p73 Independent of c-Myc Represses Transcription of Platelet-derived Growth Factor β-Receptor through Interaction with NF-Y*

We recently reported that c-Myc represses the transcription of platelet-derived growth factor (PDGF) β-receptor (Izumi, H., Molander, C., Penn, L. Z., Ishisaki, A., Kohno, K., and Funa, K. (2001) J. Cell Sci. 114, 1533–1544). We demonstrate here that the p53 family protein p73α represses PDGF β-receptor transcription essentially by the same mechanism. p73α but not p73β or p53 represses the transcription in concordance with its ability to bind NF-YC and NF-YB. None of other p73 isoforms (i.e. p73β, p73γ, p73ε), C-terminal deletion mutants of p73α, and p53 is able to bind NF-Y with the exception of p63α. This finding suggests that the sterile α-motif domain present only in p73α and p63ε is the interaction site. For the repression, the N-terminal transactivation domain of p73α is also indispensable, arguing for the importance of the activity of p73α in the mechanism. p73α binds the C-terminal HAP domain of NF-YC previously found to be the interaction site with c-Myc and TBP. Because c-Myc induces and activates p73α (Zaika, A., Irwin, M., Sansome, C., and Moll, U. M. (2001) J. Biol. Chem. 276, 11310–11316) and they bind each other (Uramoto, H., Izumi, H., Ise, T., Tada, M., Uchiumi, T., Kuwano, M., Yasumoto, K., Funa, K., and Kohno, K. (2002) J. Biol. Chem. 277, in press), we examined whether the repression by p73 is dependent on c-Myc. However, Myc-null rat fibroblasts are also susceptible to p73α-induced repression. Serum stimulation of NIH3T3 cells gradually decreased the amount of endogenous NF-Y binding to the PDGF β-receptor promoter, whereas NF-YA expression in the nuclear extracts remains unchanged. Our results indicate that serum stimulation induces c-Myc and p73α, leading to the down-regulation of PDGF β-receptor expression by repressing its transcription.

Platelet-derived growth factor (PDGF)^1^ is a serum mitogen consisting of four isoforms (A, B, C, and D) that exists as dimers (AA, BB, AB, CC, and DD) and binds two distinct PDGF receptors, α and β, with different affinities (4–7). The receptors are endowed with a split tyrosine kinase in the intracellular domains, which upon ligand binding, dimerize and become auto-phosphorylated to activate the downstream signal cascades. Thus, it is essential for cells that the receptors are activated at right time by refined control mechanisms. The mechanism of activation has been extensively studied, whereas knowledge regarding the control mechanism to cease activation is still limited. Both of these mechanisms can be disturbed to maintain the active status in cancer cells.

In normal cells, the PDGF β-receptor expression decreases rapidly after stimulation by PDGF, which is an important feedback mechanism to prevent further activation of the cells leading to uncontrolled proliferation. The attenuation on the PDGF signaling occurs at multiple levels with both short and long term effects. The receptors on the cell surface decrease by ligand-induced receptor-mediated endocytosis (8) as well as by ubiquitination and subsequent degradation (9). As soon as the ligands diminish or cells become confluent or differentiated, tyrosine phosphatases can be activated, reverting the receptor to its normal status (10, 11). As a long term effect, the transcription of the receptor itself can be repressed by increased c-Myc following stimulation by PDGF or serum (1, 12). c-myc, one of the target genes of PDGF, leads to proliferative responses of cells and, in turn, down-regulates PDGF β-receptor, resulting in the cell cycle-dependent expression of the PDGF β-receptor. A Myc-null cell line showed a high and stable level of PDGF β-receptor mRNA, which disappeared upon forced expression of c-Myc (12). We have previously shown that c-Myc represses the transcription of the receptor through binding to the NF-Y transcription factor and interferes its activation (1).

The mouse PDGF β-receptor promoter contains a CCAAT box, and the NF-Y transcription factor binds to the motif and activates the transcription (13, 14). NF-Y consists of NF-YA, NF-YB, and NF-YC subunits that are all necessary for DNA binding (15). The specific domains of NF-YA, NF-YB, and NF-YC that are needed for subunit interaction and DNA binding have conserved homologous sequences with the yeast HAP2, HAP3, and HAP5 proteins, respectively (as reviewed in Ref. 16). The repression of c-Myc on PDGF β-receptor promoter activity is dependent on the interaction of c-Myc with HAP domains of NF-YB and NF-YC (1).

We have recently found that c-Myc binds p73α, thereby either activating or repressing the target genes of Myc or p73α, respectively (3). It has been reported that c-Myc induces and activates p73α and p73β (2). These findings prompted us to examine the effect of p73 on PDGF β-receptor transcription. p73 is one of the recently identified p53-related family proteins including p63 and p51 (as reviewed in Ref. 17). p73 is similar to p53 in its ability to form oligomers, bind DNA, and activate transcription of p53-responsive genes by genotoxic stresses, leading to various actions such as induction of growth suppres-

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The abbreviations used are: PDGF, platelet-derived growth factor; HA, hemagglutinin; TAD, transactivation domain; ANOVA, analysis of variance; DNNF-YA, dominant negative NF-YA; DBD, DNA-binding domain; PDGFRβ, PDGF β-receptor.

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sion and apoptosis. However, p73 seems to play more essential roles in development and differentiation (18). There are several splicing variants of p73 (α, β, γ, δ, ε, ζ) and also variants without N-terminal transactivation domains that act as dominant negative proteins for the corresponding full-length proteins (17). Roles of these proteins in normal cells have not yet been fully elucidated. The expression levels of p73 proteins are low in normal cells, and often, only p73α and p73β can be detected. We propose that serum stimulation induces c-Myc and p73α, leading to the down-regulation of PDGF β-receptor expression by repressing its transcription in a similar manner.

EXPERIMENTAL PROCEDURES

Cell Culture—NIH3T3 mouse fibroblasts, HO15.19 (myc−/−), rat fibroblasts (19), and COS-1 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 units/ml penicillin in a 5% CO2 atmosphere at 37 °C. All expression of cDNA in cells was carried out by transient transfection with FuGENE 6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer’s protocol.

Plasmid Constructs and Antibodies—N-terminal hemagglutinin (HA) tagged pcDNA3-HA-p73α, pcDNA3-HA-p73β, pcDNA3-HA-p63α, and pcDNA3-HA-p63ß, and the dominant negative mutant (TAD) deletion mutants were provided by Dr. Melino (University of Rome, Rome, Italy) (20). pcDNA3-FLAG-p73α, β, γ, ε, and the C-terminal deletion mutants Δp73αC269 and Δp73αC242 were provided by Dr. Hjikata (University of Kyoto, Kyoto, Japan) (21). The cDNAs for wild type and dominant negative mouse NF-YA, mouse NF-YB, and human NF-YC were provided by Dr. Mantovani (University of Modena, Modena, Italy) (22, 23). We used the N-terminal FLAG-tagged pcDNA3 expression vector for NF-YA, NF-YB, NF-YC, and the deletion mutants of FLAG-NF-YC (FLAG-NF-YCA1–100, FLAG-NF-YCA1–107, FLAG-NF-YCA1–115, and FLAG-NF-YCA1–143) as well as pSG424-NF-YC, NF-YC1–107 (for review see Ref. 1), and NF-YC1–247. Anti-PDGF β-receptor polyclonal antibody (958, Santa Cruz Biotechnology, San Diego, CA), anti-FLAG antibody (M2), anti-FLAG M2-agarose affinity gel, and anti-β-tubulin antibody (Sigma) as well as anti-HA high affinity peroxidase-conjugated antibody (3F10, Roche Molecular Biochemicals) were employed. Dr. Mantovani provided the anti-NF-YA antibody (22, 23).

Promoter Reporter Assay—NIH3T3 cells were seeded in 12-well plates at a density of 2 × 104 cells/well. The following day, cells were transiently transfected with an expression plasmid, a reporter plasmid, and a mock DNA plasmid. Total amount of DNA per well was adjusted to 1 μg by the addition of mock DNA plasmid. When two expression plasmids were used, an initial denaturation step at 94 °C for 1 min, annealing at 55 °C for 45 s, and primer extension at 72 °C for 1 min, 30 s. For p73α, 2.5% cDNA was mixed with primers: 5’-AGCAGGCTCCTCCTACAGAGG-3’ and 5’-ACTCTGCGGGGATTCATCGAGG-3’. Initial denaturation at 94° for 3 min was followed by 22 cycles of denaturation at 94 °C for 2 min, annealing at 67 °C for 45 s, and primer extension at 72 °C for 1 min. For p73β and p63α and the primers described previously (24) were used. Initial denaturation at 94 °C for 4 min was followed by 25 cycles of denaturation at 94 °C for 30 min, annealing at 63 °C for 45 s, and primer extension at 72 °C for 1 min, 30 s. All of the reactions were completed with a final extension step at 72 °C for 6 min. PCR products were analyzed on 1.5% TAE agarose gel electrophoresis. The gels were stained with Sybr Gold (Molecular Probes) and scanned in a FLA2000 (Fuji).

Immunoblotting after Transfection—To see the time course of PDGF β-receptor expression following transfection of HAp73, NIH3T3 (5 × 106 cells/dish) and HO15.19 (myc−/−) cells (1 × 106 cells/dish) were seeded in eight 6-cm dishes each. They were transfected as described above for reverse transcription-PCR. The cells were harvested at 0, 12, 24, and 36 h post-transfection. They were scraped in 1 ml of phosphate-buffered saline and centrifuged for 2 min at 4 °C at 1000 × g. The cells were resuspended in 100 μl of lysis buffer (50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 120 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, and 1 mM phenylmethylsulfonyl fluoride), incubated on ice for 20 min, and centrifuged at 21,000 × g for 10 min at 4 °C. The amount of protein was determined with Bio-Rad protein assay, and 50 μg of each sample was adjusted to equal volumes (75 μl/sample), boiled, and separated by 8% SDS-PAGE. The gel was transferred onto a Hybond-P polyvinylidene difluoride membrane (Amersham Biosciences), which was cut and blotted with 0.1 μg/ml PDGF β-receptor antibody (958) or 0.5 μg/ml HA (3F10) antibody in 1% skim milk. The membrane was developed by the ECL using vendor’s protocol (Amersham Biosciences) and exposed in LAS-1000 Plus (Fuji). To estimate the p73 concentration-dependent PDGF β-receptor expression, NIH3T3 and HO15.19 fibroblasts were transfected with 0, 0.1, 0.5, or 2 μg of HAp73 expression plasmid as described for the time course experiment. The total amount of transfected DNA was adjusted to 1 μg with HA pcDNA3 vector. After another 24 h, cells were harvested, separated, and blotted in the same way as described for the time course experiment.

In Vivo Binding Assay—COS-1 cells were seeded in 6-well plates at a density of 1 × 106 cells/well. After 24 h, cells were co-transfected with 1 μg of HAp73 expression plasmid with 1 μg of FLAG-NF-YA, FLAG-NF-YB, or FLAG-NF-YC expression plasmid. After another 24 h, cells were lysed in 200 μl of lysis buffer described above. The cells were sonicated for 20 min on ice, and then was transferred into a 1.5 ml centrifuge tube and centrifuged at 21,000 × g for 10 min at 4 °C. 40 μl of the supernatant was transferred to a new tube for expression control of the transfected plasmids. The remaining 160 μl of the lysate was transferred to another tube and rotated at 4 °C with 15 μl of FLAG M2-agarose affinity gel for 2 h. The agarose beads were washed with 4 × 1 ml of lysis buffer, separated by SDS-PAGE (10%), and blotted onto Hybond-P membrane. The membranes were first blotted with anti-HA (3F10) antibody and developed by ECL, and the signal was detected in LAS-1000 Plus. After drying, the membranes were reblotted with FLAG M2-agarose antibody.

Electrophoresis Mobility Shift Assay—A single-stranded antisense oligonucleotide containing CCAAT was used as a probe. The probe was complemented with a sense strand and labeled with [32P]dATP. The template sequence was 5’-TTTGGGAGAACGGCTCAGGGCGCT/CAAGC-3’ (MCAAGC-3’). Labeled DNA was isolated from unincorporated [32P]dATP using a G-25 MicroSpinTM column (Amersham Biosciences). To obtain nuclear extracts, a total of 4 × 106 NIH3T3 cells were seeded in 6-cm dishes. Nuclear protein was extracted from the cells at 0, 4, 8, 12, 24, and 48 h after 10% serum stimulation following 8 h of culture in serum-free medium. Nuclear extracts and 4 μg of protein were preincubated in 20 μl of reaction mixture containing 12 μM Tris-HCl, pH 7.9, 0.6 mM EDTA, 20% glycerol, 1 mM dithiothreitol, 5 mM MgCl2, 0.1 μg/ml BSA, 0.1% Nonidet P-40, and 1 mg/ml poly(dIdC). Unlabeled competitors (100× labeled oligo), 6-labeled oligonucleotide, and antibodies NF-YA or p73 (0.5 μg/reaction) were added in this order. The reactions were incubated 10 min at room temperature. At the end of the incubation, 4 μl of 6× agarose dye loading-buffer was added. The reaction mixtures were resolved by electrophoresis on a 4% acrylamide gel (polyacrylamide: bisacrylamide, 37.5:1) by 20 V/cm for 2 h at 4 °C. The gel was dried and exposed to a BAS imaging plate (Fuji) and scanned in a FLA-2000 (Fuji).
**RESULTS**

**Replication of PDGF β-Receptor Promoter Activity by p73**—We used the PDGF β-receptor promoter luciferase reporter, pGL3SacI/SacI (pGL3−1900; −1900 to +23) containing CCAAT motif located −60 bp upstream of the first initiation site (Fig. 1A) (13). As shown in Fig. 1A, cotransfection of p73α decreased the luciferase activity of pGL3−1900 to 35% of that obtained with a control vector containing only HA tag. In comparison, the expression of neither p73β nor p53 did significantly and stably affect the reporter activity. The shorter MuI/SacI (pGL3−116; −116 to +23) promoter luciferase activity was also repressed by p73α to the same extent (result not shown). This shorter promoter contains Sp1 and NF-Y binding sites (28). We have previously reported that c-Myc represses PDGF β-receptor promoter (1). To see whether c-Myc is involved in the p73 repression on the promoter-luciferase activity, we used the HO15.19, c-myc−/− rat fibroblast cell line and the NIH3T3 normal mouse fibroblast cell line. In both cell lines, p73α and c-Myc significantly decreased the promoter activity (Fig. 1B). p73α repressed the promoter-luciferase activity more effectively than c-Myc in NIH3T3 cells containing c-myc. In the HO15.19, however, the activity of p73α was similar to that of c-Myc, and both together resulted in a further decrease in the transcriptional activity.

The 1.9-kb PDGF β-receptor promoter lacks a putative binding site for p73. To see whether the CCAAT-binding NF-Y transcription factor is required for the p73α-mediated repression, we co-transfected the DNA-binding defective and dominant negative NF-YA (DNNF-YA) with p73α and the promoter-luciferase plasmids. The DNNF-YA is shown to act as a dominant repressor of NF-Y-DNA complex formation and of NF-dependent transcription (23). As expected, the co-expression of DNNF-YA and the promoter-luciferase plasmids decreased half of the promoter activity. However, additional co-expression of p73α to DNNF-YA showed no further effect on the promoter activity (Fig. 1C). In fact, the overexpression of p73α yielded a comparable repression as was seen by DNNF-YA alone on the promoter. These results indicate that p73α represses the promoter activity by its effect on NF-Y, attenuating the stimulatory effect of NF-Y on the promoter (14).

**Association of p73α with NF-YB and NF-YC**—The HA-tagged p73α expression plasmid, HAp73α, HAp73β, HAp53, or HAp63α, respectively, was co-transfected with the FLAG-NF-YA, FLAG-NF-YB, FLAG-NF-YC, or control FLAG-vector expression plasmid, respectively, to COS-1 cells. Forty-eight hours after transfection, cells were lysed and immunoprecipitated with anti-FLAG M2 antibody and immunoblotted with an anti-HA antibody. As shown in Fig. 2A, p73α and p63α bound FLAG-NF-YB and FLAG-NF-YC in vivo but not FLAG-NF-YA or the control vector. Neither p73β nor p53 bound any of the NF-Y subunits. Immunoblotting of the membrane being trans-

FIG. 1. A, p73α represses PDGFRβ promoter activity. NIH3T3 cells were co-transfected with the pGL3-PDGFRβ promoter construct to-
Fig. 2. A, interaction of NF-YB and NF-YC with p73α. COS-1 cells were co-transfected with various FLAG-NF-Y-subunits or FLAG-vector alone, respectively, together with HA-tagged p73α, p73β, p53, or p63α. Cell lysate was divided and used for immunoprecipitation (IP) or immunoblotting (IB). After immunoprecipitation with anti-FLAG M2-agarose affinity gel, the samples were separated together with the cell lysates used for the reaction. Transferred membrane was immunoblotted with HA antibody (3F10, upper panel), and FLAG-M2 antibody (lower left panel). The expression of HA-tagged p73α, p73β, p53, and p63α in the same cell lysates was confirmed by HA antibody (lower right panel). B, schematic
ferred with the same cell lysate, which reacted with the anti-HA antibody, as well as the anti-FLAG M2 antibody confirmed the appropriate expression of proteins. The N-terminal region of p73α and p73β is homologous, and the only notable divergence can be found in the C-terminal area (Fig. 2B). This unique area of p73α not found in p73β is probably responsible for the binding. In addition, p63α also bound NF-Y. Because in vertebræ, this p53 family protein is the only one other than p73α that contains an area known as the SAM domain in its C terminus. As expected, N-terminal deletion mutant of p73α lacking the TAD (ΔNP73α) also bound NF-Y (result not shown).

To determine whether this area is responsible for the interaction, we examined several other p73 isoforms (β, γ, and ε) as well as the two C-terminal deletion mutants of p73α, the Δ73αC269 and Δp73αC424, together with p73α and p53 as a positive and negative control, respectively (Fig. 2C). In this experiment, we used FLAG-tagged p73 and HA-tagged NF-YC to confirm the reciprocal binding. No other p73 isoforms could bind NF-YC, and the C-terminal deletion mutants of p73α without SAM domain were unable to bind NF-YC. The binding site of NF-YC was also determined by co-immunoprecipitation using various N-terminal deletion mutants (Fig. 2, D and E). This result indicates that the C-terminal HAP5 domain of NF-YC, i.e. amino acids 108–115, is necessary for the binding. To exclude the possibility that p73α and NF-Y interacts via c-Myc, the binding assay between NF-YB or YC and p73α was repeated in HO15.19 c-myc cells (Fig. 2F). Again, also in this cell line, p73α binds the NF-Y subunits B and C, indicating that c-myc is dispensable for this binding.

The binding of p73α to NF-YC Is Indispensable for the Repression of NF-YC Transactivation—The transactivation domain of NF-YC subunit has the highest activity of all three NF-Y subunits, whereas NF-YB contains very little such activity (29). Therefore, p73α effect was examined on its activity of the C-terminal transactivation domain of NF-YC (Fig. 2G). The deletion mutant containing the transactivation domain was coupled downstream of the DNA-binding domain (DBD) of GAL4 transcription factor (Fig. 2H) (1). As reported, the full-length NF-YC fused to DBD of GAL4 transcription factor did not significantly activate transcription from the GAL4-TATAPromoter, whereas the corresponding constructs containing only the TAD are active (29). When the N-terminal deletion mutant of NF-YC retaining the C-terminal HAP domain needed for the p73 binding (GAL4-DBD-NF-YC1–107) was co-transfected with p73α, the transactivation activity was diminished to half of that obtained by co-transfection of the control HA-vector, indicating that p73 interferes with the activation of NF-YC (Fig. 2G). In agreement, the activity of the GAL4-DBD-NF-YC1–248 being unable to bind p73α was not affected by co-transfection of p73α.

Transactivation Domain of p73α Is Necessary for Repression of PDGF β-Receptor Promoter—We determined the binding site of p73α to its C-terminal SAM domain. To see whether the TAD of p73α is necessary for the repression, we transfected the ΔNP73α lacking the TAD (ΔNP73α), HA-vector alone, or the full-length p73α together with the PDGF β-receptor promoter luciferase construct in NIH3T3 cells and measured its activity. As shown in the Fig. 3A, there was no repression, but rather activation was observed when TAD was deleted from p73α. This finding was further confirmed by the concentration-dependent increase of promoter activity by the addition of presentation of various p73 isoforms. Each box indicates translated exons 2–14 in all p73 alternative splicing variants. Stretched boxes indicate divergent amino acid sequences because of framenshifts. Only the p73α of ΔNP73 is indicated at the bottom. The TAD, DBD, oligomerization (OD), and SAM domains are shown. C, interaction of FLAG-tagged various p73 isoforms with HA-NF-YC. COS-7 cells were co-transfected with FLAG-p73α, β, γ, ε, p73α C-terminal deletion mutants (p73αΔC269 and p73αΔC424), p53, or FLAG-vector, respectively, together with HA-tagged NF-YC. Immunoprecipitation and immunoblotting were performed as described in A. Immunoblotting with HA antibody (upper panel) and FLAG-M2 antibody (middle panel) are shown. The same cell lysates were blotted with HA antibody (lower panel). E, schematic presentation of NF-YC and its N-terminal deletion mutants with their p73α binding ability. HAP domain is indicated by a black box. F, interaction of HAp73α with FLAG-tagged NF-YC and NF-YB in HO15.16 c-myc–/– cells. COS-1 cells were co-transfected with FLAG-NF-YA, FLAG-NF-YB or FLAG-vector alone, respectively, together with HA-tagged p73α or vector alone. In vivo binding is shown as described in A. Bands located above the FLAG-NF-YB are nonspecific and seen in all lines. G, p73α represses the activity of NF-YC transactivation domain. NIH3T3 cells were co-transfected with GAL4-TATA luciferase construct (GAL4-TATA luc) and pSV4024 full-length (FL), Δ1–107, or Δ1–247 NF-YC and p73α or vector alone. ***p < 0.0001. Error bars indicate mean ± S.D. of triplicate samples. A representative result of three repeated experiments is shown. H, schematic presentation of NF-YC-GAL4DBD fusion constructs.
Fig. 4. A and B, p73α represses PDGFRβ protein expression in NIH3T3 (A) and HO15.19 (c-Myc−/−) (B) fibroblasts. Cells were transfected with HA-p73α (left panel) or HA-vector alone (right panel). Cells were lysed at 0, 12, 24, and 36 h after transfection. Cell lysates with equal amounts of protein were run on a SDS-PAGE gel. The membrane was immunoblotted with PDGFRβ antibody and HA antibody (3F10) as well as with β-tubulin antibody as a standard. C and D, p73α concentration-dependent expression of PDGFRβ in NIH3T3 (C) and HO15.19 (c-Myc−/−) (D) fibroblasts. Cells were transfected with 0, 0.1, 0.5, or 2 μg of HA-p73α, and the total amount of transfected DNA was corrected with HA-vector. Cells were lysed at 24 h after transfection. Immunoblotting was done as described above.

ΔNp73α to the full-length p73α. ΔNp73α alone stimulated the promoter activity.

**Effects of p73 on the Expression of the PDGF β-Receptor**—We demonstrated that p73α repressed the transcriptional activity of PDGF β-receptor promoter. We next examined whether this repression is reflected in the mRNA and protein expression of PDGF β-receptor by reverse transcription-PCR and immunoblotting. When HA-tagged p73α was transfected in NIH3T3 cells, the expression of p73α mRNA increased as judged by the p73α PCR product. It became detectable at 4 h to increase gradually up to 24 h, remaining at the same level at 36 h. On the contrary, the PDGF β-receptor PCR product being clearly expressed already at 0 and 4 h gradually decreased at 8, 12, and 36 h. (Fig. 3B). On the other hand, the transfection of the control HA-vector without p73α did not affect the level of PDGF receptor mRNA. In agreement with this finding, p73α protein was detected already at 12 h, and the expression increased up to 36 h (Fig. 4A). By contrast, the receptor expression rapidly decreased already at 12 h and remained as a faint band during the whole observation period. Control HA-vector transfection did not alter the receptor expression level that remained at a high level. The product of the control expression vector with only HA tag migrates too fast to be detected in the immunoblots. A similar marked decrease of the PDGF β-receptor expression was seen in the p73α expressing c-myc-deficient HO15.19 cells (Fig. 4B). This cell line has normally higher expression of the receptor than c-myc containing cell lines, its parental line TGR (data not shown) and NIH3T3. The p73α concentration-dependent receptor expression was further examined by transfecting increasing amounts of p73α expression plasmid in each of these cell lines. As expected, both cell lines showed a gradual decrease of PDGF β-receptor expression when the p73α expression increased (Fig. 4, C and D).

**DISCUSSION**

The CCAAT motif upstream of the initiation site of PDGF β-receptor gene is essential for the basal transcription activity and also for the c-Myc-induced repression (1). We herein demonstrate that p73α, a p53 family suppressor gene product, represses the activity of the 1.9-kb SacI/SacI PDGF β-receptor promoter in a concentration-dependent manner. It is consid-
ered that all the NF-Y subunits are necessary for the stable binding of NF-Y complex to DNA. However, NF-YA seems to be the important subunit to create the DNA binding surface (15). The overexpression of DNNF-YA was shown not only to prevent NF-Y complex from binding to CCAAT box (23) but also to attenuate the p73\(^{\beta}/H9251\) effect on the PDGF\(^{\beta}/H9252\)-receptor promoter activity. Thus, these results suggest that NF-Y binding is crucially involved in the p73\(^{\beta}/H9251\)-induced repression on PDGF\(^{\beta}/H9252\)-receptor promoter. Co-immunoprecipitation of p73\(^{\beta}/H9251\) with NF-Y subunits and their deletion mutants revealed that p73\(^{\beta}/H9251\) but not other isoforms, splicing variants, or p53 bound the HAP domain of NF-YB and NF-YC. The binding capacity of these molecules agrees with their capacity of the transcription repression. The C-terminal p73\(^{\alpha}\) contains both proline-rich area and the SAM domain known as interacting domains. In fact, no other p73 isoforms bind NF-YC, but p73\(^{\beta}\) that does not either bind NF-Y or repress the transcription also possesses the proline-rich domain. Thus, our results indicate that p73\(^{\beta}/H9251\) may bind NF-Y via its hydrophobic SAM domain, consisting of five helices (32). In fact, we found that p63\(^{\alpha}\), another p53 member in vertebrates that possesses the SAM domain, also binds NF-YC. The difference in the transcriptional activities between various C-terminal isoforms of p73 and p63 (33) may be partly attributed to the differences in their interacting proteins. Interestingly, the TAD of p73\(^{\alpha}\) is indispensable for the repression because the \(\Delta Np73\alpha\) devoid of TAD is incapable of repressing the promoter. This was also confirmed by the DDPp73\(^{\alpha}\) lacking both TAD and the DNA-binding domain but retaining the intact C-terminal domain (result not shown). The expression of \(\Delta Np73\alpha\) not only fails to repress the promoter but also increases the activity in a dominant negative manner. It is shown that p73\(^{\alpha}\) can homodimerize and heterodimerize with any of the p73 isoforms (33) via their oligomerization domains. Although p73\(^{\alpha}\) does not efficiently oligomerize with p53, \(\Delta Np73\alpha\) can bind p53, inactivating the function of p53 (34). Furthermore, \(\Delta Np73\alpha\) inhibits p53 by competition for DNA binding (35). Although we could not show any direct repression of p53 on the promoter, it is possible that by decreasing Myc,
p73 indirectly affects the level of the PDGF receptor. It is also possible that Np73α binds wild-type p73α more efficiently and inactivates wild-type p73α.

Structurally, p73 is a typical transcription factor, activating common target genes of p53, i.e., p21, MDM2, and Bax (36), through binding to the p53-responsive DNA sequence. Although p73α represses the transcription of the PDGF β-receptor, the promoter of which has no p73α binding site. The repression occurs not by a direct interaction with DNA but via protein-protein interaction with NF-Y. The binding site of p73 to c-Myc is close to its own DNA-binding domain (amino acids 227–312), being distinct from the area needed for the interaction of p73α with NF-Y (3). This area is conserved in all p73 isoforms and therefore is unlikely to be involved in the p73α repression on the PDGF β-receptor promoter because p73β lacks this activity. p73 is also not required for the repression of Myc on the promoter, because the p73 binding site of Myc, the C-terminal HLH-LZ domain, is dispensable for the repression of Myc on the PDGF β-receptor. The Myc-null HO15.19 cells responded as sensitively as normal Myc-positive fibroblasts to the p73α repression of various genes other than by its direct binding to DNA.

Similar to the case of Myc, we could not see any change in electrophoresis mobility shift assay when using nuclear extracts taken from p73α overexpressing cells or by using in vitro synthesized proteins, arguing against sequestering of NF-Y by p53 in the nucleus remained unaffected. NF-YA is the only common target genes of p53, transcription of various genes other than by its direct binding to DNA.

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