p73 β (Figure 3B, lane 5). On the other hand, p73 ϵ , like p73 α , repressed the transcriptional activity of p73 β (Figure 3B, lane 6). Augmentation of the transcriptional activation of p53 by co-expression of p73 ϵ , as well as of p73 α ,

was observed (Figure 3B, lanes 8 and 11). These results suggest that p73 α and p73 ϵ suppress the transactivation ability of p73 β by interacting with it. Therefore it seems that the transcriptional activity of p73 is regulated by interactions among p73 variants, and that the functions of p73 molecules will be determined by the expression pattern of p73 variants in cells.

Transcriptional suppression by the C-terminal structure of p73 α and p73 ϵ

In order to confirm that the C-terminal region of p73 ϵ , as well as of p73 α , which was reported to be a dominant-negative p73 mutant [25], plays a role in the transcriptional repression of p73 β through molecular interactions, the effect of the C-terminal region of each variant on the transcriptional activity of p73 β was examined using a reporter gene assay using pCAST2Bluc (Figure 4A). Co-transfection of the expression plasmids for the C-terminal region of p73 ϵ , and of p73 α , with p73 β resulted in a decrease in luciferase activity (Figure 4B, lanes 3 and 8). However, co-production of β C, γ C, α CAC or α CAN (which is the C-terminal region without the OD) did not have a repressive effect on p73 β (Figure 4B, lanes 4–7). As we confirmed that p73 β interacted with α C and ϵ C, but not with α CAN, in co-immunoprecipitation experiments (results not shown), it is likely that α C and ϵ C suppress the transcriptional activation of p73 β through interaction with it.

Suppression of the p73 β -induced increase in endogenous p21 protein by co-expression of p73 ϵ , but not p73 α

To examine whether transcriptional regulation of endogenous gene expression by p73 variants is observed, we investigated the levels of production of p21 protein in Saos-2 cells by immunoblotting after transfection with expression plasmids encoding Myc-tagged p73 variants and p53. As expected from the results of a previous study [3], p73 α induced p21 production to the same extent as p73 β (Figure 5A), in contrast with the results of the reporter gene assay in Figure 1(C). This may imply that transactivation of endogenous genes by p73 α is regulated through complicated interactions with other transcription factors. We also observed that p73 γ induced p21 gene expression. However, p73 ϵ did not increase the p21 protein level, consistent with the results of the reporter gene assay using the p53BCS promoter. Moreover, we observed that the level of p21 protein induced by p73 β was reduced by co-expression of p73 ϵ (Figure 5B, lane 6). This suppression appeared to be exerted by the C-terminal portion of p73 ϵ (p73 ϵ C) (Figure 5B, lane 10). p73 α C also had a suppressive effect on p21 induction by p73 β (Figure 5B, lane 7), although full-length p73 α and α CAN had no effect (Figure 5B, lanes 3 and 11). On the other hand, p73 ϵ , ϵ C and α C did not suppress the induction of p21 expression by p53 (Figure 5C). These observations suggest that p73-mediated gene expression is regulated by the mutual interactions of its splicing variants in the cell.

DISCUSSION

We reported previously that transactivation of the p53BCS promoter by p73 α and p73 ϵ was much weaker than that by p73 β [10]. In the present study, we show that p73 α and p73 ϵ have the potential to suppress the transcriptional activity of p73 β through their C-terminal regions *in trans* (Figure 3 and 4). The fact that C-terminal deletion mutants of p73 α showed significantly higher transcriptional activity than wild-type p73 α (Figure 1) suggests that the C-terminal structure of p73 α also suppresses the activity

of this transcription *in cis* ([22] and the present paper). As the C-terminal 110 amino acids of p73 α (positions 527–636) are shared by p73 ϵ (positions 446–555), this structure is likely to have an important role in the suppressive effects of these variants both *in cis* and *in trans*.

The transcriptional activity of p73 in the cells seems to be regulated in a more complex manner. Lower transcriptional activity of p73 α compared with p73 β has been shown for expression of the endogenous MDM2 and GADD45 genes [3]. As shown in Figure 5, however, p73 α induced the gene expression of p21 to the same extent as p73 β , as reported previously [3]. Similar results were obtained with reporter assay systems using reporter plasmids containing the promoter of the MDM2 or p21 gene upstream of the luciferase gene (results not shown). This suggests that the ability of the C-terminus of p73 α to repress transcription is ineffective for the promoter of the p21 gene. In contrast with p73 α , the level of induction of p21 by p73 ϵ was quite low, as was the activity in the reporter assay using pCAST2Bluc. This suggests that the mechanism of transcriptional regulation by p73 ϵ is different from that by p73 α .

We demonstrated some quantitative differences among the exogenous p73 variants and mutants expressed in Saos-2 cells (Figures 1B, 3A, 3B, 5A and 5B). There was an approximate inverse correlation between expression level and transcriptional potency for the p73 variants and deletion mutants, except for p73 α ΔC505 (Figure 1B). Moreover, the level of production of p73 β was seen to be increased by co-expression with p73 α (Figure 3A, lanes 5–8), and the amount of p73 α was conversely reduced by co-expression with p73 β (Figure 3A, lanes 5–8, compare with the expression of p73 α alone, lanes 1–4). Based on these observations, it is suggested that a gene that is responsible for the degradation of p73s may be a target of p73 β . This is by analogy with p53, which is degraded by MDM2, whose gene is transcriptionally activated by p53 [26,27].

In the present study, the results of immunoprecipitation assays and two-hybrid assays in mammalian cells showed that four p73 variants, p73 α , p73 β , p73 γ and p73 ϵ , interact with each other. Moreover, it was suggested that the transcriptional activity of each p73 splicing variant is regulated by interactions among the variants. These results indicate that the level of induction of p73-responsive gene expression was low when p73 ϵ (and p73 α in some cases) was produced at high levels, even in the presence of p73 β . It has also been reported that ΔN-p73 (p73 lacking the N-terminal region) in mice produced from an alternative promoter such as ΔN-p63 [28] suppressed the transcriptional activity of p73 α [9]. This suggests that regulation of the function of p73 by inter-variant associations is a physiologically significant event. Therefore the pattern of expression of p73 variants, as well as the level of expression of a given p73 variant, seems to be important for the function of the p73 gene. It has been reported that the mRNA expression pattern of p73 variants differs in normal human tissues and cell lines [10,11]. The differential expression of p73 variants in ovarian tumours was also reported [29]. Although inactivation of p73 due to alterations in the p73 gene has not been detected frequently in human cancer cells, changes in the pattern of expression of p73 variants can modify the expression of p73 target genes, and such modifications may contribute to the development of cancer. Therefore, to investigate the contribution of p73 to cancer development, DNA damage, cell differentiation, etc., the pattern of expression of p73 variants, as well as the total level of expression and mutation of the p73 gene, should be taken into consideration.

With regard to the mechanism of transcriptional suppression by the C-terminal region of p73 α and p73 ϵ , it was reported recently that the DNA-binding activity of C-terminal deletion

mutants of p73 α for the p53-responsive element was higher than that of wild-type p73 α , and that the difference in DNA-binding activity reflected the difference in transcriptional activity between these mutants and the wild type [22]. In the present experiments, the transcriptional activity of p73 α and p73 ϵ fused with the GAL4 DBD on the promoter containing GAL4-responsive elements was also much weaker than that of p73 β (Figure 1D), indicating that the difference in transactivation potential of these variants is independent of the DBD of p73. These results suggested two possible mechanisms for the transcriptional activities of p73 splicing variants: one is that the C-terminal regions of p73 α and p73 ϵ affected the DNA-binding capacity of the respective GAL4 DBD-fused proteins to the GAL4-responsive element; and the other is that some novel mechanism, in addition to the differences in DNA-binding activity, results in the transcriptional suppression. In order to elucidate the mechanism, we are now investigating the DNA-binding activity of p73 variants to p53BCS and the GAL4 DBD. Investigation of the mechanism of transcriptional regulation by the C-terminus of p73 may yield new insights into the regulatory machinery of transcription.

This work was supported by grants-in-aid for cancer research and for the second-term comprehensive 10-year strategy for cancer control from the Ministry of Health and Welfare, Japan, by grants-in-aid for scientific research from the Ministry of Education, Science and Culture, Japan, and by the program for the promotion of fundamental studies in health sciences of the Organization for Drug ADR Relief, R&D Promotion, and Product Review of Japan.

REFERENCES

- Kaghad, M., Bonnet, H., Yang, A., Creancier, L., Biscan, J. C., Valent, A., Minty, A., Chalon, P., Lelias, J. M., Dumont, X. et al. (1997) Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. *Cell* **90**, 809–819.
- Jost, C. A., Marin, M. C. and Kaelin, Jr, W. G. (1997) p73 is a human p53-related protein that can induce apoptosis. *Nature (London)* **389**, 191–194.
- Lee, C. W. and La Thangue, N. B. (1999) Promoter specificity and stability control of the p53-related protein p73. *Oncogene* **18**, 4171–4181.
- Zhu, J., Jiang, J., Zhou, W. and Chen, X. (1998) The potential tumor suppressor p73 differentially regulates cellular p53 target genes. *Cancer Res.* **58**, 5061–5065.
- Kaelin, Jr, W. G. (1999) The p53 gene family. *Oncogene* **18**, 7701–7705.
- Herath, N. I., Kew, M. C., Whitehall, V. L., Walsh, M. D., Jass, J. R., Khanna, K. K., Young, J., Powell, L. W., Leggett, B. A. and Macdonald, G. A. (2000) p73 is up-regulated in a subset of hepatocellular carcinomas. *Hepatology* **31**, 601–605.
- Tokuchi, Y., Hashimoto, T., Kobayashi, Y., Hayashi, M., Nishida, K., Hayashi, S., Imai, K., Nakachi, K., Ishikawa, Y., Nakagawa, K. et al. (1999) The expression of p73 is increased in lung cancer, independent of p53 gene alteration. *Br. J. Cancer* **80**, 1623–1629.
- Zaika, A. I., Kovalev, S., Marchenko, N. D. and Moll, U. M. (1999) Overexpression of the wild type p73 gene in breast cancer tissues and cell lines. *Cancer Res.* **59**, 3257–3263.
- Yang, A., Walker, N., Bronson, R., Kaghad, M., Oosterwegel, M., Bonnin, J., Vagner, C., Bonnet, H., Dikkes, P., Sharpe, A. et al. (2000) p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours. *Nature (London)* **404**, 99–103.
- Ueda, Y., Hijikata, M., Takagi, S., Chiba, T. and Shimotohno, K. (1999) New p73 variants with altered C-terminal structures have varied transcriptional activities. *Oncogene* **18**, 4993–4998.
- De Laurenzi, V., Costanzo, A., Barcaroli, D., Terrinoni, A., Falco, M., Annicchiarico-Petruzzelli, M., Levvero, M. and Melino, G. (1998) Two new p73 splice variants, γ and δ , with different transcriptional activity. *J. Exp. Med.* **188**, 1763–1768.
- De Laurenzi, V. D., Catani, M. V., Terrinoni, A., Corazzari, M., Melino, G., Costanzo, A., Levvero, M. and Knight, R. A. (1999) Additional complexity in p73: induction by mitogens in lymphoid cells and identification of two new splicing variants ϵ and ζ . *Cell Death Differ.* **6**, 389–390.
- Bork, P. and Koonin, E. V. (1998) Predicting functions from protein sequences – where are the bottlenecks? *Nat. Genet.* **18**, 313–318.
- Chi, S. W., Ayed, A. and Arrowsmith, C. H. (1999) Solution structure of a conserved C-terminal domain of p73 with structural homology to the SAM domain. *EMBO J.* **18**, 4438–4445.
- Schultz, J., Ponting, C. P., Hofmann, K. and Bork, P. (1997) SAM as a protein interaction domain involved in developmental regulation. *Protein Sci.* **6**, 249–253.
- Levine, A. J. (1997) p53, the cellular gatekeeper for growth and division. *Cell* **88**, 323–331.
- Ko, L. J. and Prives, C. (1996) p53: puzzle and paradigm. *Genes Dev.* **10**, 1054–1072.
- Marin, M. C., Jost, C. A., Irwin, M. S., DeCaprio, J. A., Caput, D. and Kaelin, Jr, W. G. (1998) Viral oncoproteins discriminate between p53 and the p53 homolog p73. *Mol. Cell. Biol.* **18**, 6316–6324.
- Davison, T. S., Vagner, C., Kaghad, M., Ayed, A., Caput, D. and Arrowsmith, C. H. (1999) p73 and p63 are homotetramers capable of weak heterotypic interactions with each other but not with p53. *J. Biol. Chem.* **274**, 18709–18714.
- Kiyono, T., Hiraiwa, A., Ishii, S., Takahashi, T. and Ishibashi, M. (1994) Inhibition of p53-mediated transactivation by E6 of type 1, but not type 5, 8, or 47, human papillomavirus of cutaneous origin. *J. Virol.* **68**, 4656–4661.
- Hijikata, M., Mizushima, H., Akagi, T., Mori, S., Kakiuchi, N., Kato, N., Tanaka, T., Kimura, K. and Shimotohno, K. (1993) Two distinct proteinase activities required for the processing of a putative nonstructural precursor protein of hepatitis C virus. *J. Virol.* **67**, 4665–4675.
- Ozaki, T., Naka, M., Takada, N., Tada, M., Sakiyama, S. and Nakagawara, A. (1999) Deletion of the COOH-terminal region of p73 α enhances both its transactivation function and DNA-binding activity but inhibits induction of apoptosis in mammalian cells. *Cancer Res.* **59**, 5902–5907.
- Diller, L., Kassel, J., Nelson, C. E., Gryka, M. A., Litwak, G., Gebhardt, M., Bressac, B., Ozturk, M., Baker, S. J., Vogelstein, B. and Friend, S. H. (1990) p53 functions as a cell cycle control protein in osteosarcomas. *Mol. Cell. Biol.* **10**, 5772–5781.
- Takada, N., Ozaki, T., Ichimiya, S., Todo, S. and Nakagawara, A. (1999) Identification of a transactivation activity in the COOH-terminal region of p73 which is impaired in the naturally occurring mutants found in human neuroblastomas. *Cancer Res.* **59**, 2810–2814.
- Irwin, M., Marin, M. C., Phillips, A. C., Seelan, R. S., Smith, D. I., Liu, W., Flores, E. R., Tsai, K. Y., Jacks, T., Vousden, K. H. and Kaelin, Jr, W. G. (2000) Role for the p53 homologue p73 in E2F-1-induced apoptosis. *Nature (London)* **407**, 645–648.
- Haupt, Y., Maya, R., Kazanietz, A. and Oren, M. (1997) Mdm2 promotes the rapid degradation of p53. *Nature (London)* **387**, 296–299.
- Kubbutat, M. H., Jones, S. N. and Vousden, K. H. (1997) Regulation of p53 stability by Mdm2. *Nature (London)* **387**, 299–303.
- Yang, A., Kaghad, M., Wang, Y., Gillett, E., Fleming, M. D., Dotsch, V., Andrews, N. C., Caput, D. and McKeon, F. (1998) p63, a p53 homolog at 3q27–29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. *Mol. Cell* **2**, 305–316.
- Zwahlen, D., Tschann, M. P., Grob, T. J., Peters, U. R., Fink, D., Haenggi, W., Altermatt, H. J., Cajot, J. F., Tobler, A., Fey, M. F. and Aepli, S. (2000) Differential expression of p73 splice variants and protein in benign and malignant ovarian tumours. *Int. J. Cancer* **88**, 66–70.

Received 29 January 2001/29 March 2001; accepted 17 April 2001