Hme1, that may be down-regulated in neoplastic mammary cells, Cell Growth Differ. 3 (1992) 507–513.
[19] A.T. Ferguson, E. Evron, C.B. Umbricht, T.K. Pandita, T.A. Chan, H. Hermeking, J.R. Marks, A.R. Lambers, P.A. Futreal, M.R. Stampfer, S. Sukumar, High frequency of hypermethylation at the 14-3-3s locus leads to gene silencing in breast cancer, Proc. Natl. Acad. Sci. USA 97 (2000) 6049–6054.
[20] C. Laronga, H.Y. Yang, C. Neel, M.H. Lee, Association of the cyclin-dependent kinases and 14-3-3σ negatively regulates cell cycle progression, J. Biol. Chem. 275 (2000) 23106–23112.
[21] H. Hermeking, C. Lengauer, K. Polyak, T.C. He, L. Zhang, S. Thiagalingam, K.W. Kinzler, B. Vogelstein, 14-3-3σ is a p53-regulated inhibitor of G2/M progression, Mol. Cell 1 (1997) 3–11.
[22] H.-Y. Yang, Y.-Y. Wen, C.-H. Chen, G. Lozano, M.-H. Lee, 14-3-3σ positively regulates p53 and suppresses tumor growth, Mol. Cell. Biol. 23 (2003) 7096–7170.
[23] K. Ando, T. Ozaki, H. Yamamoto, K. Furuya, M. Hosoda, S. Hayashi, M. Fukuzawa, A. Nakagawara, Polo-like linase 1 (Plk1) inhibits p53 function by physical interaction and phosphorylation, J. Biol. Chem. 279 (2004) 25549–25561.
[24] M. Vayssade, H. Haddada, L. Faridoni-Laurens, S. Tourpin, A. Valent, J. Benard, J.C. Ahomadegbe, p73 functionally replaces p53 in adriamycin-treated, p53-deficient breast cancer cells, Int. J. Cancer 116 (2005) 860–869.
[25] J. Gray-Bablin, J. Zalvide, M.P. Fox, C.J. Knickerbocker, J.A. DeCaprio, K. Keyomarsi, Cyclin E, a redundant cyclin in breast cancer, Proc. Natl. Acad. Sci. USA 93 (1996) 15215–15220.
[26] G. Ragnarsson, G. Eiriksodottir, J.T. Johannsdottir, J.G. Jonasson, V. Egilsson, S. Ingvarsson, Loss of heterozygosity at chromosome 1p in different solid human tumours: association with survival, Br. J. Cancer 79 (1999) 1468–1474.
[27] H. Yang, R. Zhao, M.-H. Lee, 14-3-3σ, a p53 regulator, suppresses tumor growth of nasopharyngeal carcinoma, Mol. Cancer Ther. 5 (2006) 253–260.
[28] M. Rossi, V. De Laurenzi, E. Munarriz, D.R. Green, Y.C. Liu, K.H. Vousden, G. Cesareni, G. Melino, The ubiquitin-protein ligase Itch regulates p73 stability, EMBO J. 24 (2005) 836–848.
[29] A. Costanzo, P. Merlo, N. Pediconi, M. Fulco, V. Sartorelli, P.A. Cole, G. Fontemaggi, M. Fanciulli, L. Schiltz, G. Blandino, C. Balsano, M, Levrero, DNA damage-dependent acetylation of p73 dictates the selective activation of apoptotic target genes, Mol. Cell 9 (2002) 175–186.
[30] M.S. Irwin, K. Kondo, M.C. Marin, L.S. Cheng, W.C. Hahn, W.G. Kaelin Jr., Chemosensitivity linked to p73 function, Cancer Cell 3 (2003) 403–410.

Available online at www.sciencedirect.com
SCIENCE DIRECT
ELSEVIER
Biochemical and Biophysical Research Communications 347 (2006) 60–66
BBRC
www.elsevier.com/locate/ybbrc

p73 and MDM2 confer the resistance of epidermoid carcinoma to cisplatin by blocking p53

Syunji Hayashi [a,b], Toshinori Ozaki [a], Kaori Yoshida [a], Mitsuchika Hosoda [a], Satoru Todo [b], Shin-ichi Akiyama [c], Akira Nakagawara [a,*]

[a] *Division of Biochemistry, Chiba Cancer Center Research Institute, Chuoh-ku, Chiba 260-8717, Japan*
[b] *Department of General Surgery, Hokkaido University School of Medicine, Kita-ku, Sapporo 060-8638, Japan*
[c] *Department of Molecular Oncology, Graduate School of Medical and Dental Science, Kagoshima University, Kagoshima 890-8520, Japan*

Received 4 June 2006
Available online 27 June 2006

Abstract

p73 responds to DNA damage and exerts its pro-apoptotic function. However, p73 might contribute to the development of drug-resistance in certain tumor cells. In this study, we found that p73 and MDM2 correlate with cisplatin-resistant phenotype of human epidermoid carcinoma-derived cells. p73 and MDM2 were kept at low levels in the cisplatin-sensitive KB-3-1 cells, whereas p53 was induced to be phosphorylated at Ser-15 in response to cisplatin. In contrast, p73 and MDM2 were expressed at higher levels, and cisplatin-mediated p53 phosphorylation was undetectable in the cisplatin-resistant KCP-4 cells. Enforced expression of p73 in KB-3-1 cells caused an accumulation of unphosphorylated form of p53 and MDM2, and conferred the cisplatin resistance. Collectively, our results suggest that a loss of the cisplatin sensitivity is at least in part due to a lack of cisplatin-induced p53 phosphorylation, and p73 might cooperate with MDM2 to be involved in this process.

Keywords: Cisplatin; KB-3-1; MDM2; p53; p73

p53 plays a pivotal role in a variety of biological processes including cell cycle arrest and DNA damage-induced apoptosis. p53 is a sequence-specific nuclear transcription factor, and has an ability to transcriptionally activate its downstream target genes such as $p21^{WAF1}$, *MDM2*, *Bax*, and *PUMA*, whose products trigger cell cycle arrest and/or apoptosis [1,2]. The biological activities of p53 are tightly linked to its sequence-specific transactivation function, and more than 90% of p53 mutations found in human tumor tissues are located within its highly conserved DNA-binding domain. In response to genotoxic stresses, p53 is phosphorylated at multiple sites including Ser-15 and Ser-20, and thereby the latent form of p53 is converted to the active form for its function. DNA damage-induced phosphorylation of p53 increases its half-life and nuclear concentration by preventing its interaction with MDM2, which acts as an E3 ubiquitin ligase for p53 and directly mediates the ubiquitination-dependent proteolytic degradation of p53 [3–5].

There are several potential molecular mechanisms responsible for acquisition of drug resistance, including reduced drug uptake, increased drug inactivation and accelerated DNA damage repair [6,7]. These mechanisms, individually or collectively, reduce the apoptotic response of tumor cells following drug exposure and thus contribute to the development of drug resistance. Alternatively, it has been shown that p53 status is one of the critical determinants of cellular sensitivity to DNA-damaging agents [8,9]. Alterations in p53 caused by mutations result in a poor response to DNA-damaging agents. *p53* mutations found in tumor tissues lead to a significant accumulation of mutant forms of p53, because mutant forms of p53 lack

* Corresponding author. Fax: +81 43 265 4459.
E-mail address: akiranak@chiba-cc.jp (A. Nakagawara).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.06.095

an ability to transactivate the expression of *MDM2* [10]. Wild-type p53 functions as a tetramer, and mutant forms of p53 have an ability to hetero-oligomerize with wild-type p53. As a result of this heteromeric association, mutant forms of p53 inhibit the transactivation as well as pro-apoptotic function of wild-type p53. Thus, the defects in the p53-mediated apoptotic pathway play an important role in tumorigenesis and also render tumor cells resistant to anti-cancer drug.

p73 shares remarkable structural and functional similarities with p53 [11]. Contrary to the initial prediction based on the *in vitro* studies, mutations of *p73* in a wide variety of human tumors appear to be rare [12], and p73-deficient mice displayed a specific neuronal disorder as well as a defective immunological response but showed no increased susceptibility to spontaneous tumorigenesis [13]. In addition, p73 is expressed as multiple functionally distinct isoforms that differ at their NH_2- and COOH-termini, arising from alternative promoter utilization and differential mRNA splicing, respectively [14,15]. Among them, ΔNp73, which lacks an NH_2-terminal transactivation domain, has an oncogenic potential [16] and exhibits dominant-negative behavior toward wild-type p73 as well as p53 [17]. Intriguingly, *ΔNp73* itself is transactivated by p73, thus forming an autoregulatory feedback loop for inhibiting p73 function [18,19].

Recently, it has been shown that p73 or p63 (another member of p53 family) is required for the p53-dependent apoptosis in response to DNA damage [20]. On the other hand, enforced expression of p73 reduced the transcriptional activity of p53 by inhibiting its DNA-binding ability [21], and is also associated with the resistance to DNA-damaging agents in ovarian cancer cells that carry wild-type *p53* [22]. In the present study, we have found that overexpression of p73 as well as MDM2 correlates quite well with the cisplatin-resistant phenotype of human epidermoid carcinoma cells, and the cisplatin-resistant phenotype might be due to a lack of the induction of p53 phosphorylation at Ser-15 in response to cisplatin.

Materials and methods

Cell culture and transfection. Human epidermoid carcinoma KB-3-1, a cisplatin-resistant subline KCP-4, and a cisplatin-sensitive KCP-4R derived from KCP-4 were cultured in minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen) and penicillin (100 IU/ml)/streptomycin (100 μg/ml). Human breast carcinoma cell lines MCF-7 and MDA-MB-231 were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FBS and antibiotic mixture. Human pancreas carcinoma cell lines JHP1, MIAPaCa, Panc-1, SW1990, and AsPC-1; human non-small cell lung carcinoma cell lines A549 and H1299; human colon carcinoma cell lines COLO320, SW480 were grown in RPMI 1640 supplemented with 10% heat-inactivated FBS and antibiotic mixture. Cells were maintained in an incubator at 37 °C with humidified 5% CO_2 and 95% air. For transient transfection, cells were seeded in 6-well plates at a density of 5×10^4/ml. The day after plating, transfection was performed using the calcium phosphate/DNA precipitation procedure.

Semi-quantitative reverse transcription-PCR analysis. Total RNA was extracted from the indicated cells in the presence or absence of cisplatin by using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. The first strand cDNA was generated from 1 μg of total RNA using random primers and a SuperScript II reverse transcriptase (Invitrogen) at 42 °C for 1 h. When the reaction was complete, the cDNA was used for PCR amplification with rTaq DNA polymerase (Takara). Primer sequences were designed as follows: forward, 5′-ATTTGATGCTGTCCC CGGACGATATTGAAC-3′, and reverse, 5′-ACCCTTTTTGGACTTC AGGTGGCTGGAGTG-3′ for *p53*; forward, 5′-CCGGGAGAACTTT GAGATCC-3′, and reverse, 5′-ATCTTCAGGGCCCCCAGGTC-3′ for *p73*; forward, 5′-CGCCTACCATGCTGTACGTCA-3′, and reverse, 5′-G TGCTGGACTGCTGGAAAGT-3′ for *ΔNp73*; forward, 5′-GTCCCAG AGCACACAGACAA-3′, and reverse, 5′-GAGGAGCCGTTCTGAAT CTG-3′ for *p63*; forward, 5′-CTGGAAAACAATGCCCAGAC-3′, and reverse, 5′-GGGTGATGGAGAGAGAGCAT-3′ for *ΔNp63*; forward, 5′-ATGAAATTCACCCCCTTTCC-3′, and reverse, 5′-CCCTAGGCTG TGCTCACTTC-3′ for $p21^{WAF1}$; forward, 5′-ACTTGAGCCGAGGAG TTCAA-3′, and reverse, 5′-TTGCTCTGTCACCTGGACTG-3′ for *MDM2*; forward, 5′-ACTTGAGCCGAGGAGTTCAA-3′, and reverse, 5′-TCCCGGCAAAAACAAATAAG-3′ for *GADD45*; forward, 5′-TTT GCTTCAGGGTTTCATCC-3′, and reverse, 5′-CAGTTGAAGTTGCC GTCAGA-3′ for *Bax*; forward, 5′-AGGTGGACCTGTTTCGTGAC-3′, and reverse, 5′-ACCCTGTGATCCACCAGAAG-3′ for *MRP1*; forward, 5′-TGCTTCCTGGGGATAATCAG-3′, and reverse, 5′-CACGGATAA CTGGCAAACCT-3′ for *MRP2*; forward, 5′-GGCGTCTATGCTGC TTTAGG-3′, and reverse, 5′-CCTTGGAGAAGCAGTTCAGG-3′ for *MRP3*; forward, 5′-TGCTCT GGAGTGTGCATTTC-3′, and reverse, 5′-AAGCCGAGTACGGAC TCTCA-3′ for *ATP7B*; forward, 5′-TGGG ACGAAGAAAAGGAATG-3′, and reverse, 5′-GATCAGGCAGGTT AGCAAGC-3′ for *MLH1*; forward, 5′-GCCATTTTGGAGAAAGGA CA-3′, and reverse 5′-CTCACATGGCACAAAACACC-3′ for *MSH2*; forward, 5′-CTCCCACTGCTGCCTTAGTC-3′, and reverse, 5′-CTTGC AAGCAATGGTGAAGA-3′ for *XPA*; forward, 5′-GCGGCAGAGAT TCTTGGTAG-3′, and reverse 5′-GGCCCCAGACATAGAACTCA-3′ for *XPB*; forward, 5′-CCATGAGGACACACACAAGG-3′, and reverse 5′-ACAACCACCCTCCAAGACAG-3′ for *XPC*; forward, 5′-AGCCAC GTTCCCCTATGTG-3′, and reverse, 5′-ACAGCTTCCTTTCAGCCA AA-3′ for *XPE*; forward, 5′-TGCGTGAATTTCGAAGTGAG-3′, and reverse, 5′-TGGAGATGCACTGGCTGTAG-3′ for *XPF*; forward, 5′-C AGACACAGCTCCGAATTGA-3′, and reverse, 5′-TTCTGGGTTTTT CGTTTTGC-3′ for *XPG*; forward, 5′- ACCTGACCTGCCGTCTAGA A-3′, and reverse, 5′-TCCACCACCCTGTTGCTGTA-3′ for *GAPDH*. *GAPDH* was used as a control for each PCR for semi-quantitative purposes. PCR products were electrophoresed on 1.5% agarose gels containing ethidium bromide and visualized by UV-induced fluorescence.

Recombinant adenovirus and infection. Adenovirus-p73α and adenovirus-LacZ were generated as described previously [18]. For adenovirus gene transfer, KB-3-1 cells were incubated with the indicated multiplicity of infection of adenovirus constructs at 37 °C for 48 h.

Western blot analysis. Cells were extracted directly with the lysis buffer containing 25 mM Tris–HCl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1% Triton X-100, 1 mM PMSF, and protease inhibitor mixture (Sigma). Total protein concentrations were determined using the Bradford protein assay according to the instructions of the vendor (Bio-Rad). Equal amounts of protein (50–100 μg) were boiled for 5 min in an SDS sample buffer consisting of 62.5 mM Tris–HCl, pH 6.8, 2% SDS, 2% β-mercaptoethanol, and 0.01% bromophenol blue, subjected to 10% SDS–PAGE under reducing conditions, and then electro-transferred onto Immobilon-P membranes (Millipore) using a semi-dry transfer apparatus at room temperature for 1 h. Nonspecific antigen–antibody binding on the membranes was blocked by incubating in TBS-T [50 mM Tris–Cl (pH 7.6), 100 mM NaCl, and 0.1% Tween 20] containing 5% non-fat dried milk at room temperature for 1 h. The membranes were then incubated for 1 h with antibodies against p73 (Ab-4; NeoMarkers), p53 (DO-1; Oncogene Research Products), MDM2 (SMP14; Santa Cruz Biotechnologies), $p21^{WAF1}$ (H-164; Santa Cruz Biotechnologies), p53 phosphorylated at Ser-15 (Cell Signaling), p53 phosphorylated at Ser-20 (Cell Signaling), p53 phosphorylated at Ser-392 (Cell Signaling), ΔNp73 (18), or actin (20–33, Sigma) in TBS-T, followed by an incubation with the appropriate sec-

ondary antibody conjugated with horseradish peroxidase (Jackson ImmunoResearch Laboratories) for 1 h at room temperature. The membranes were washed extensively with TBS-T and protein bands were visualized by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Cell survival assays. Cell viability was determined by a modified 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) assay. In brief, KB-3-1 cells were seeded at a density of 5×10^3 cells/96-well microtiter plates with 100 μl of complete medium and allowed to attach in a 37 °C incubator. The next day, the medium was changed and cells were exposed to cisplatin at a final concentration as indicated for 48 h. For the MTT assay, 10 μl MTT solution was added to each well for 3 h at 37 °C. The absorbance readings for each well were carried out at 570 nm using the microplate reader (Model 450, Bio-Rad).

Results

Expression of p53 family genes in various human cancerous cell lines

We examined the expression levels of *p53* family genes including *p53*, *p73*, *ΔNp73*, *p63*, and *ΔNp63* in various human cell lines derived from cancers of multiple origins. Total RNA was purified from the indicated cell lines and subjected to the semi-quantitative RT-PCR analysis. The levels of *GAPDH* mRNA were comparable among these cell lines. Under our experimental conditions, the expression of *p53* was undetectable in *p53*-deficient AsPC-1 and H1299 cells, whereas *p53* was readily detectable in the remaining cell lines (Fig. 1). Relatively higher levels of *p63* expression were observed in MIAPaCa, Panc-1, A549, and MB-231 cells. The expression of *ΔNp63* was detected in the majority of pancreas carcinoma cell lines, but not in the other tumor-derived cell lines except MCF-7 cells. Additionally, *p73* was expressed at higher levels in KCP-4, MIAPaCa, Panc-1, H1299, MCF-7, and SW480 cells, whereas *ΔNp73* was undetectable in all of the cell lines that we examined. It is worth noting that, in a sharp contrast to the cisplatin-resistant human epidermoid carcinoma KCP-4 cells, *p73* was undetectable or remained extremely low level in the cisplatin-sensitive parental KB-3-1 or incomplete revertant KCP-4R cells, respectively. These results showed that the elevated level of *p73* expression is closely associated with the acquisition of cisplatin resistance in human epidermoid carcinoma KB cell lines, and also suggested that p73 could play an important role in the development of resistance to cisplatin in certain cell types. We focused subsequent studies on KB cell lines.

Cisplatin-induced Ser-15 phosphorylation of p53 is impaired in the cisplatin-resistant KCP-4 cells

As described previously [23,24], the cisplatin-resistant KCP-4 cells were isolated by culturing their parental KB-3-1 cells in the presence of cisplatin after EMS mutagenesis. The cisplatin-sensitive incomplete revertant KCP-4R cells were derived from KCP-4 cells. Consistent with the previous results [23,24], KCP-4 cells were highly resistant to cisplatin as compared with the parental KB-3-1 cells (Fig. 2A). Under our experimental conditions, KCP-4R cells exhibited about 3-fold higher resistance to cisplatin than KB-3-1 cells. To examine whether the differential cytotoxic response of these cells to cisplatin could correlate with the differential expression of *p73* in more detail, cells were exposed to cisplatin at a final concentration of 3 or 90 μM, and the expression levels of *p73*, *p53*, and their direct transcriptional target genes including *MDM2*, $p21^{WAF1}$, and *Bax* were analyzed by RT-PCR at various time points. At 3 μM of cisplatin, KB-3-1 and KCP-4R cells underwent apoptosis, whereas KCP-4 cells did not (Fig. 2A). At 90 μM cisplatin, KCP-4 cells showed an apoptotic response to cisplatin (data not shown). As shown

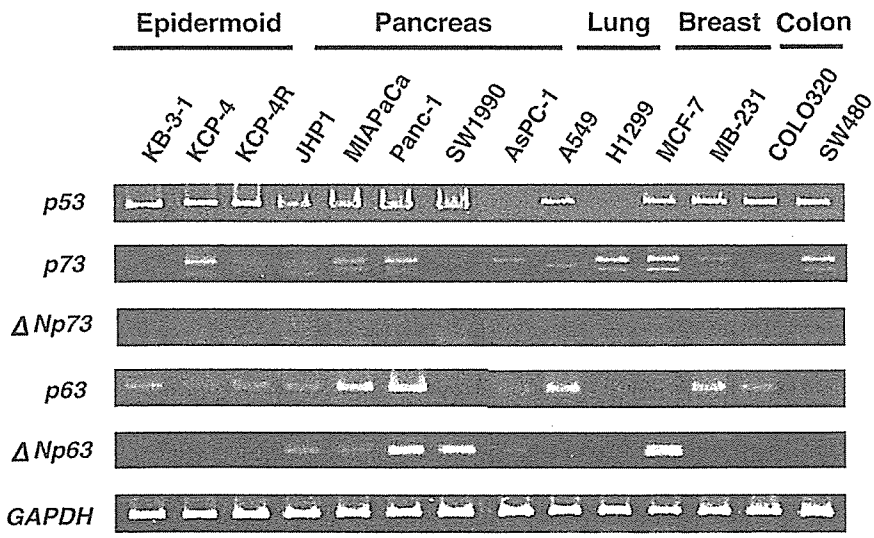

Fig. 1. The expression of *p53* family genes in various human cancerous cell lines. Total RNA prepared from the indicated cell lines was subjected to semi-quantitative reverse transcription-PCR to examine the expression levels of *p53*, *p73*, *ΔNp73*, *p63* and *ΔNp63*. The PCR-amplified products were analyzed by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. RNA samples were also amplified using *GAPDH* gene primers as an internal control.

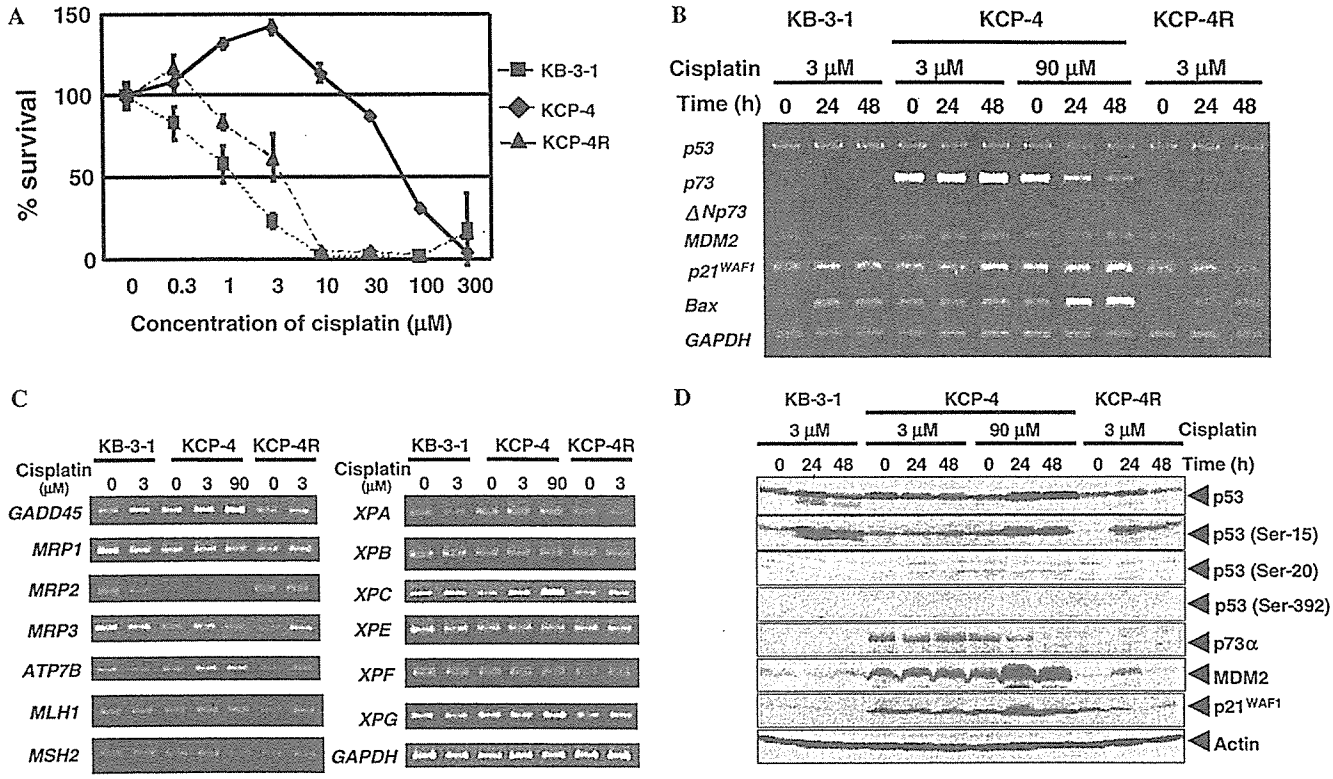

Fig. 2. *p73* expression is up-regulated in the cisplatin-resistant KCP-4 cells. (A) Concentration-response curves of KB-3-1, KCP-4, and KCP-4R cells to cisplatin. Cells were seeded at a density of 5×10^3 cells/96-well microtiter plates, incubated in the absence of cisplatin for 24 h, and subsequently exposed to various concentrations of cisplatin for 48 h. Cell viability was determined by MTT assay as described in Materials and methods. Data are presented as mean values ± the standard deviation (SD) of three independent experiments. (B) RT-PCR analysis. KB-3-1, KCP-4, and KCP-4R cells were left untreated or exposed to cisplatin at a final concentration of 3 or 90 μM. At the indicated time periods after the treatment with cisplatin, total RNA was purified and subjected to RT-PCR to examine the expression levels of *p53*, *p73*, *ΔNp73*, *MDM2*, $p21^{WAF1}$, and *Bax*. PCR with *GAPDH* primers is shown as an internal control. (C) RT-PCR analysis of the multi-drug resistance-associated genes, the copper-transporting ATPase gene, DNA-damage recognition protein genes, and DNA repair-related genes. KB-3-1, KCP-4, and KCP-4R cells were treated with the indicated concentrations of cisplatin for 48 h, and total RNA was subjected to RT-PCR. (D) Immunoblot analysis. KB-3-1, KCP-4, and KCP-4R cells were treated with cisplatin (3 or 90 μM) for 0, 24, and 48 h, followed by immunoblotting with the indicated antibodies. Anti-actin immunoblot was used as a loading control.

in Fig. 2B, the expression levels of *p53*, $p21^{WAF1}$ and *MDM2* remained almost constant in KB-3-1, KCP-4, and KCP-4R cells regardless of cisplatin treatment. On the other hand, during the cisplatin-induced apoptosis, the expression level of *Bax* was significantly increased in KB-3-1 and KCP-4 cells. Similarly, *Bax* was also induced in KCP-4R cells in response to cisplatin but to a lesser degree as compared with KB-3-1 and KCP-4 cells. Of note, *p73* was maintained at higher levels in KCP-4 cells exposed to cisplatin at 3 μM, however, *p73* was markedly down-regulated in KCP-4 cells in response to 90 μM of cisplatin. At 3 μM cisplatin, *p73* was undetectable in KB-3-1 cells, whereas the expression level of *p73* was slightly increased in KCP-4R cells in response to cisplatin. Next, we examined the expression levels of the multi-drug resistance-associated (MRP) genes (*MRP1*, *MRP2*, and *MRP3*), the copper-transporting P-type ATPase gene (*ATP7B*), DNA damage recognition protein genes (*MLH1* and *MSH2*), and the DNA repair-related genes (*GADD45*, *XPA*, *XPB*, *XPC*, *XPE*, *XPF*, and *XPG*) in response to cisplatin. As shown in Fig. 2C, there was no significant correlation between the expression levels of these genes and the cisplatin resistance.

To confirm the differential expression of p73 at protein level, whole cell lysates prepared from KB-3-1, KCP-4, and KCP-4R cells exposed to cisplatin (3 or 90 μM) for the indicated time periods were subjected to immunoblot analysis. Consistent with the RT-PCR analysis, p73α was maintained at higher levels in KCP-4 cells treated with cisplatin at 3 μM, however, its expression level was strongly reduced in KCP-4 cells exposed to cisplatin at 90 μM in a time-dependent manner (Fig. 2D). After treatment with 90 μM cisplatin, KCP-4 cells underwent apoptosis. On the other hand, p73α was undetectable at protein level in the cisplatin-sensitive KB-3-1 and KCP-4R cells. Thus, there exists a clear correlation between the degree of cisplatin sensitivity and the decrease in the expression level of p73α in KCP-4 cells. The steady-state expression level of p53 was much lower in KB-3-1 and KCP-4R cells than in KCP-4 cells. Cisplatin treatment resulted in a remarkable accumulation of p53 in KB-3-1 and KCP-4R cells. In KCP-4 cells, 3 μM cisplatin had no effect on p53, however, p53 was significantly induced in the presence of 90 μM cisplatin. The cisplatin-mediated accumulation of p53 was associated with its remarkable induction of phosphorylation at Ser-15, whereas the cisplatin-dependent

phosphorylation of p53 at Ser-20 or Ser-392 was undetectable. Intriguingly, MDM2 as well as $p21^{WAF1}$ was maintained at higher levels in KCP-4 cells as compared with KB-3-1 and KCP-4R cells, and their amounts were increased in response to 90 μM cisplatin. Since 90 μM cisplatin had no detectable effect on *MDM2* and $p21^{WAF1}$ transcripts (Fig. 2B), their up-regulation might be due to their increased protein stability. According to the expression study, it is likely that the accumulation of p53 phosphorylated at Ser-15 is required for the cisplatin-mediated apoptosis in KB cells, and the cisplatin-resistant phenotype might be caused at least in part by a defect in the induction of active p53 even in the presence of cisplatin.

Ectopic expression of p73 inhibits cisplatin-mediated induction of p53 phosphorylation at Ser-15 and increases resistance to cisplatin

As described above, we found that, in the cisplatin-resistant KCP-4 cells, the steady-state expression level of p73α correlates quite well with those of p53 as well as MDM2. Recently, it has been reported that p73α has an ability to stabilize p53 at protein level independent of its transcriptional activity [25]. To determine whether p73 could contribute to the development of resistance to cisplatin, the cisplatin-sensitive KB-3-1 cells were infected with the recombinant adenovirus for LacZ or p73α. As shown in Fig. 3A, ectopic expression of p73α was associated with a strong accumulation of p53 and MDM2, but phosphorylation at Ser-15 of p53 was undetectable. Under our experimental conditions, mRNA levels of *p53* and *MDM2* remained unchanged even in the presence of exogenous p73α (data not shown). Twenty-four hours after infection, cells were left untreated or exposed to the indicated concentrations of cisplatin for another 48 h, and their cisplatin sensitivity was examined by cell survival assay. As shown in Fig. 3B, KB-3-1 cells expressing exogenous p73α displayed an increased resistance to cisplatin, with a 1.5- to 3-fold higher IC_{50} as compared with those of cells expressing LacZ. Intriguingly, cisplatin-mediated induction of p53 phosphorylation at Ser-15 was inhibited by exogenous p73α (Fig. 3C).

To further investigate the role of p73 in the regulation of the cisplatin resistance, we have constructed an expression plasmid for small interfering RNA (siRNA) targeting p73. KCP-4 cells were transfected with the empty plasmid or with the expression plasmid for p73 siRNA. Twenty-four hours after transfection, cells were left untreated or treated with cisplatin at a final concentration of 5 μM for the indicated time periods, and then cell survival assay was performed. As shown in Fig. 4A, RT-PCR analysis demonstrated that a significant reduction of the endogenous *p73*

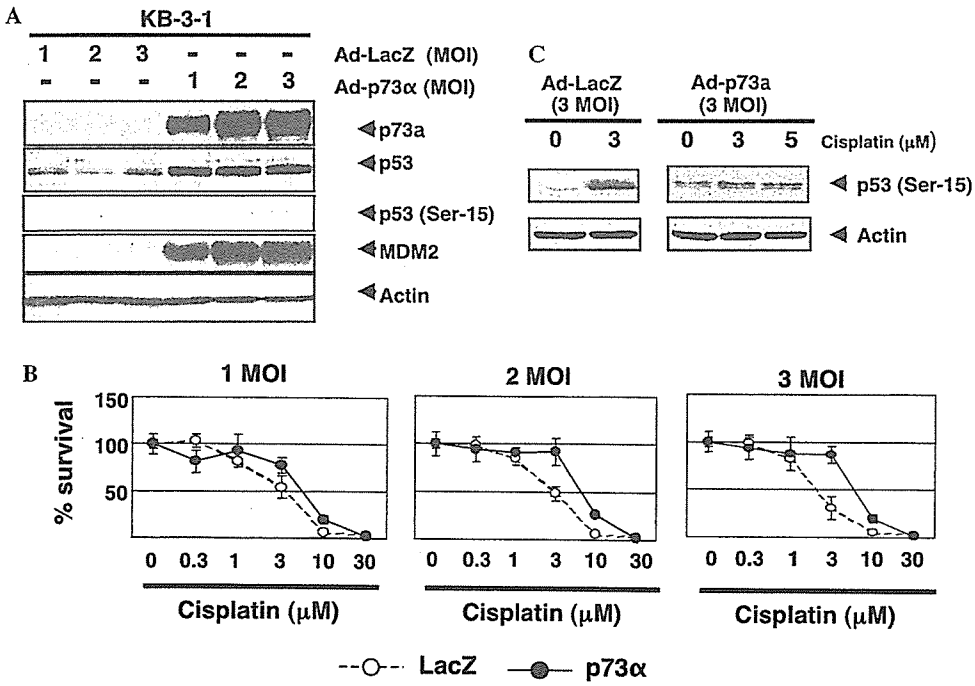

Fig. 3. Enforced expression of p73 increases resistance to cisplatin in KB-3-1 cells. (A) Immunoblot analysis. KB-3-1 cells were infected with the recombinant adenovirus encoding LacZ or p73α (MOI = 1, 2 or 3) for 72 h. Whole cell lysates were prepared from KB-3-1 cells infected with the recombinant adenovirus, and analyzed for the expression of p73α, p53, p53 phosphorylated at Ser-15 and MDM2 by immunoblotting. Anti-actin immunoblot was used as a loading control. (B) Cell survival assay. KB-3-1 cells (5×10^3 cells/well) were infected with the recombinant adenovirus for LacZ or p73α (MOI = 1, 2 or 3). Twenty-four hours after infection, cells were left untreated or treated with the indicated concentrations of cisplatin for 48 h, and then their viability was determined by MTT assay. The graphs indicate relative viability based on the percent viable cells compared to the control infection (adenovirus-LacZ). (C) Immunoblotting. KB-3-1 cells infected with the adenovirus encoding LacZ or p73α (MOI = 3) were exposed to the indicated concentrations of cisplatin for 24 h, and whole cell lysates were analyzed for the amounts of p53 phosphorylated at Ser-15. Anti-actin immunoblot was used as a loading control.

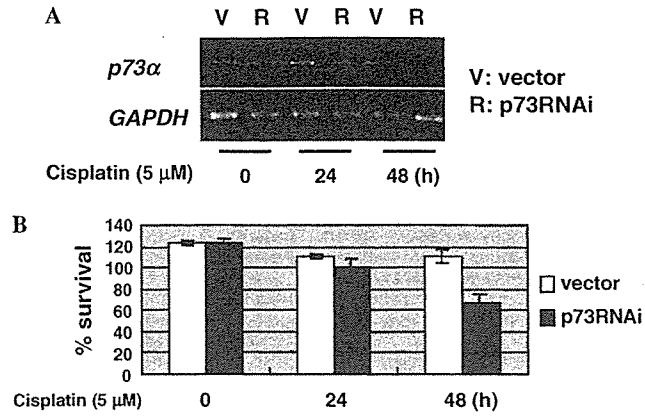

Fig. 4. p73 siRNA treatment increases cisplatin sensitivity of KCP-4 cells. (A) Down-regulation of *p73α* in KCP-4 cells transfected with p73 siRNA plasmid. The empty plasmid or p73 siRNA plasmid was introduced into KCP-4 cells. Twenty-four hours after transfection, cells were exposed to cisplatin at a final concentration of 5 μM. At the indicated time periods after the treatment with cisplatin, total RNA was purified and subjected to RT-PCR to examine the expression level of *p73α*. Amplification of *GAPDH* was used as an internal control. (B) Cell survival assay. KCP-4 cells were transfected with the empty plasmid or p73 siRNA plasmid. Twenty-four hours after transfection, cells were treated with cisplatin (5 μM) for the indicated times, and then their viability was determined by MTT assay.

mRNA was detected in cells transfected with the expression plasmid for p73 siRNA, whereas no detectable effect on the expression levels of *p73* was observed in the control cells transfected with the empty plasmid. Under our experimental conditions, the control KCP-4 cells were resistant to cisplatin at a final concentration of 5 μM (Fig. 4B). In contrast, the down-regulation of the endogenous *p73* levels in KCP-4 cells resulted in an increased cell killing by cisplatin. Thus, it is likely that p73 might contribute to the cisplatin-resistant phenotype of KB cells at least in part through the inhibition of the cisplatin-mediated induction of p53 phosphorylation at Ser-15.

Discussion

As described previously [23], cisplatin was actively effluxed from the cisplatin-resistant KCP-4 cells but not from the cisplatin-sensitive KB-3-1 cells, however, there was no clear correlation between the degree of cisplatin resistance and the decreased accumulation of cisplatin. In addition, Komatsu et al. reported that overexpression of the copper-transporting P-type ATPase (ATP7B) renders KB-3-1 cells resistant to cisplatin, whereas the expression level of endogenous ATP7B in KCP-4 cells is almost identical to that in KB-3-1 as well as the revertant KCP-4R cells [26], suggesting that there could exist another unknown molecular mechanism(s) behind the acquisition of cisplatin resistance in human epidermoid KB carcinoma cells.

Our cell-based study demonstrated that p73 and MDM2 are significantly associated with the cisplatin-resistant phenotype of KCP-4 cells. Vikhanskaya et al. described that the enforced expression of p73 results in the resistance to DNA-damaging agents through the up-regulation of a variety of DNA repair-related genes [21]. Under our experimental cell systems, however, there was no clear correlation between the expression levels of *p73* and DNA repair-related genes. MDM2 overexpression has been shown to induce the expression of multi-drug resistance (MDR) P-glycoprotein, and thereby conferring resistance to DNA-damaging agents [27]. As described previously [23,28], P-glycoprotein was undetectable in KB-3-1 as well as KCP-4 cells, suggesting that p73-mediated up-regulation of the DNA repair-related genes and MDM2-dependent induction of P-glycoprotein might not be responsible for the acquisition of cisplatin resistance of KB cells.

According to our present results, p53 was highly phosphorylated at Ser-15 in KB-3-1 and KCP-4R cells exposed to cisplatin. Of particular interest was a lack of the induction of p53 phosphorylation at Ser-15 in KCP-4 cells in response to cisplatin. Rodicker and Putzer described that a loss of p53 pro-apoptotic activity in pancreatic cancer cells might be due to the lack of p53 phosphorylation at Ser-46 [29]. At a higher concentration of cisplatin (90 μM), KCP-4 cells underwent apoptosis in association with a significant down-regulation of p73 as well as a remarkable induction of p53 phosphorylation at Ser-15. Accumulating evidence suggests that the stress-induced phosphorylation of p53 at Ser-15 causes its stabilization as well as increase in its sequence-specific DNA-binding activity [1,2]. It has been shown that cisplatin preferentially activates ATR and thereby enhancing p53 function [30,31]. Although it remains unclear which is the primary p53 Ser-15 kinase that actually phosphorylates p53 in response to cisplatin, our present results strongly suggest that p53 phosphorylation at Ser-15 is required, at least in part, for the cisplatin-mediated apoptosis in KB cells.

Adenovirus-mediated overexpression of p73α in KB-3-1 cells resulted in a remarkable accumulation of MDM2 at protein level and a decrease in cisplatin-sensitivity. Of note, cisplatin-mediated induction of p53 phosphorylation at Ser-15 was inhibited significantly by exogenous p73α in KB-3-1 cells. These results strongly indicate that p73 and/or MDM2 might be involved in an acquisition of resistance to cisplatin in KB cells. Mounting evidence suggests that MDM2 binds to the NH_2-terminal transactivation domain of p53 and thereby inhibiting p53 phosphorylation at Ser-15 [1,2]. Thus, it is likely that p73-mediated stabilization of MDM2 could block the cisplatin-induced phosphorylation of p53 at Ser-15 in KCP-4 cells. In support of this notion, Wang et al. described that MDM2 is stabilized in the presence of p73 in human lung carcinoma-derived H1299 cells [32]. Additionally, Yu et al. showed that siRNA-mediated knockdown of MDM2 increases the cisplatin sensitivity in colorectal adenocarcinoma cells [33]. In another report, overexpression of p73 attenuates the transcriptional activity of p53 by sequestrating p53 from its DNA-binding site through competitive binding with p73 [20]. Since the precise molecular mechanism(s)

behind the p73-dependent stabilization of MDM2 is unclear, further studies are needed to elucidate the functional role of p73 and MDM2 in the acquisition of cisplatin resistance.

Acknowledgments

This work was supported in part by a Grant-in-Aid from the Ministry of Health, Labor and Welfare for Third Term Comprehensive Control Research for Cancer, a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and a Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science.

References

[1] C. Prives, P.A. Hall, The p53 pathway, J. Pathol. 187 (1999) 112–126.
[2] K.H. Vousden, X. Lu, Live or let die: the cell's response to p53, Nat. Rev. Cancer 2 (2002) 594–604.
[3] Y. Haupt, R. Maya, A. Kazaz, M. Oren, Mdm2 promotes the rapid degradation of p53, Nature 387 (1997) 296–299.
[4] M.H.G. Kubbutat, S.N. Jones, K.H. Vousden, Regulation of p53 stability by Mdm2, Nature 387 (1997) 299–303.
[5] R. Honda, H. Tanaka, H. Yasuda, Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53, FEBS Lett. 420 (1997) 25–27.
[6] T.C. Hamilton, P.J. O'Dwyer, R.F. Ozols, Platinum analogues in preclinical and clinical development, Curr. Opin. Oncol. 5 (1993) 1010–1016.
[7] Z.H. Siddik, Cisplatin: mode of cytotoxic action and molecular basis of resistance, Oncogene 22 (2003) 7265–7279.
[8] S.W. Lowe, Cancer therapy and p53, Curr. Opin. Oncol. 7 (1995) 547–553.
[9] F. Bunz, P.M. Hwang, C. Torrance, T. Waldman, Y. Zhang, L. Dillehay, J. Williams, C. Lengauer, K.W. Kinzler, B. Vogelstein, Disruption of p53 in human cancer cells alters the responses to therapeutic agents, J. Clin. Investig. 104 (1999) 263–269.
[10] T. Soussi, C. Beroud, Assessing TP53 status in human tumours to evaluate clinical outcome, Nat. Rev. Cancer 1 (2001) 233–240.
[11] M. Kaghad, H. Bonnet, A. Yang, L. Creancier, J.C. Biscan, A. Valent, A. Minty, P. Chalon, J.M. Lelias, X. Dumont, P. Ferrara, F. McKeon, D. Caput, Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers, Cell 90 (1997) 809–819.
[12] S. Ikawa, A. Nakagawara, Y. Ikawa, p53 family genes: structural comparison, expression and mutation, Cell Death Differ. 6 (1999) 1154–1161.
[13] A. Yang, N. Walker, R. Bronson, M. Kaghad, M. Oosterwegel, J. Bonnin, C. Vagner, H. Bonnet, P. Dikkes, A. Sharpe, F. McKeon, D. Caput, p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours, Nature 404 (2000) 99–103.
[14] G. Melino, V. De Laurenzi, K.H. Vousden, p73: friend or foe in tumorigenesis, Nat. Rev. Cancer 2 (2002) 605–615.
[15] T. Ozaki, A. Nakagawara, p73, a sophisticated p53 family member in the cancer world, Cancer Sci. 96 (2005) 729–737.
[16] T. Stiewe, S. Zimmermann, A. Frilling, H. Esche, B.M. Putzer, Transactivation-deficient ΔTA-p73 acts as an oncogene, Cancer Res. 62 (2002) 3598–3602.
[17] C.D. Pozniak, S. Radinovic, A. Yang, F. McKeon, D.R. Kaplan, F.D. Miller, An anti-apoptotic role for the p53 family member, p73, during developmental neuron death, Science 289 (2000) 304–306.
[18] T.J. Grob, U. Novak, C. Maisse, D. Barcaroli, A.U. Luthi, F. Pimia, B. Hugli, H.U. Graber, L.V. De, M.F. Fey, G. Melino, A. Tobler, Human ΔNp73 regulates a dominant negative feedback loop for TAp73 and p53, Cell Death Differ. 8 (2001) 1213–1223.
[19] T. Nakagawa, M. Takahashi, T. Ozaki, K. Watanabe, S. Todo, H. Mizuguchi, T. Hayakawa, A. Nakagawara, Autoinhibitory regulation of p73 by ΔNp73 to modulate cell survival and death through a p73-specific target element within the *ΔNp73* promoter, Mol. Cell. Biol. 22 (2002) 2575–2585.
[20] E.R. Flores, K.Y. Tsai, D. Crowley, S. Senqupta, A. Yang, F. McKeon, T. Jacks, p63 and p73 are required for p53-dependent apoptosis in response to DNA damage, Nature 416 (2002) 560–564.
[21] F. Vikhanskaya, M. D'Incalci, M. Broggini, p73 competes with p53 and attenuates its response in a human ovarian cancer cell line, Nucleic Acids Res. 28 (2000) 513–519.
[22] F. Vikhanskaya, S. Marchini, M. Marabese, E. Galliera, M. Broggini, p73α overexpression is associated with resistance to treatment with DNA-damaging agents in a human ovarian cancer cell line, Cancer Res. 61 (2001) 35–938.
[23] R. Fujii, M. Mutoh, K. Niwa, K. Yamada, T. Aikou, M. Nakagawa, M. Kuwano, S. Akiyama, Active efflux system for cisplatin in cisplatin-resistant human KB cells, Jpn. J. Cancer Res. 85 (1994) 426–433.
[24] R. Fujii, M. Mutoh, T. Sumizawa, Z. Chen, A. Yoshimura, S. Akiyama, Adenosine triphosphate-dependent transport of leukotriene C4 by membrane vesicles prepared from cisplatin-resistant human epidermoid carcinoma cells, J. Natl. Cancer Inst. 86 (1994) 1781–1785.
[25] F. Miro-Mur, A. Meiller, H. Haddada, E. May, p73α expression induces both accumulation and activation of wt-p53 independent of the p73α transcriptional activity, Oncogene 22 (2003) 5451–5456.
[26] M. Komatsu, T. Sumizawa, M. Mutoh, Z.-C. Chen, K. Terada, T. Furukawa, X.-L. Yang, H. Gao, N. Miura, T. Sugiyama, S. Akiyama, Copper-transporting P-type adenosine triphosphatase (ATP7B) is associated with cisplatin resistance, Cancer Res. 60 (2000) 1312–1316.
[27] H.A. Cocker, S.M. Hobbs, N. Tiffin, K. Pritchard-Jones, C.R. Pinkerton, L.R. Kelland, High levels of the MDM2 oncoprotein in paediatric rhabdomyosarcoma cell lines may confer multidrug resistance, Br. J. Cancer 85 (2001) 1746–1752.
[28] M. Kool, M. De Haas, G.L. Scheffer, M.J.T. Van Eijk, J.A. Juijn, F. Baas, P. Borst, Analysis of expression of *cMOAT* (*MRP2*), *MRP3*, *MRP4*, and *MRP5*, homologues of the multidrug resistance-associated protein gene (*MRP1*), in human cancer cell lines, Cancer Res. 57 (1997) 3537–3547.
[29] F. Rodicker, B.M. Putzer, p73 is effective in p53-null pancreatic cancer cells resistant to wild-type TP53 gene replacement, Cancer Res. 63 (2003) 2737–2741.
[30] H. Zhao, H. Piwnica-Worms, ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1, Mol. Cell. Biol. 21 (2001) 4129–4139.
[31] E. Appella, C.W. Anderson, Post-translational modifications and activation of p53 by genotoxic stresses, Eur. J. Biochem. 268 (2001) 2764–2772.
[32] X.-Q. Wang, W.M. Ongkeko, A.W.S. Lau, K.M. Leung, R.Y.C. Poon, A possible role of p73 on the modulation of p53 level through MDM2, Cancer Res. 61 (2001) 1598–1603.
[33] Y. Yu, P. Sun, L.C. Sun, G.Y. Liu, G.H. Chen, L.H. Shang, H.B. Wu, J. Hu, Y. Li, Y.L. Mao, G.J. Sui, X.W. Sun, Downregulation of MDM2 expression by RNAi inhibits LoVo human colorectal adenocarcinoma cells growth and the treatment of LoVo cells with mdm2siRNA3 enhances the sensitivity to cisplatin, Biochem. Biophys. Res. Commun. 339 (2006) 71–78.

Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 351 (2006) 57–63
BBRC
www.elsevier.com/locate/ybbrc

The intracellular domain of the amyloid precursor protein (AICD) enhances the p53-mediated apoptosis

Toshinori Ozaki [a,1], Yuanyuan Li [a,1], Hironobu Kikuchi [a], Taisuke Tomita [b], Takeshi Iwatsubo [b], Akira Nakagawara [a,*]

[a] *Division of Biochemistry, Chiba Cancer Center Research Institute, Chiba 260-8717, Japan*
[b] *Department of Neuropathology and Neuroscience, Graduate School of Pharmaceutical Sciences, University of Tokyo, Tokyo 113-0033, Japan*

Received 29 September 2006
Available online 9 October 2006

Abstract

Amyloid precursor protein (APP)-derived intracellular domain (AICD) has a cytotoxic effect on neuronal cells and also participates in the regulation of gene transactivation. However, the precise molecular mechanisms behind the AICD-mediated apoptosis remain unknown. In this study, we have demonstrated that AICD interacts with p53 and enhances its transcriptional and pro-apoptotic functions. p53 was induced to be accumulated and associated with APP in response to cisplatin. Indeed, APP-C57 was co-immunoprecipitated with the endogenous p53. Enforced expression of APP-C57 or APP-C59 in U2OS cells bearing wild-type p53 led to an increase in number of apoptotic cells, whereas they had undetectable effects on p53-deficient H1299 cells, suggesting that AICD contributes to the activation of the p53-mediated apoptotic pathway. Consistent with this notion, the p53-mediated transcriptional activation and apoptosis were significantly enhanced by co-expression with APP-C57 or APP-C59. Thus, our present results strongly suggest that AICD triggers apoptosis through the p53-dependent mechanisms.

Keywords: AICD; Apoptosis; APP; Fe65; γ-Secretase; p53

Amyloid precursor protein (APP) is a type I transmembrane glycoprotein with a large extracellular domain, a single hydrophobic transmembrane region, and a short cytoplasmic tail [1]. APP is cleaved sequentially by α-, β-, and γ-secretases, which results in the generation of the large soluble NH_2-terminal ectodomain, small hydrophobic extracellular amyloid-β (Aβ, 40- and 42-residues) peptide, and APP intracellular domain (AICD, 57- and 59-residue-long COOH-terminal fragments) [2]. Among them, Aβ has been believed to be one of the major neurodegenerative agents in Alzheimer's disease (AD) [3]. Indeed, Aβ rapidly aggregates into fibrils and the extracellular fibrillar Aβ can promote apoptosis in cultured neurons [4]. Alternatively, AICD has been initially identified in brains of AD patients [5] and AICD itself induced apoptosis in human H4 neuroglioma cells [6]. However, the precise molecular mechanisms by which AICD exerts its pro-apoptotic activity remain to be determined. It has been shown that AICD is stabilized and translocated into the nucleus by collaboration with the adaptor protein Fe65 [6–8], raising a possibility that APP transduces signal through the release and translocation of AICD into nucleus. Intriguingly, Cao and Sudhof found that AICD interacts with Fe65 as well as Tip60 thereby regulating the transcription [9]. In support with this notion, Baek et al. [10] described that the nuclear AICD/Fe65/Tip60 complex can displace N-CoR co-repressor complex and activate the transcription of KAI1 gene. Telese et al. [11] demonstrated that the nucleosome assembly factor SET is required for the nuclear AICD/Fe65/Tip60 complex-mediated transactivation of KAI1 gene. Although these observations suggest that AICD participates in the transcriptional

* Corresponding author. Fax: +81 43 265 4459.
E-mail address: akiranak@chiba-cc.jp (A. Nakagawara).
[1] These authors contributed equally to this work.

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.09.162

regulation in combination with Fe65 and Tip60, the physiological target(s) that is activated by this nuclear complex remains to be identified. As described [12–14], the activation of tumor suppressor p53 might contribute to the genesis of AD and other neurodegenerative diseases of the adult central nervous system, however, the signal(s) responsible for the activation of p53 during this process is not known. Of note, mutant form of APP derived from familial AD (FAD) enhances the p53-dependent transactivation [15,16]. In addition, Legube et al. [17] found that Tip60 associates with p53 and functions as a p53 co-activator. Thus, it is likely that there could exist a functional interaction between APP and p53. In the present study, we found that AICD interacts with p53 and enhances its transcriptional and pro-apoptotic functions, suggesting that AICD-mediated activation of p53 might be one of the cytotoxic mechanisms exerted by AICD in neuronal cells.

Materials and methods

Cell culture and transfection. U2OS and H4 cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotic mixture. H1299 and SH-SY5Y cells were grown in RPMI-1640 supplemented with 10% heat-inactivated FBS and antibiotic mixture. Transfection was performed using LipofectAMINE 2000 (Invitrogen).

Immunofluorescence. Cells were fixed in 3.7% formaldehyde and permeabilized with 0.2% Triton X-100. Cells were then incubated with anti-FLAG antibody (M2, Sigma) followed by an incubation with FITC-conjugated secondary antibody (Invitrogen) and observed under Fluoview laser scanning confocal microscope (Olympus).

Immunoblotting. Lysates were subjected to SDS–PAGE and transferred onto Immobilon-P membranes (Millipore). Membranes were incubated with anti-p53 (DO-1, Oncogene Research Products), anti-$p21^{WAF1}$ (Ab-1, Oncogene Research Products), anti-phosphorylated from of p53 at Ser-15 (Cell Signaling), anti-APP, or anti-actin antibody (20–33, Sigma), and developed with an ECL system (Amersham Biosciences).

Immunoprecipitation. Precleared lysates were incubated with the indicated antibodies followed by incubation with protein G–Sepharose beads. Immune complexes were washed with lysis buffer, eluted in 2× SDS-sample buffer, and separated by SDS–PAGE. Gels were transferred onto Immobilon-P membranes, and immunoblotted.

Cell fractionation. Cells were lysed in lysis buffer containing 10 mM Tris–Cl, pH 7.5, 1 mM EDTA, 0.5% NP-40, and protease inhibitor cocktail. Lysates were centrifuged to separate soluble (cytoplasmic) from insoluble (nuclear) fraction. Nuclear and cytoplasmic fractions were analyzed by immunoblotting with anti-Lamin B (Ab-1, Oncogene Research Products), or anti-tubulin α antibody (Ab-2, NeoMarkers), respectively.

Cell viability assay. Cells were transferred to fresh medium containing cisplatin, incubated for 24 h, and 10 μl MTT solution was added to each well. After 1 h of incubation at 37 °C, absorbance readings for each well were performed at 570 nm using the microplate reader (Model 450, Bio-Rad).

Apoptosis assay. U2OS cells were transfected with GFP expression plasmid together with expression plasmid for APP-C57-FLAG or APP-C59-FLAG. Forty-eight hours after transfection, transfected cells were identified by the presence of green fluorescence. Cell nucleus was stained with DAPI.

Luciferase reporter assay. U2OS cells were transfected with p53-responsive luciferase reporter ($p21^{WAF1}$ or MDM2), pRL-TK *Renilla* luciferase cDNA, and p53 expression plasmid along with or without the increasing amounts of expression plasmid for APP-C57-FLAG or APP-C59-FLAG, and subjected to dual-luciferase assay (Promega).

Colony formation assay. H1299 cells were transfected with empty plasmid, p53 expression plasmid, or with p53 expression plasmid plus expression plasmid for APP-C57-FLAG or APP-C59-FLAG. Forty-eight hours after transfection, cells were transferred to the fresh medium containing G418 (400 μg/ml). After 14 days of selection, the plates were stained with Giemsa's solution.

Results

APP is associated with endogenous p53

To test whether there could exist an interaction between APP and p53 during the neuronal apoptosis, human neuroblastoma SH-SY5Y cells bearing wild-type p53 were exposed to cisplatin (CDDP). In accordance with the previous observations [18], SH-SY5Y cells underwent apoptosis in response to CDDP (data not shown). Next, we examined the protein levels of p53 and APP during the CDDP-mediated apoptosis. As shown in Fig. 1, p53 was induced in cells exposed to CDDP in association with an up-regulation of $p21^{WAF1}$ which is one of the p53-targets. In addition, a remarkable phosphorylation of p53 at Ser-15 was detected in response to CDDP. Protein levels of APP remained unchanged regardless of CDDP treatment. Of note, the immunoprecipitation of cell lysates with anti-APP antibody which recognizes the extreme COOH-terminal region of APP [19] resulted in a co-immunoprecipitation of p53 with APP. These observations suggest that APP and/or APP intracellular domain (AICD) might be associated with endogenous p53.

Interaction between AICD and p53

To examine whether AICD could interact with p53, we generated expression plasmids encoding APP-C57 and APP-C59 tagged with FLAG peptide on their

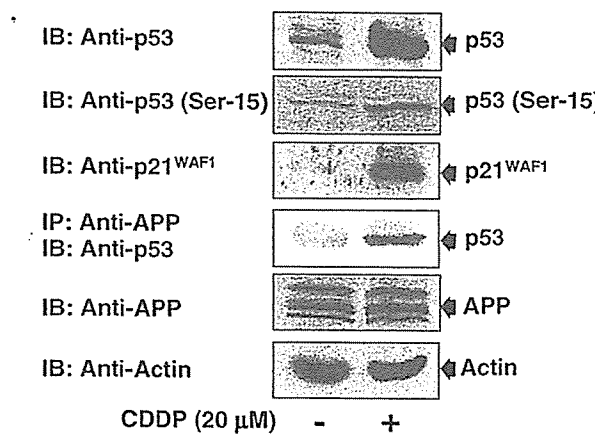

Fig. 1. Interaction between APP and endogenous p53. SH-SY5Y cells were treated with CDDP (20 μM) or left untreated. Twenty-four hours after CDDP treatment, cell lysates were subjected to immunoblotting with the indicated antibodies. Immunoblotting for actin is shown as a control for protein loading. For immunoprecipitation, equal amounts of cell lysates (1 mg of protein) were immunoprecipitated with anti-APP antibody and the immunoprecipitates were processed for immunoblotting with anti-p53 antibody.

COOH-termini (APP-C57-FLAG and APP-C59-FLAG, respectively). Human neuroglioma H4 cells were transfected with APP-C57-FLAG or APP-C59-FLAG expression plasmid and transfected cells were fixed followed by staining with anti-FLAG antibody. Consistent with the previous reports [6,8,20], APP-C57-FLAG and APP-C59-FLAG were detected both in cytoplasm and nucleus (Fig. 2A). Similar results were also obtained in immunoblotting using cytoplasmic and nuclear fractions prepared from H4 cells expressing APP-C57-FLAG or APP-C59-FLAG (Fig. 2B).

To verify the interaction between AICD and p53, cell lysates prepared from H4 cells co-transfected with expression plasmids for APP-C57-FLAG and p53 were immunoprecipitated with the normal mouse serum (NMS) or anti-p53 antibody followed by immunoblotting with anti-APP or anti-p53 antibody. As shown in Fig. 2C, APP-C57-FLAG was efficiently co-immunoprecipitated with p53. Similar to SH-SY5Y cells, H4 cells underwent apoptosis in response to CDDP in association with a significant induction of p53 (data not shown). To confirm the interaction between AICD and the endogenous p53, H4

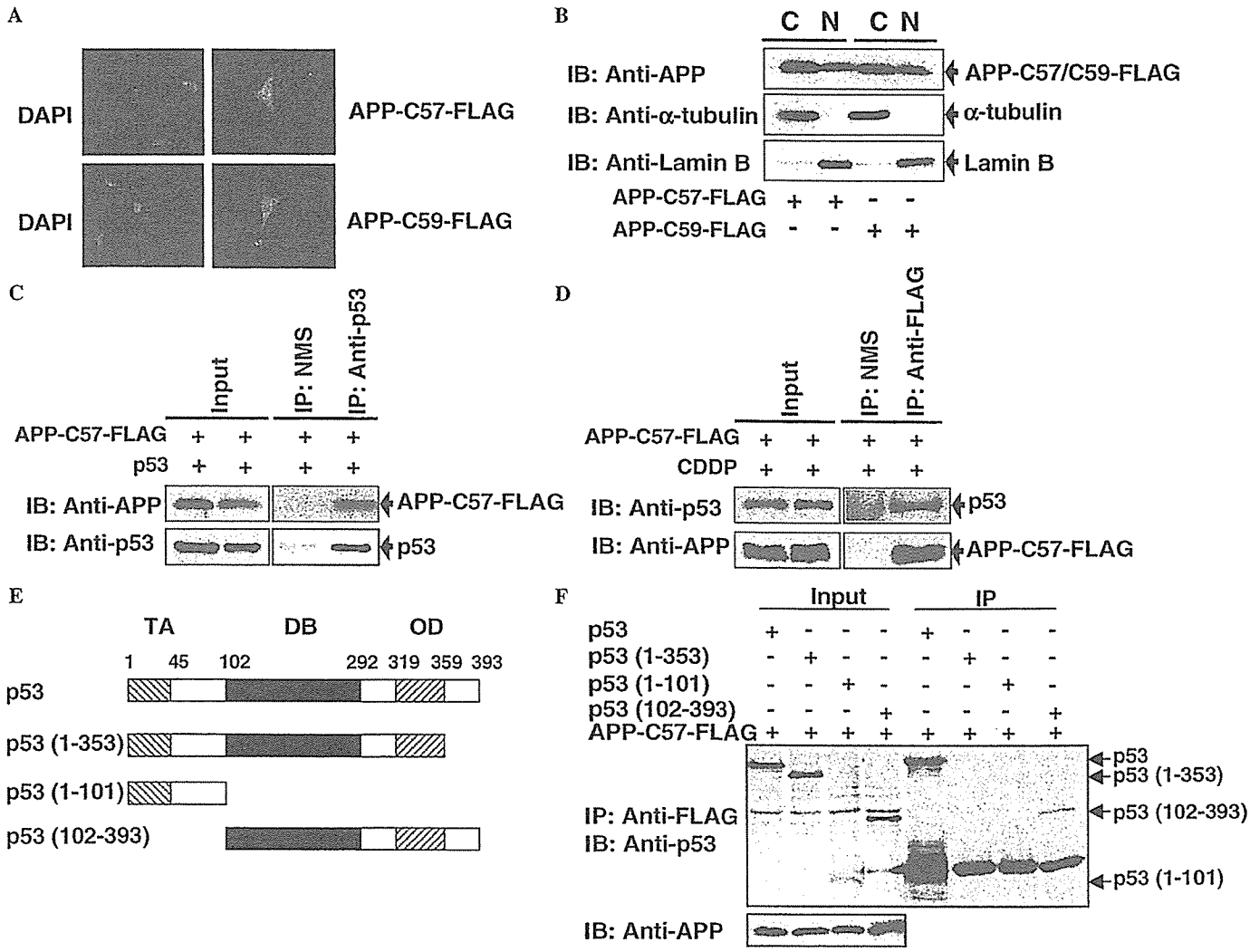

Fig. 2. Complex formation between nuclear AICD and p53 in cells. (A,B) Subcellular localization of AICD. H4 cells were transfected with the expression plasmid for APP-C57-FLAG or APP-C59-FLAG. Forty-eight hours after transfection, cells were fixed and incubated with anti-FLAG antibody. Cell nuclei were stained with DAPI (A). For subcellular fractionation, H4 cells were transfected as in (A). Transfected cells were fractionated into cytoplasmic (C) and nuclear (N) fractions. Each fraction was subjected to immunoblotting with anti-APP antibody. α-Tubulin and lamin B were used as cytoplasmic and nuclear markers, respectively. (C,D) AICD interacts with p53. H4 cells were co-transfected with expression plasmids encoding APP-C57-FLAG plus p53. Forty-eight hours after transfection, cell lysates were immunoprecipitated with normal mouse serum (NMS) or with anti-p53 antibody followed by immunoblotting with anti-APP or with anti-p53 antibody (C). For the interaction of AICD with the endogenous p53, H4 cells were transfected with the expression plasmid for APP-C57-FLAG. Twenty-four hours after transfection, cells were exposed to CDDP (20 μM) for 24 h. Cell lysates were subjected to immunoprecipitation with NMS or anti-FLAG antibody. The immunoprecipitates were analyzed by immunoblotting with anti-p53 or anti-APP antibody (D). (E,F) The COOH-terminal region of p53 is required for the interaction with AICD. H1299 cells were co-transfected with the expression plasmid for APP-C57-FLAG together with the indicated expression plasmids for p53 deletion mutants (E). Cell lysates were subjected to the immunoprecipitation with anti-APP and immunoblotted with anti-p53 antibody (F).

cells were transfected with the APP-C57-FLAG expression plasmid followed by exposure to CDDP for 24 h. As seen in Fig. 2D, the endogenous p53 was co-immunoprecipitated with APP-C57-FLAG. APP-C59-FLAG was also co-immunoprecipitated with p53 (data not shown). To identify the region(s) of p53 required for the interaction with AICD, p53-deficient H1299 cells were co-transfected with the APP-C57-FLAG expression plasmid together with the indicated expression plasmids for p53 deletion mutants (Fig. 2E). Immunoprecipitation demonstrated that APP-C57-FLAG binds to the COOH-terminal region of p53 (Fig. 2F). Thus, it is likely that nuclear AICD can interact with p53 and might modulate its function.

AICD promotes apoptosis in a p53-dependent manner

To examine whether the AICD-mediated apoptosis could be dependent on p53, H1299 cells were co-transfected with the constant amount of GFP expression plasmid together with the expression plasmid for p53, APP-C57-FLAG or APP-C59-FLAG. Forty-eight hours after transfection, transfected cells were identified by the presence of green fluorescence and the number of GFP-positive cells with apoptotic nuclei was scored. Consistent with the previous observations [21], enforced expression of p53 led to a significant induction of apoptosis (Fig. 3A). In contrast, APP-C57-FLAG and APP-C59-FLAG did not promote apoptosis. The ectopically expressed APP-C57-FLAG and APP-C59-FLAG induced apoptosis in U2OS cells (Fig. 3B). Since U2OS cells carry wild-type p53, our present results showed a good correlation between an ability of AICD to induce apoptosis and the p53 status.

AICD enhances the transcriptional and pro-apoptotic activities of p53

To address whether AICD could enhance the transcriptional activity of p53, U2OS cells were co-transfected with the constant amount of p53 expression plasmid and the luciferase reporter construct containing p53-responsive promoter derived from $p21^{WAF1}$ or MDM2 gene together with or without the increasing amounts of the expression plasmid for APP-C57-FLAG or APP-C59-FLAG. As shown in Fig. 4A, APP-C57-FLAG enhanced the p53-mediated transcriptional activity toward $p21^{WAF1}$ and MDM2 promoters in a dose-dependent manner. Similar results were also obtained in cells expressing APP-C59-FLAG (Fig. 4B).

Next, we examined a possible effect of AICD on the pro-apoptotic activity of p53. H1299 cells were transfected with the expression plasmid for p53, APP-C57-FLAG or APP-C59-FLAG. Following selection in G418, there was a drastic reduction of colony formation after transfection with p53 expression plasmid as compared with the empty plasmid, whereas APP-C57-FLAG or APP-C59-FLAG alone had undetectable effects (Fig. 4C). Intriguingly, co-expression of p53 with APP-C57-FLAG or APP-C59-FLAG reduced the colony formation as compared with p53 alone (Fig. 4D and E). Taken together, our present findings strongly suggest that AICD transduces apoptotic signals from cell surface to cell nucleus and might act as a co-activator of p53.

Discussion

AICD modulates gene transactivation in collaboration with Fe65 and Tip60 [9–11] and induces apoptosis in certain cells, which is dependent on its nuclear access [6]. Thus, it is likely that AICD has a potential role in transducing an apoptotic signal from cell surface to the nucleus, however, the detailed molecular mechanisms behind the AICD-mediated apoptotic response remain to be determined. In this study, we demonstrated that AICD interacts with p53 and enhances its transcriptional and pro-apoptotic functions. Our present findings provide a novel insight into understanding how γ-secretase cleavage of APP could lead to the neurodegeneration.

Cytosolic AICD has a short half-life and its stability is enhanced through the interaction with Fe65 [7,8]. Fe65 is an adaptor protein containing a central WW domain and two COOH-terminal phosphotyrosine-binding domains (PTB1 and PTB2) [22], and has a transactivation potential

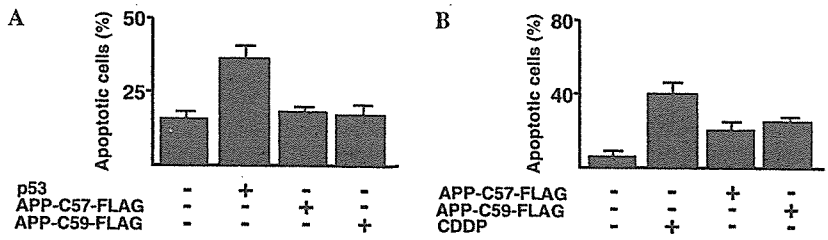

Fig. 3. AICD induces apoptosis in a p53-dependent manner. (A) AICD has undetectable effect on H1299 cells. H1299 cells were co-transfected with the constant amount of GFP expression plasmid (200 ng) together with the empty plasmid (800 ng), expression plasmid for p53 (200 ng), APP-C57-FLAG (800 ng) or APP-C59-FLAG (800 ng). Forty-eight hours after transfection, transfected cells were identified by the presence of green fluorescence. Cell nuclei were stained with DAPI to reveal nuclear condensation and fragmentation. The number of GFP-positive cells with apoptotic nuclei was scored. (B) AICD induces apoptosis in U2OS cells. U2OS cells were co-transfected with the constant amount of GFP expression plasmid (200 ng) along with the empty plasmid (800 ng), 800 ng of the expression plasmid encoding APP-C57-FLAG or APP-C59-FLAG. Twenty-four hours after transfection, cells were treated with 20 μM of CDDP for 24 h or left untreated and then the number of apoptotic cells was measured as in (A).

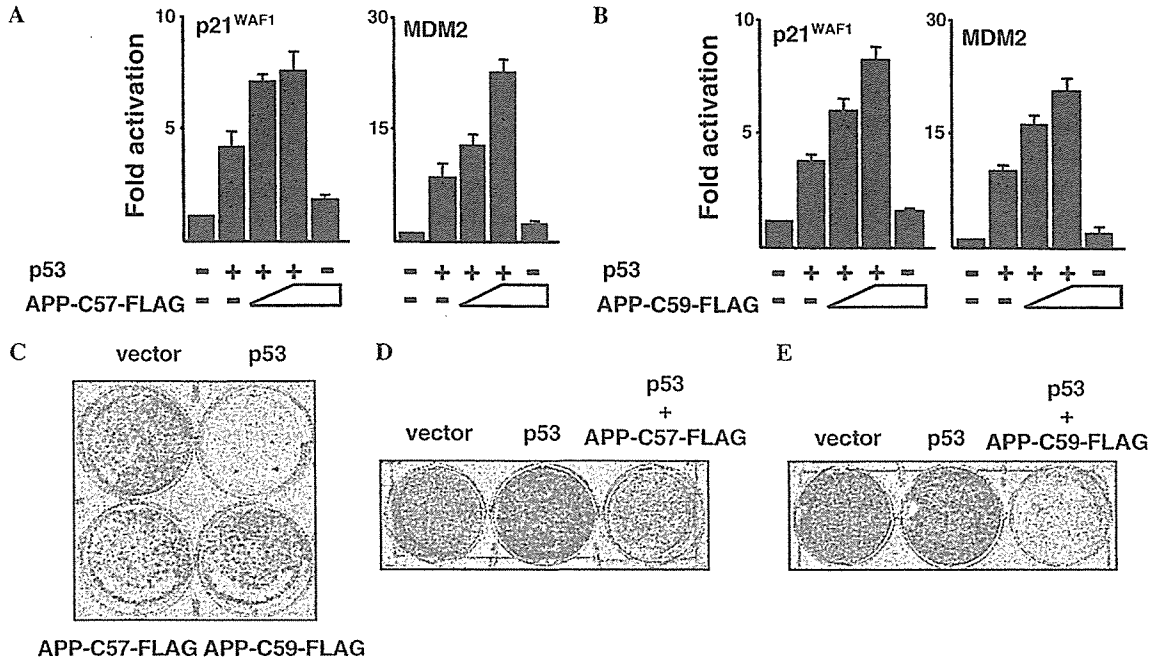

Fig. 4. AICD enhances the p53-mediated transcriptional and pro-apoptotic activities. (A,B) Luciferase reporter assay. U2OS cells were co-transfected with the constant amount of the expression plasmid for p53 (25 ng), p53-responsive luciferase reporter construct carrying the $p21^{WAF1}$ or MDM2 promoter (100 ng), *Renilla* luciferase cDNA (10 ng) together with or without the increasing amounts of the expression plasmid for APP-C57-FLAG (100 and 200 ng) (A) or APP-C59-FLAG (100 and 200 ng) (B). Forty-eight hours after transfection, cells were lysed and their luciferase activities were analyzed. Results are shown as fold-induction of the firefly luciferase activity compared with control cells. (C–E) Colony formation assay. H1299 cells were transfected with pcDNA3 (1 μg), the expression plasmid for p53 (200 ng), APP-C57-FLAG (800 ng) or APP-C59-FLAG (800 ng). Total amount of the expression plasmids was kept constant (1 μg) with pcDNA3. At 48 h post-transfection, cells were maintained in the culture medium containing G418 (400 μg/ml). After 2 weeks of selection, the plates were stained with Giemsa's solution (C). To examine the effect of APP-C57 or APP-C59 on p53, H1299 cells were transfected with the constant amount of the expression plasmid for p53 (50 ng) together with or without the expression plasmid encoding APP-C57-FLAG (400 ng) (D) or APP-C59-FLAG (400 ng) (E). Forty-eight hours after transfection, cells were kept in the medium containing G418 for 2 weeks and surviving colonies were stained as described in (C).

depending on its WW domain [23]. Tip60 with a histone acetyltransfecrase activity acts as a co-activator for AICD/Fe65 complex [9]. Tip60 alone has no transactivation function [9]. Additionally, Tip60 is part of a large nuclear protein complex, which possesses a DNA-binding activity [24]. To understand the molecular mechanisms underlying the nuclear AICD/Fe65/Tip60-mediated transcriptional regulation, it is necessary to identify nuclear protein(s) with a sequence-specific DNA-binding activity. According to our present results, AICD interacts with p53 and enhances its transcriptional activity, suggesting that p53 is one of the sequence-specific transcription factors in the nuclear AICD/Fe65/Tip60 complex. Of note, Legube et al. [17] described that Tip60 is associated with p53 and functions as a co-activator for p53. Deletion analysis revealed that AICD binds to the COOH-terminal region of p53. Since the p53 COOH-terminal region has an inhibitory effect on its DNA-binding activity, it is possible that AICD reduces its inhibitory effect.

Alternatively, Kim et al. [25] reported that AICD forms a complex with Fe65 and CP2/LSF/LBP1 family, and induces the expression of glycogen synthase kinase 3β (GSK3β). According to their results, AICD-mediated up-regulation of GSK3β led to neuronal apoptosis. Intriguingly, Watcharasit et al. [26] demonstrated that nuclear GSK3β interacts with p53 and promotes its apoptotic response. Therefore, it is possible that AICD contributes to the formation of p53/GSK3β complex, thereby enhancing the p53-mediated pro-apoptotic activity. Additionally, c-Abl binds to Fe65 and stimulates the AICD/Fe65-mediated transactivation [27]. Considering that c-Abl enhances the transcriptional activity of p53 [28], it is likely that c-Abl might be involved in the AICD-dependent activation of p53.

Recently, Esposito et al. [16] reported that the inhibition of β-secretase cleavage of FAD-linked APP mutant significantly reduces the p53-mediated transcriptional activation. They also described that the treatment of FAD-associated APP mutant-expressing cells with γ-secretase inhibitor can confer resistance to apoptotic stimuli. APP missense mutations found in FAD led to an increased production of Aβ42, which might be due to the increased cleavage of APP by γ-secretase [29]. The accumulation of Aβ42 caused the neuronal apoptosis and this process was mediated through the activation of p53/Bax cell death pathway [30], suggesting that there could exist a functional interaction between intracellular Aβ42 and proximal effector(s) of this pathway. However, the precise molecular mechanisms by which p53 is activated by intracellular Aβ42 remain to be clarified. During the preparation of our

manuscript, Alves da Costa et al. [31] described that AICD enhances the transcriptional activity of p53 through the up-regulation of p53 at mRNA level. Under our experimental conditions, AICD had negligible effects on the mRNA level of p53 as examined by RT-PCR (data not shown). It might be due to the different cell systems. Collectively, it is likely that the intracellular Aβ and/or nuclear AICD might induce neuronal apoptosis at least in part through the activation of the p53-dependent pro-apoptotic pathway.

Acknowledgments

This work was supported in part by a Grant-in-Aid from the Ministry of Health, Labour and Welfare for Third Term Comprehensive Control Research for Cancer, a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and a Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science.

References

[1] J. Kang, H.G. Lemaire, A. Unterbeck, J.M. Salbaum, C.L. Masters, K.H. Grzeschik, G. Multhaup, K. Beyreuther, B. Muller-Hill, The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor, Nature 325 (1987) 733–736.

[2] S.F. Lichtenthaler, C. Haass, Amyloid at the cutting edge: activation of alpha-secretase prevents amyloidogenesis in an Alzheimer disease mouse model, J. Clin. Invest. 113 (2004) 1384–1387.

[3] D.J. Selkoe, Alzheimer's disease: genes, proteins, and therapy, Physiol. Rev. 81 (2001) 741–766.

[4] C.J. Pike, D. Burdick, A.J. Walencewicz, C.G. Glabe, C.W. Cotman, Neurodegeneration induced by beta-amyloid peptides in vitro: the role of peptide assembly state, J. Neurosci. 13 (1993) 1676–1687.

[5] B. Passer, L. Pellegrini, C. Russo, R.M. Siegel, M.J. Lenardo, G. Schettini, M. Bachmann, M. Tabaton, L. D'Adamio, Generation of an apoptotic intracellular peptide by gamma-secretase cleavage of Alzheimer's amyloid beta protein precursor, J. Alzheimers Dis. 2 (2000) 289–301.

[6] A. Kinoshita, C.M. Whelan, O. Berezovska, B.T. Hyman, Neurodegeneration induced by beta-amyloid peptides in vitro: the role of peptide assembly state, J. Biol. Chem. 277 (2002) 28530–28536.

[7] P. Cupers, I. Orlans, K. Craessaerts, W. Annaert, B. De Strooper, The amyloid precursor protein (APP)-cytoplasmic fragment generated by gamma-secretase is rapidly degraded but distributes partially in a nuclear fraction of neurones in culture, J. Neurochem. 78 (2001) 1168–1178.

[8] W.T. Kimberly, J.B. Zheng, S.Y. Guenette, D.J. Selkoe, The intracellular domain of the beta-amyloid precursor protein is stabilized by Fe65 and translocates to the nucleus in a notch-like manner, J. Biol. Chem. 276 (2001) 40288–40292.

[9] X. Cao, T.C. Sudhof, A transcriptionally correction of trascriptively active complex of APP with Fe65 and histone acetyltransferase Tip60, Science 293 (2001) 115–120.

[10] S.H. Baek, K.A. Ohgi, D.W. Rose, E.H. Koo, C.K. Glass, M.G. Rosenfeld, Exchange of N-CoR corepressor and Tip60 coactivator complexes links gene expression by NF-kappaB and beta-amyloid precursor protein, Cell 110 (2002) 5–67.

[11] F. Telese, P. Bruni, A. Donizetti, D. Gianni, C. D'Ambrosio, A. Scaloni, N. Zambrano, M.G. Rosenfield, T. Russo, Transcription regulation by the adaptor protein Fe65 and the nucleosome assembly factor SET, EMBO Rep. 6 (2005) 77–82.

[12] R.S. Slack, D.J. Belliveau, M. Rosenberg, J. Atwal, H. Lochmuller, R. Aloyz, A. Haghighi, B. Lach, P. Seth, E. Cooper, F.D. Miller, Adenovirus-mediated gene transfer of the tumor suppressor, p53, induces apoptosis in postmitotic neurons, J. Cell Biol. 135 (1996) 1085–1096.

[13] H. Xiang, D.W. Hochman, H. Saya, T. Fujiwara, P.A. Schwartzkroin, R.S. Morrison, Evidence for p53-mediated modulation of neuronal viability, J. Neurosci. 16 (1996) 6753–6765.

[14] P.E. Hughes, T. Alexi, M. Dragunow, A role for the tumour suppressor gene p53 in regulating neuronal apoptosis, Neuroreport 8 (1997) v–xii.

[15] X. Xu, D. Yang, T. Wyss-Coray, J. Yan, L. Gan, Y. Sun, L. Mucke, Wild-type but not Alzheimer-mutant amyloid precursor protein confers resistance against p53-mediated apoptosis, Proc. Natl. Acad. Sci. USA 96 (1999) 7547–7552.

[16] L. Esposito, L. Gan, G.Q. Yu, C. Essrich, L. Mucke, Intracellularly generated amyloid-beta peptide counteracts the antiapoptotic function of its precursor protein and primes proapoptotic pathways for activation by other insults in neuroblastoma cells, J. Neurochem. 91 (2004) 1260–1274.

[17] G. Legube, L.K. Linares, S. Tyteca, C. Caron, M. Scheffner, M. Chevillard-Briet, D. Trouche, Role of the histone acetyl transferase Tip60 in the p53 pathway, J. Biol. Chem. 279 (2004) 44825–44833.

[18] T. Nakagawa, M. Takahashi, T. Ozaki, K. Watanabe, S. Todo, H. Mizuguchi, T. Hayakawa, A. Nakagawara, Autoinhibitory regulation of p73 by Delta Np73 to modulate cell survival and death through a p73-specific target element within the Delta Np73 promoter, Mol. Cell. Biol. 22 (2002) 2575–2585.

[19] K. Takio, M. Hasegawa, K. Titani, Y. Ihara, Identification of beta protein precursor in newborn rat brain, Biophys. Biochem. Res. Commun. 160 (1989) 1296–1301.

[20] Y. Gao, S.W. Pimplinkar, The gamma -secretase-cleaved C-terminal fragment of amyloid precursor protein mediates signaling to the nucleus, Proc. Natl. Acad. Sci. USA 98 (2001) 14979–14984.

[21] C.J. Di Como, C. Gaiddon, C. Prives, p73 function is inhibited by tumor-derived p53 mutants in mammalian cells, Mol. Cell. Biol. 19 (1999) 1438–1449.

[22] J.P. Borg, J. Ooi, E. Levy, B. Margolis, The phosphotyrosine interaction domains of X11 and FE65 bind to distinct sites on the YENPTY motif of amyloid precursor protein, Mol. Cell. Biol. 16 (1996) 6229–6241.

[23] X. Cao, T.C. Sudhof, Dissection of amyloid-beta precursor protein-dependent transcriptional transactivation, J. Biol. Chem. 279 (2004) 24601–24611.

[24] T. Ikura, V.V. Ogryzko, M. Grigoriev, R. Groisman, J. Wang, M. Horikoshi, R. Scully, J. Qin, Y. Nakatani, Involvement of the TIP60 histone acetylase complex in DNA repair and apoptosis, Cell 102 (2002) 463–473.

[25] H.S. Kim, E.M. Kim, J.P. Lee, C.H. Park, S. Kim, J.H. Seo, K.A. Chang, E. Yu, S.J. Jeong, Y.H. Chong, Y.H. Suh, C-terminal fragments of amyloid precursor protein exert neurotoxicity by inducing glycogen synthase kinase-3 beta expression, FASEB J. 17 (2003) 1951–1953.

[26] P. Watcharasit, G.N. Bijur, J.W. Zmijewski, L. Song, A. Zmijewska, X. Chen, G.V.W. Johnson, R.S. Jope, Glycogen synthase kinase-3beta (GSK3beta) binds to and promotes the actions of p53, Proc. Natl. Acad. Sci. USA 99 (2002) 7951–7955.

[27] M.S. Perkinton, C.L. Standen, K.F. Lau, S. Kesavapany, H.L. Byers, M. Ward, D.M. McLoughlin, C.C.J. Miller, The c-Abl tyrosine kinase phosphorylates the Fe65 adaptor protein to stimulate Fe65/amyloid precursor protein nuclear signaling, J. Biol. Chem. 279 (2004) 22084–22091.

[28] A. Goga, X. Liu, T.M. Hambuch, K. Senechal, E. Major, A.J. Berk, O.N. Witte, C.L. Sawyers, p53 dependent growth suppression by the c-Abl nuclear tyrosine kinase, Oncogene 11 (1995) 791–799.
[29] D.J. Selkoe, Amyloid beta-protein and the genetics of Alzheimer's disease, J. Biol. Chem. 271 (1996) 18295–18298.
[30] Y. Zhang, R. McLaughlin, C. Goodyer, A. LeVlanc, Selective cytotoxicity of intracellular amyloid beta peptide1–42 through p53 and Bax in cultured primary human neurons, J. Cell Biol. 156 (2002) 519–529.
[31] C. Alves da Costa, C. Sunyach, R. Parddossi-Piquard, J. Sevalle, B. Vincent, N. Boyer, T. Kawarai, N. Girardot, P. St George-Hyslop, F. Checler, Presenilin-dependent γ-secretase-mediated control of p53-associated cell death in Alzheimer's disease, J. Neurosci. 26 (2002) 6377–6385.

Oncogene (2006) 25, 7608–7617

www.nature.com/onc

ORIGINAL ARTICLE

NF-κB regulates the stability and activity of p73 by inducing its proteolytic degradation through a ubiquitin-dependent proteasome pathway

H Kikuchi[1,2,3], T Ozaki[1,3], K Furuya[1], T Hanamoto[1,2], M Nakanishi[1], H Yamamoto[1], K Yoshida[1], S Todo[2] and A Nakagawara[1]

[1]*Division of Biochemistry, Chiba Cancer Center Research Institute, Chiba, Japan and* [2]*Department of General Surgery, Hokkaido University School of Medicine, Kita-ku, Sapporo, Japan*

Nuclear factor kappa B (NF-κB), which exists as heterodimeric complexes composed of p50 and p65, has been shown to play an important role in cell survival processes. In the present study, we found for the first time that NF-κB has an ability to induce the ubiquitin-dependent proteasomal degradation of proapoptotic p73α. The activation of NF-κB in tumor necrosis factor α (TNF-α)-stimulated H1299 cells resulted in a significant reduction in the amounts of the endogenous p73α. Consistent with these results, TNF-α-mediated down-regulation of p73α was observed in wild-type (WT) mouse embryonic fibroblasts (MEFs) but not in p65-deficient MEFs. Ectopic expression of NF-κB decreased a half-life of p73α by increasing its ubiquitination levels, and thereby inhibiting the transcriptional activity as well as proapoptotic function of p73α, whereas NF-κB had undetectable effects on p53. Immunoprecipitation experiments demonstrated that, under our experimental conditions, NF-κB does not bind to p73α in mammalian cultured cells. In contrast to WT p65, the COOH-terminal deletion mutant of p65 (p65ΔC) failed to reduce the expression levels of p73α, suggesting that NF-κB-mediated proteolytic degradation of p73α requires the transcriptional activity of NF-κB. Taken together, our present results imply that NF-κB-mediated degradation of proapoptotic p73 is a novel inhibitory mechanism of p73 that regulates cell survival and death.

Oncogene (2006) **25**, 7608–7617. doi:10.1038/sj.onc.1209748; published online 4 September 2006

Keywords: apoptosis; NF-κB; p53; p73; ubiquitination

Introduction

p73 and p63 share a significant amino-acid sequence similarity to p53, and exist as multiple isoforms arising from alternative splicing termed the TA variants (Kaghad *et al.*, 1997; Yang *et al.*, 1998), or from alternative promoter usage termed the ΔN variants (Yang and McKeon, 2000; Melino *et al.*, 2002; Stiewe and Putzer, 2002). Recently, it has been shown that p53 is also expressed as multiple variants (Bourdon *et al.*, 2005). These TA variants function in a manner similar to p53 by inducing G1 cell cycle arrest or apoptosis in certain cancerous cells through transactivating an overlapping set of p53/p73-responsive downstream effectors such as $p21^{WAF1}$ and BAX (Jost *et al.*, 1997; Kaghad *et al.*, 1997; Gressner *et al.*, 2005). Among these TA variants of p73, p73α has an extended COOH-terminal region including SAM (sterile α motif) domain and the extreme COOH-terminus. In marked contrast to p53, p73 is rarely mutated in human tumors despite an extensive search (Ikawa *et al.*, 1999). The initial genetic studies demonstrated that p73-deficient mice exhibit neurological defects, but do not develop any spontaneous tumors (Yang *et al.*, 2000), suggesting that p73 does not function as a classical tumor suppressor. Intriguingly, Flores *et al.* (2002) found that the indirect contribution of p73 or p63 is required for the p53-dependent apoptosis. Thus, it is likely that p73 cooperates with p53 to induce apoptosis and/or exerts its proapoptotic activity in a p53-independent manner.

ΔNp73, which lacks the NH_2-terminal transactivation domain, has an oncogenic potential, and acts in a dominant-negative fashion toward wild-type (WT) p53 as well as p73 (Pozniak *et al.*, 2000; Yang *et al.*, 2000; Stiewe *et al.*, 2002). Pozniak *et al.* (2000) demonstrated that ΔNp73 is predominantly expressed in sympathetic neurons, and inhibits p53-dependent neuronal apoptosis. Of note, we and others found that p73 directly binds to the canonical p53/p73-responsive element within the ΔNp73 promoter region, and strongly upregulates the expression of its own negative regulator ΔNp73, indicating that there exists the negative feedback regulation of p73 by its transcriptional target ΔNp73 (Grob *et al.*, 2001; Nakagawa *et al.*, 2002; Zaika *et al.*, 2002).

p73 is kept at extremely low level in mammalian cells, keeping p73 in an inactive state. Previous studies revealed that p73 is induced to be accumulated in response to a wide variety of DNA-damaging agents (Irwin *et al.*, 2003). For example, cisplatin or ionizing radiation triggers phosphorylation of p73 at Tyr-99

Correspondence: Dr A Nakagawara, Division of Biochemistry, Chiba Cancer Center Research Institute, 666-2 Nitona, Chuoh-ku, Chiba 260-8717, Japan.
E-mail: akiranak@chiba-cc.jp
[3]These authors contributed equally to this work.
Received 7 February 2006; revised 28 April 2006; accepted 10 May 2006; published online 4 September 2006

mediated by nonreceptor tyrosine kinase c-Abl, increasing its stability at protein level and proapoptotic activity (Agami *et al.*, 1999; Gong *et al.*, 1999). Alternatively, it has been shown that the prolyl isomerase Pin1 as well as the transcriptional coactivator Yes-associated protein YAP enhances p73 acetylation mediated by p300 in response to DNA damage, and thereby increasing p73 stability (Mantovani *et al.*, 2004; Strano *et al.*, 2005). These observations suggest that the stress-induced chemical modifications of p73 play a critical role in regulating p73 stability and activity. Accumulating evidence strongly suggests that p73 protein level is regulated in a ubiquitin-mediated proteasomal degradation pathway (Balint *et al.*, 1999; Lee and La Thangue, 1999; Bernassola *et al.*, 2004). Unlike p53, MDM2 binds to the NH_2-terminal transactivation domain of p73 and inhibits its transcriptional activity but does not target p73 for ubiquitin-mediated degradation (Balint *et al.*, 1999; Zeng *et al.*, 1999), implying that the protein stability of p73 is regulated through a pathway distinct from that of p53.

The activation of nuclear factor κB (NF-κB) has been shown to play an important role in the control of cell survival processes, which protects cells from a wide variety of apoptotic stresses (Beg and Baltimore, 1996; Van Antwerp *et al.*, 1996; Wang *et al.*, 1996, 1998). For example, camptothecin-mediated activation of NF-κB provides an antiapoptotic function (Huang *et al.*, 2000), and inhibition of NF-κB results in radiosensitization (Yamagishi *et al.*, 1997). Tumor necrosis factor alpha (TNF-α) activates the NF-κB-mediated cellular protective mechanism against the proapoptotic effect of TNF-α through the induction of the NF-κB-target genes that are involved in the inhibition of apoptosis (Beg and Baltimore, 1996; Van Antwerp *et al.*, 1996; Wang *et al.*, 1996). In addition to the antiapoptotic effect of NF-κB, NF-κB also contributes to cellular transformation and oncogenesis. Consistent with this notion, constitutive high levels of NF-κB activity were detectable in various human tumors (Bayon *et al.*, 2003). Intriguingly, Wan and DeGregori (2003) reported that NF-κB promotes T-cell survival in response to antigenic stimulation through the downregulation of p73; however, the precise molecular mechanism behind the NF-κB-mediated reduction of p73 is less well understood.

In the present study, we have found for the first time that NF-κB activation promotes the ubiquitin-dependent proteasomal turnover of p73, and the transcriptional activity of NF-κB is required for the NF-κB-mediated degradation of p73. Our present findings provide an evidence that NF-κB-mediated degradation of p73 might be a novel inhibitory mechanism of p73 function.

Results

Ectopic expression of NF-κB decreases p73α level

We first asked whether NF-κB could affect the expression level of proapoptotic p73. To this end, p53-deficient H1299 cells were co-transfected with the constant amount of the expression plasmid for FLAG-p73α together with or without the increasing amounts of the HA-p65 plus HA-p50 expression plasmids. At 48 h after transfection, whole-cell lysates were prepared, and immunoblot analysis revealed that the expression level of FLAG-p73α is significantly reduced in cells co-expressing HA-p65 and HA-p50 in a dose-dependent manner (Figure 1a). Under our experimental conditions, *p73α* mRNA level remained unchanged even in the presence of the exogenous HA-p65 and HA-p50. Similar results were also obtained in COS7 cells (Figure 1b). Next, we examined the effect of ectopically expressed NF-κB on the endogenous p73. COS7 cells were co-transfected with or without the increasing amounts of the expression plasmids encoding HA-p65 plus HA-p50. At 48 h after transfection, whole-cell lysates were immunoprecipitated with the normal mouse serum (NMS) or with the anti-p73 antibody, followed by immunoblotting with the anti-p73 antibody. As shown in Figure 1c and d, the endogenous p73α was significantly decreased at protein level in the presence of the ectopically expressed HA-p65 and HA-p50.

NF-κB specifically downregulates p73α

We tested whether NF-κB could reduce the expression levels of the other p53 family members including p53 and p63. Figure 2a shows the domain structures of p53, p73α, p73β and p63α. Whole-cell lysates prepared from H1299 cells transiently co-transfected with the indicated combinations of the expression plasmids were analysed for the expression levels of FLAG-p53, FLAG-p73β and p63α. In a sharp contrast to p73α, the enforced expression of NF-κB had marginal effects on the levels of FLAG-p53, FLAG-p73β and p63α (Figure 2b–d). Under our experimental conditions, NF-κB had undetectable effect on ΔNp73 (data not shown). These results strongly suggest that NF-κB-mediated downregulation is highly specific to p73α.

TNF-α-mediated activation of NF-κB results in a downregulation of the endogenous p73α

We sought to examine whether the activation of the endogenous NF-κB could affect the expression level of the endogenous p73. H1299 cells stably transfected with the NF-κB luciferase reporter plasmid (Muta and Takeshige, 2001) were treated with TNF-α as indicated, and their luciferase activity was determined. As shown in Figure 3a–c, TNF-α treatment enhanced the NF-κB-dependent transcriptional activation, and p65 as well as p50 translocated into cell nucleus in response to TNF-α as examined by immunoblotting and indirect immunofluorescence staining. As expected, immunoprecipitation experiments demonstrated that the expression level of the endogenous p73α protein is significantly reduced in response to TNF-α, whereas *p73α* mRNA level remained unchanged in cells exposed to TNF-α (Figure 3d). TNF-α treatment had no significant effects on the viability of cells (data not shown). These results suggest that the TNF-α-mediated activation of NF-κB leads to a

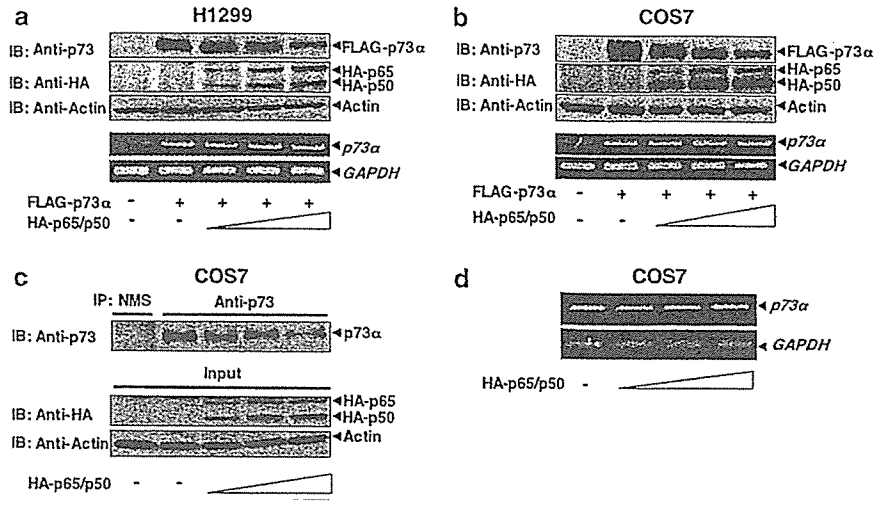

Figure 1 NF-κB decreases the amount of p73α. (a and b) Downregulation of p73α by NF-κB. H1299 (a) or COS7 (b) cells were co-transfected with the constant amount of the expression plasmid for FLAG-p73α (0.5 μg) together with or without the increasing amounts of the HA-p65 and HA-p50 expression plasmids (0.5, 1.0 and 1.5 μg). At 48 h after transfection, whole-cell lysates or total RNA were subjected to immunoblotting or RT–PCR analysis, respectively. Expression of FLAG-p73α, HA-p65 and HA-p50 was verified by the indicated antibodies. Actin was used to confirm that an equivalent amount of protein was loaded into each lane. For RT–PCR, ethidium bromide staining of *GAPDH* confirmed equivalent loading. (c and d) Downregulation of the endogenous p73α by the enforced expression of NF-κB. COS7 cells were co-transfected with or without the increasing amounts of HA-p65 and p50 expression plasmids. At 48 h after transfection, whole-cell lysates or total RNA were processed for the immunoprecipitation with the NMS or with the anti-p73 antibody, or subjected to RT–PCR, respectively.

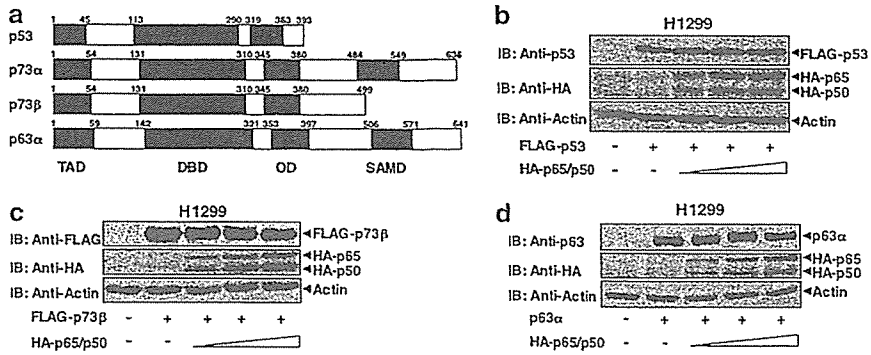

Figure 2 NF-κB-dependent downregulation is specific to p73α. (**a**) Domain structure of p53 family members. The transactivation domain (TAD), DNA-binding domain (DBD), oligomerization domain (OD), and SAM domain are indicated. (**b–d**) NF-κB does not affect the expression levels of p53, p73β and p63α. H1299 cells were co-transfected with the constant amount of the expression plasmid for FLAG-p53 (**b**), FLAG-p73β (**c**) or p63α (**d**) along with or without the increasing amounts of the expression plasmids for HA-p65 and HA-p50. At 48 h after transfection, whole-cell lysates were analysed by immunoblotting with the indicated antibodies. The *lower panels* show actin to demonstrate equal loading of the gels.

reduction in the amounts of proapoptotic p73α at protein level. In contrast to TNF-α, cisplatin treatment had negligible effects on the transcriptional activity of NF-κB, and immunoprecipitation experiments revealed that the endogenous p73α is induced to be accumulated in cells exposed to cisplatin (Supplementary Figure S1).

To confirm our hypothesis that the downregulation of p73α is dependent on NF-κB, we further investigated whether the expression level of the endogenous p73α could be altered in the absence of heterodimeric complex of NF-κB. To this end, we used mouse embryonic fibroblasts (MEFs) derived from p65 knockout mice (Figure 4a) (Beg *et al.*, 1995). Whole-cell lysates were prepared from p65 knockout and WT MEFs exposed to TNF-α (20 ng/ml) or left untreated. Immunoprecipitation experiments demonstrated that TNF-α-mediated reduction of the endogenous p73α is observed in WT MEFs but not in p65 knockout MEFs (Figure 4b and c), indicating that the presence of functional NF-κB complex is required for the downregulation of p73α.

NF-κB stimulates the ubiquitin-dependent proteasomal degradation of p73α

To determine whether NF-kB could modulate p73α turnover, we sought to examine the half-life of p73α in the presence or absence of the exogenously expressed NF-κB using cycloheximide blockade. COS7 cells were

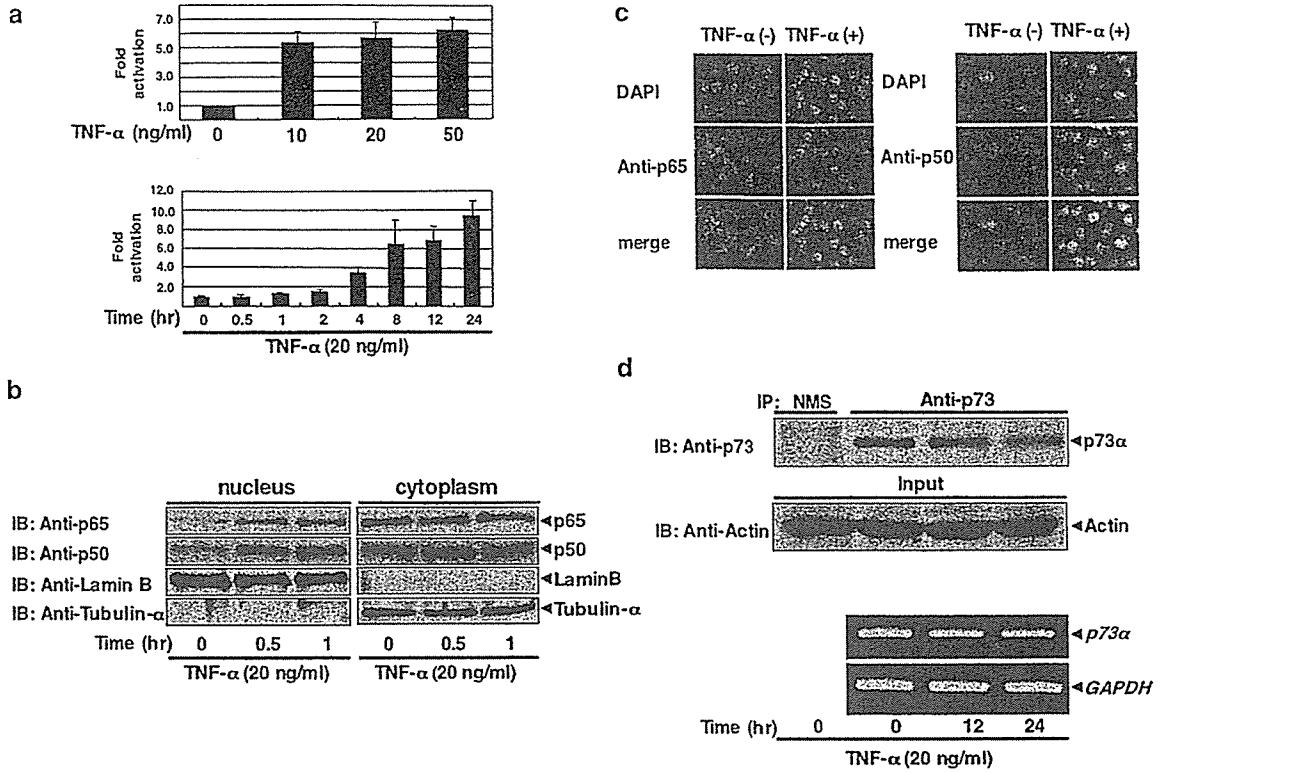

Figure 3 TNF-α treatment causes a downregulation of the endogenous p73α. (a) TNF-α treatment enhances the transcriptional activity of NF-κB. H1299 cells stably transfected with the luciferase reporter plasmid carrying 1xκB site and *Renilla* luciferase construct were exposed to the indicated concentrations of TNF-α for 24 h (upper panel) or treated with 20 ng/ml of TNF-α for the indicated time periods (lower panel). At the indicated time periods after the treatment with TNF-α, luciferase assays were performed. (**b** and **c**) Nuclear translocation of p65 and p50 in response to TNF-α. H1299 cells exposed to TNF-α (20 ng/ml) for the indicated time periods were separated into nuclear and cytoplasmic fractions. The aliquots of these fractions were analysed by immunoblotting with the anti-p65 (1st panel) or with the anti-p50 (2nd panel) antibody. These fractions were also analysed for nucleus-specific Lamin B (3rd panel) and cytoplasm-specific α-tubulin.(4th panel) to show the validity of our fractionation technique (**b**). H1299 cells were treated with TNF-α (20 ng/ml) for 1 h or left untreated. Cells were fixed and then incubated with the anti-p65 (left panels) or with the anti-p50 (right panels) antibody. Cell nuclei were stained by DAPI. Cellular localization was detected by fluorescence microscopy. The merged images indicate the nuclear translocation of p65 and p50 in response to TNF-α (**c**). (**d**) TNF-α treatment decreases the expression level of the endogenous p73α. H1299 cells were treated with TNF-α (20 ng/ml) for the indicated time periods or left untreated. Whole-cell lysates or total RNA were subjected to immunoprecipitation with the indicated antibodies or RT–PCR, respectively.

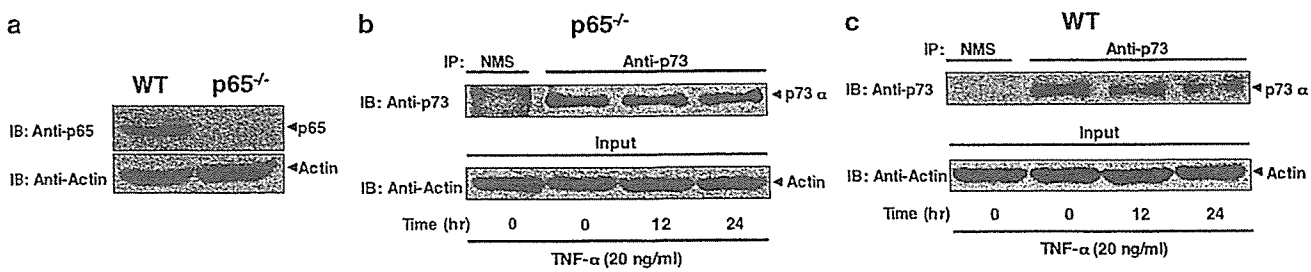

Figure 4 NF-κB-dependent reduction of p73 in response to TNF-α. (**a**) Expression of the endogenous p65 in MEFs. Whole-cell lysates from WT and p65 knockout MEFs were analysed for p65 by immunoblotting. (**b** and **c**) TNF-α-dependent downregulation of the endogenous p73α is observed in WT but not in p65 knockout MEFs. p65 knockout (**b**) and WT (**c**) MEFs were treated with TNF-α (20 ng/ml) for indicated time periods or left untreated. Whole-cell lysates were subjected to immunoprecipitation with NMS or with the anti-p73 antibody, followed by immunoblotting with the anti-p73 antibody.

co-transfected with the constant amount of the expression plasmid for FLAG-p73α along with or without the constant amount of the expression plasmids encoding HA-p65 and HA-p50. At 24 h after transfection, cells were exposed to cycloheximide (100 μg/ml). At the indicated time points, whole-cell lysates were subjected to immunoblotting with the anti-p73 antibody. Consistent with the previous observations (Lee and La Thangue, 1999), transiently expressed FLAG-p73α had a half-life of about 3 h (Figure 5a). When FLAG-p73α was co-expressed with HA-p65 and HA-p50, the degradation rate of FLAG-p73α was faster than that

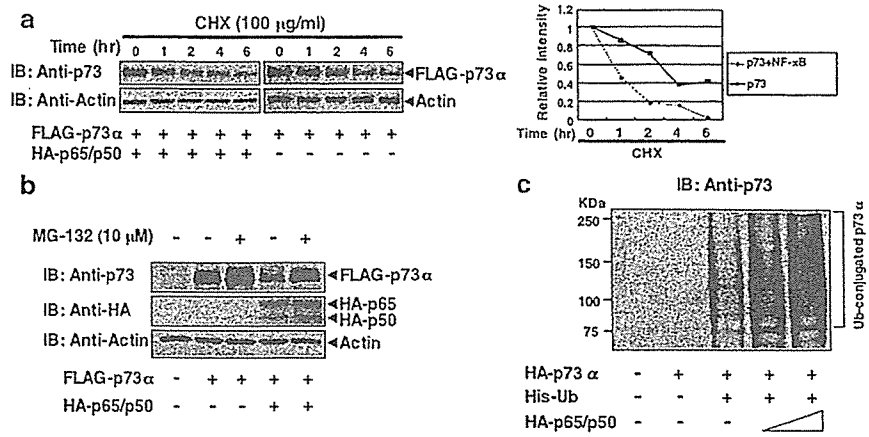

Figure 5 NF-κB-induced reduction of p73 is regulated by a ubiquitin–proteasome pathway. (a) NF-κB decreases a half-life of p73α. COS7 cells were co-transfected with the constant amount of the FLAG-p73α expression plasmid together with or without the expression plasmids for HA-p65 and HA-p50. At 24 h after transfection, cells were exposed to cycloheximide (100 μg/ml). At the indicated time points after the addition of cycloheximide, whole-cell lysates were analysed for FLAG-p73α by immunoblotting. Actin was used for equal protein loading. Densitometry was used to quantify the amounts of FLAG-p73α, which normalized to actin. (b) NF-κB-mediated degradation of p73α is blocked by proteasomal inhibitor. COS7 cells were transfected either with FLAG-p73α alone or with FLAG-p73α, HA-p65 and HA-p50. At 40 h after transfection, cells were treated with or without MG-132 (10 μM) for 6 h. Whole-cell lysates were subjected to immunoblotting with the anti-p73, anti-HA or with anti-actin antibody. (c) NF-κB increases the ubiquitination levels of p73. COS7 cells were co-transfected with the constant amount of the expression plasmids for HA-p73α and His-Ub together with or without the increasing amounts of the HA-p65 and HA-p50 expression plasmids. At 40 h post-transfection, cells were treated with MG-132 (10 μM) for 6 h. Whole-cell lysates were then prepared, and ubiquitinated materials were recovered by Ni^{2+}-NTA-agarose beads, followed by immunoblotting with the anti-p73 antibody.

in cells expressing FLAG-p73α alone (a half-life of about 1 h). In addition, NF-κB had undetectable effects on the half-life of ΔNp73 (data not shown).

According to the previous reports (Balint *et al*., 1999; Lee and La Thangue, 1999; Bernassola *et al*., 2004), p73 protein level is regulated through the ubiquitin-mediated proteasomal degradation pathway. We then determined the effects of proteasomal inhibitor MG-132 on the expression level of p73α. As expected, proteasome inhibition resulted in a stabilization of FLAG-p73α (Figure 5b). Of note, the addition of MG-132 blocked the NF-κB-mediated degradation of FLAG-p73α, indicating that NF-κB-mediated degradation of p73α is regulated at least in part through the proteasomal pathway. To ask whether NF-κB could affect the ubiquitination levels of p73α, COS7 cells were co-transfected with the constant amount of the HA-p73α expression plasmid and the expression plasmid for His-tagged ubiquitin together with or without the increasing amounts of the expression plasmids for HA-p65 and HA-p50. After the treatment with MG-132 for 6 h, whole-cell lysates were prepared, and the ubiquitinated materials were recovered with Ni^{2+}-agarose beads followed by immunoblotting with the anti-p73 antibody. As shown in Figure 5c, ubiquitin-conjugated p73α was significantly increased upon the co-expression of HA-p73α with HA-p65 and HA-p50. Thus, it is likely that NF-κB mediates the ubiquitin-dependent proteasomal turnover of p73α.

NF-κB inhibits the transcriptional activity of p73α but not of p53

To evaluate whether NF-κB could influence the transcriptional activity of p73α, H1299 cells were co-transfected with the constant amount of the expression plasmid for FLAG-p73α, and the luciferase reporter construct controlled by the p53/p73-responsive element from the *BAX* or *MDM2* promoter together with or without the increasing amounts of the expression plasmids for HA-p65 and HA-p50. At 48 h after transfection, the luciferase activities were measured. As shown in Figure 6a, co-expression of FLAG-p73α with HA-p65 and HA-p50 significantly reduced the p73α-mediated transcriptional activation of *BAX* and *MDM2* promoters in a dose-dependent manner, and HA-p65 and HA-p50 had negligible effects on the reporter gene activities. Consistent with these results, RT–PCR analysis revealed that enforced expression of HA-p65 and HA-p50 inhibits the p73α-mediated transactivation of the endogenous *BAX*. In contrast, p53-mediated transcriptional activation was not affected in cells co-transfected with the HA-p65 and HA-p50 expression plasmids (Figure 6b).

NF-κB inhibits the proapoptotic activity of p73α

To further confirm the inhibitory effects of NF-κB on the p73 function, we examined the possible effects of NF-κB on the proapoptotic activity of p73α. H1299 cells were co-transfected with the indicated combinations of the expression plasmids. At 48 h after the transfection, cells were fixed, stained with propidium iodide, and the numbers of cells with sub-G1 DNA content were measured. In accordance with the previous report (Jost *et al*., 1997), expression of FLAG-p73α resulted in an increase in number of cells with sub-G1 DNA content (Figure 6c). Co-expression of FLAG-p73α with HA-p65 and HA-p50 decreased number of cells with sub-G1 DNA content in a dose-dependent manner. Additionally,