

p19^{ras} Accelerates p73 β -mediated Apoptosis through a Caspase-3 Dependent Pathway

Sang-Min Jang[†], Jung-Woong Kim[†], and Kyung-Hee Choi*

Department of Life Science (BK21 program), College of Natural Sciences, Chung-Ang University, Seoul 156-756, Korea

Abstract: p19^{ras} is an alternative splicing variant of the proto-oncogene c-H-ras pre-mRNA of p21^{ras}. In contrast to p21^{ras}, p19^{ras} does not have a C-terminal CAAX motif that targets the plasma membrane and is localized to both the cytoplasm and nucleus. We found that p19^{ras} activated the transcriptional activity of p73 β through protein-protein interactions in the nucleus. p73 is known to play an important role in cellular damage responses such as apoptosis. Although p73 is a structural and functional homologue of p53, p73-mediated apoptosis has not yet been clearly elucidated. In this study, we demonstrate that the interaction between p19^{ras} and p73 β accelerated p73 β -induced apoptosis through a caspase-3 dependent pathway. Treatment with DEVD-CHO, a caspase inhibitor, also strengthened p73 β -mediated apoptosis through a caspase-3 dependent pathway. Furthermore, the enhanced transcriptional activity of endogenous p73 β by treatment with Taxol was amplified by p19^{ras} overexpression, which markedly increased caspase-3 dependent apoptosis in the p53-null SAOS2 cancer cell line. Our findings indicate a functional linkage between p19^{ras} and p73 in caspase-3 mediated apoptosis of cancer cells.

Key words: p19^{ras}, p73 β , caspase-3, apoptosis

INTRODUCTION

p73, a member of the p53 gene family, has at least four splicing variants expressed in various tissue-specific manners, including p73 α , p73 β , p73 γ , and p73 δ , as well as

the transcriptionally active TAp73 and the amino-terminally truncated and inactive DNp73 (Ozaki et al., 2005; Ueda et al., 1999; Zwahlen et al., 2000). p73b lacks a carboxy-terminal tail and most of the sterile a motif (SAM) domain, which is a protein-protein interaction domain found in p73-related and other non-p73 proteins. (Liu et al., 2005) p73 β transactivates a variety of p53/p73 target genes and induces apoptotic cell death in certain cancerous cells more effectively than full-length p73 α (Lee et al., 1999; Zhu et al., 1998). p73 also has p53-like functions such as inducing tumor cell apoptosis and participating in cell cycle checkpoint control through the transactivation of an overlapping set of p53- and p73-target genes. (Jost et al., 1997; Kaelin, 1999; Melino et al., 2003; Moll et al., 2004; Kim et al., 2007; Kim et al., 2008) Recent studies show that p73 offers an alternative cancer therapeutic target in p53 down-regulated cancer cells (Peirce et al., 2009; Vilgelm et al., 2008). Because p73 is also activated by therapeutic agents that target p53, increasing the transcriptional activity of p73 through protein interactions could enhance apoptosis in many tumors.

We previously reported that p19^{ras} is a novel p73 β binding protein that activates the transcriptional activity of p73 β by blocking MDM2-mediated p73 β inhibition (Jeong et al., 2006). p19^{ras} is an alternative splicing variant of the proto-oncogene c-H-ras pre-mRNA in conjunction with p21^{ras} (Cohen et al., 1989). In contrast to p21^{ras}, p19^{ras} does not have a C-terminal CAAX motif that targets the cellular plasma membrane and is localized in both the cytoplasm and nucleus (Guil et al., 2003; Reuther et al., 2000; Takai et al., 2001). In addition, p19^{ras} has little GTP binding affinity, as it lacks two important GTP-binding sites located in the C-terminus end of p21^{ras}. Furthermore, we found that the p19^{ras}-p73 β interaction enables p73 β to transcriptionally activate the target gene, Bax. Thus, the p19^{ras}-p73 β interaction amplifies p73 β -induced apoptosis, including Bax translocation, cytochrome *c* release, and loss of

Abbreviations: SAM, sterile a motif; MDM2, mouse double minute 2; PBS, phosphate-buffered saline; PI, propidium iodide; SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulphonyl fluoride; ROS, reactive oxygen species

[†]These authors contributed equally to this work.

*To whom correspondence should be addressed.
Tel: +82-2-820-5209; Fax: +82-2-824-7302;
E-mail: khchoi@cau.ac.kr

mitochondrial transmembrane potential ($\Delta\Psi_m$) (Kim et al., 2008.) These differences between p19^{ras} and 21^{ras} suggest a novel pathway of Ras signaling that occurs in both the nucleus and cytoplasm, involving transcriptional regulation, cell cycle control, and apoptotic responses. However, little is known about the precise physiological consequences of the interaction between p19^{ras} and p73 β .

In this study, we examined the biological and physiological functions of the interaction between p19^{ras} and p73 β , which increased induction of apoptotic cell death through a caspase-3 pathway. Furthermore, Taxol-enhanced endogenous p73 β was amplified by p19^{ras}, markedly increasing cellular apoptosis through caspase-3 activation in the p53-null SAOS2 cancer cell line.

MATERIALS AND METHODS

Cell culture and transfection

HEK 293 and SAOS2 cells were obtained from the American type culture collection (ATCC: Manassas, VA) and maintained in DMEM or RPMI1640 supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and penicillin-streptomycin (50 U/mL). Transient transfection was performed by Lipofectamine 2000 (Invitrogen) with different plasmid DNA, according to the manufacturer's instructions.

Flow cytometry and DNA fragmentation

After cells were trypsinized, approximately 1×10^6 cells were collected by centrifugation at 3,000 rpm for 5 min. Cells were then washed in phosphate-buffered saline (PBS), followed by resuspension and fixation in 70% ethanol for approximately 2 hr. Cellular DNA was then stained by the addition of 10 mg of propidium iodide (PI) and apoptotic cells were visualized by Annexin V staining. Cells were analyzed using a FACScan flow cytometer with Cellquest software (Becton Dickinson, Franklin Lakes, NJ).

Enzymatic assay of caspase-3

Cleavage of the caspase-3 substrate I (*N*-acetyl-DEVD-*p*-nitroaniline) (Calbiochem, San Diego, CA) was used as a measure of caspase-3 activity. *p*-nitroaniline was used as the standard. Cleavage of the substrate was monitored at 405 nm, and the specific activity was expressed in picomoles of the product (nitroaniline) per minute per milligram of protein.

Western blotting

HEK 293 and SAOS2 cells were seeded in 100 mm plates at an initial density of 2×10^6 cells and allowed to grow for 12 hr. The cells were treated with 50 nM Taxol for 48 hr, and lysed in a buffer containing 1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.1% SDS, 1% Nonidet P-

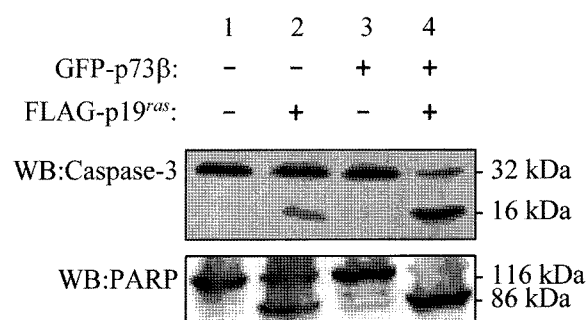


Fig. 1. p19^{ras} amplified p73 β -triggered caspase-3 activation and PARP cleavage. The cleavages of caspase-3 induced by p19^{ras} interaction with p73 β were measured by Western blot analysis using specific antibodies against caspase-3 and PARP. Proteolytic cleavages of poly(ADP-ribose) polymerase (PARP) were also analyzed as apoptotic controls.

40, and 1 mM PMSF. Boiled samples were analyzed by Western blotting using the appropriate antibodies to detect protein expression. The FLAG, GFP, and p19^{ras} proteins were detected by their respective primary antibodies (anti-FLAG: purchased from Santa-Cruz, anti-p19^{ras} polyclonal antibodies were prepared in-house from rabbits). The polyclonal anti-b-actin, p73, and caspase-3 antibodies were purchased from Santa Cruz Biotechnology.

Statistical analysis

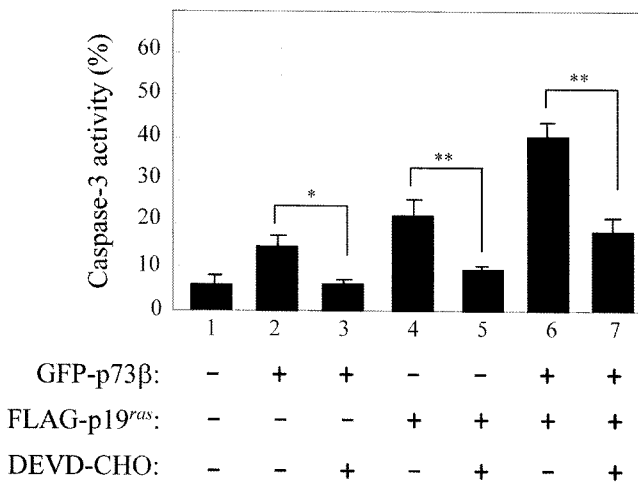
One-factor analysis of variance was used to compare values obtained in three or more groups, followed by Turkey's post hoc test. $P < 0.05$ was considered to imply statistical significance.

RESULTS AND DISCUSSION

p19^{ras} interaction with p73 β induced the production of executive caspase-3 and cleaved poly(ADP-ribose) polymerases (PARP)

Previously, we found that p19^{ras} directly interacts with p73 β in the nucleus, activating p73 β -dependent transcriptional activity (Jeong et al., 2006). In addition, p19^{ras} enhances p73 β -induced apoptotic responses, including Bax mitochondrial translocation, cytochrome *c* release, increased production of reactive oxygen species (ROS), and loss of mitochondrial transmembrane potential ($\Delta\Psi_m$) through the protein-protein interaction of p19^{ras} and p73 β (Kim et al., 2008) Once the mitochondrial outer membrane has been permeabilized by Bax translocation, cytochrome *c* diffuses from the intermembrane space into the cytosol, where it promotes caspase activation (Liu et al., 1996; Rodriguez et al., 1999; Zou et al., 1999). To investigate whether p19^{ras} interaction with p73 β would effectively induce apoptosis through a caspase-3 dependent pathway, HEK293 cells were co-transfected with p19^{ras} and GFP-p73 β expression plasmids. Compared to cells transfected with p73 β or p19^{ras}

A.



GFP-p73β:	-	+	+	-	-	+	+
FLAG-p19 ^{ras} :	-	-	-	+	+	+	+
DEVD-CHO:	-	-	+	-	+	-	+

B.

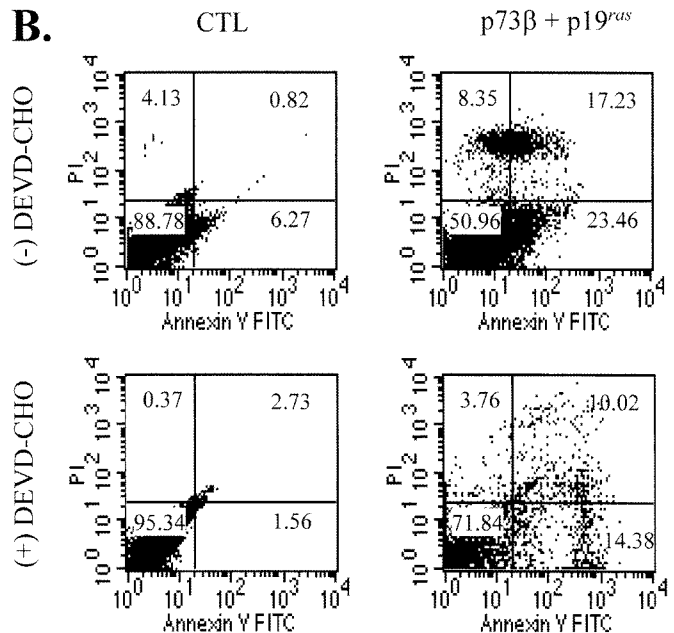


Fig. 2. p19^{ras} potentiates p73β-induced apoptosis through a caspase-3 dependent pathway. (A) Effects of caspase-3 activation on apoptosis induced by p19^{ras} interaction with p73β were determined in the presence or absence of the caspase-3 inhibitor, z-DEVD-CHO. All values are expressed as mean±SD. Statistically significant differences in caspase-3 activities were determined by Turkey's post hoc test, indicated by asterisks (**P*<0.05; ***P*<0.01). *P*<0.05 was considered to imply statistical significance. (B) Effects of z-DEVD-CHO on apoptosis induced by p19^{ras} interaction with p73β were examined by Annexin V and PI staining in HEK293 cells.

alone, co-transfected cells displayed a significant increase in executive caspase-3 (16 kDa) expression (Fig. 1, upper panel; lane 4). Western blot analysis also revealed that transfection of the cells with p19^{ras} or p73β alone only slightly increased the cleavage of PARP, a known caspase substrate. Co-transfection with both p19^{ras} and p73β induced much greater PARP cleavage (Fig. 1, lower panel; lane 4). These results demonstrate that p19^{ras} amplified the p73β-triggered caspase-3 dependent pathway and PARP activation, thereby activating apoptotic cell death.

p19^{ras} accelerates p73β-induced apoptosis through a caspase-3 dependent pathway

To investigate whether p19^{ras} enhances p73β-induced apoptosis through a caspase-3 dependent pathway, HEK293 cells were pretreated with the caspase-3 inhibitor DEVD-CHO for 12 hr. As shown in Fig. 2, DEVD-CHO treatment significantly reduced p73β-mediated caspase-3 activation (Fig. 2A; lane3). The caspase-3 activity amplified by co-transfection with the p19^{ras} and p73β plasmids (Fig. 2A; lane 6) was also markedly inhibited by DEVD-CHO pretreatment (Fig. 2A; lane 7). To further investigate whether p19^{ras} interaction with p73β effectively induces apoptotic cell death through a caspase-3 dependent pathway, SAOS2 cells were pretreated with DEVD-CHO for 12 hr before co-transfection with the p19^{ras} and p73β expression plasmids (Fig. 2B). Co-expression with p19^{ras} and p73β resulted in a prominent increase of apoptotic

patterns of 17.23% (Fig. 2B; upper right). DEVD-CHO treatment significantly reduced p73β-mediated apoptosis (by 10.02%) (Fig. 2B; lower right). Taken together, these results indicate that p19^{ras} interaction with p73β potentiates a synergistic activation of p73β-induced apoptosis in a caspase-3 dependent manner in SAOS2 cells.

In our previous report, we demonstrated that p19^{ras} interacts with p73β and activates its transcriptional activity. We also found that p19^{ras} interaction with p73β amplifies p73β-induced apoptosis, including Bax translocation, cytochrome *c* release, and increased production of ROS. In this study, we further showed that p19^{ras} effectively enhanced p73β-mediated apoptotic cell death through a caspase-3 pathway in HEK293 and SAOS2 osteosarcoma cells. These results suggest fundamental differences in the function and regulation of p19^{ras} and p21^{ras}.

p19^{ras} amplified p73β-mediated cellular apoptotic responses induced by Taxol treatment in a caspase-3 dependent manner

p73β expression can be induced by the microtubule dynamic inhibitor Taxol, which upregulates both p73 mRNA and the stability of p73 (Lin et al., 2004; Oh et al., 2008). In our previous report, we found that endogenous p73β and p19^{ras} proteins are also noticeably induced by Taxol treatment in a time-dependent manner.(Kim et al., 2008) To investigate whether p19^{ras} amplifies p73β-mediated apoptosis through a caspase-3 dependent pathway

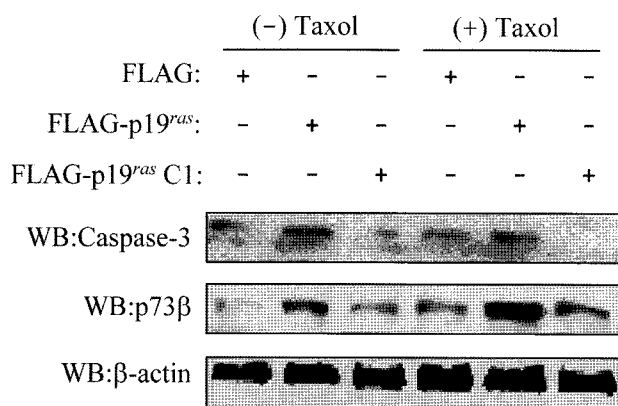


Fig. 3. p19^{ras} enhances Taxol-induced apoptosis through a caspase-3 pathway in osteosarcoma SAOS2 cells. SAOS2 cells were transiently transfected with either p19^{ras}- or p19^{ras}C1-expressing, or corresponding control plasmids and then treated with 50 nM Taxol for 24 hr. Whole cell lysates were measured by Western blot analysis using indicated antibodies. β-Actin proteins were used as loading controls after stripped and reprobed.

in vivo, we treated p19^{ras} or p19^{ras}C1-transfected cells with Taxol, and then detected cellular caspase-3 levels and endogenous p73β expression by Western blotting (Fig. 3). p19^{ras} increased pro-apoptotic Bax expression, cytochrome *c* release, ROS production (our previous data), and caspase-3 activation (Fig. 3). These changes were not detected in p19^{ras}C1-transfected cells either treated or untreated with Taxol. Taken altogether, these results demonstrate that the endogenous expression of p19^{ras} and p73β is significantly amplified by Taxol treatment. Taxol-enhanced endogenous p73β transcriptional activities are amplified by p19^{ras}, leading to a marked increase in cellular apoptosis through a caspase-3 pathway in the p53-null SAOS2 cancer cell line.

Despite more than three decades of intensive study, the function of Ras in cell physiology has yet to be fully understood. We observed both biological and physiological functions of the interaction between p19^{ras} and p73β, which triggers p73β-mediated apoptosis through a caspase-3 pathway. These results support the potential benefit of low-dose Taxol treatment with gene therapy using p19^{ras}. Further investigation is needed to support this possibility and elucidate the role of p19^{ras} in cancer cells.

ACKNOWLEDGMENTS

This research was supported by a Chung-Ang University Research Scholarship Grant in 2009.

REFERENCES

Cohen JB, Broz SD, and Levinson AD (1989) Expression of the H-ras proto-oncogene is controlled by alternative splicing. *Cell* 58: 461-472.

Guil S, Gattoni R, Carrascal M, Abian J, Stevenin J, and Bach-

Elias M (2003) Roles of hnRNP A1, SR proteins, and p68 helicase in c-H-ras alternative splicing regulation. *Mol Cell Biol* 23: 2927-2941.

Jeong MH, Bae J, Kim WH, Yoo SM, Kim JW, Song PI, and Choi KH (2006) p19^{ras} interacts with and activates p73 by involving the MDM2 protein. *J Biol Chem* 281: 8707-8715.

Jost CA, Marin MC, and Kaelin WG, Jr. (1997) p73 is a simian [correction of human] p53-related protein that can induce apoptosis. *Nature* 389: 191-194.

Kaelin WG, Jr. (1999) The emerging p53 gene family. *J Natl Cancer Inst* 91: 594-598.

Kim JW, Kim WH, Jeong MH, Jang SM, Song KH, Park SI, Song PI, Kang KH, and Choi KH (2008) p19(ras) amplifies p73beta-induced apoptosis through mitochondrial pathway. *Biochem Biophys Res Commun* 373: 146-150.

Kim JW, Lee SY, Jeong MH, Jang SM, Song KH, Kim CH, Kim YJ, and Choi KH (2008) Interaction of microtubule-associated protein 1B light chain (MAP1B-LC1) and p53 represses transcriptional activity of p53. *Animal Cells and Systems* 12: 69-75.

Kim WH, Kim JW, Jang SM, Song KH, Ham SW, and Choi KH (2007) Naphthoquinone analog-induced G1 arrest is mediated by cdc25A inhibition and p53-independent p21 induction in human hepatocarcinoma cells. *Integrative Biosciences* 11: 9-15

Lee CW and La Thangue NB (1999) Promoter specificity and stability control of the p53-related protein p73. *Oncogene* 18: 4171-4181.

Lin KW, Nam SY, Toh WH, Dulloo I, and Sabapathy K (2004) Multiple stress signals induce p73beta accumulation. *Neoplasia* 6: 546-557.

Liu G and Chen X (2005) The C-terminal sterile alpha motif and the extreme C terminus regulate the transcriptional activity of the alpha isoform of p73. *J Biol Chem* 280: 20111-20119.

Liu X, Kim CN, Yang J, Jemmerson R, and Wang X (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome *c*. *Cell* 86: 147-157.

Melino G, Lu X, Gasco M, Crook T, and Knight RA (2003) Functional regulation of p73 and p63: development and cancer. *Trends Biochem Sci* 28: 663-670.

Moll UM and Slade N (2004) p63 and p73: roles in development and tumor formation. *Mol Cancer Res* 2: 371-386.

Oh YK, Lee HJ, Jeong MH, Rhee M, Mo JW, Song EH, Lim JY, Choi KH, Jo I, Park SI, Gao B, Kwon Y, and Kim WH (2008) Role of activating transcription factor 3 on TAp73 stability and apoptosis in paclitaxel-treated cervical cancer cells. *Mol Cancer Res* 6: 1232-1249.

Ozaki T and Nakagawara A (2005) p73, a sophisticated p53 family member in the cancer world. *Cancer Sci* 96: 729-737.

Peirce SK and Findley HW (2009) The MDM2 antagonist nutlin-3 sensitizes p53-null neuroblastoma cells to doxorubicin via E2F1 and TAp73. *Int J Oncol* 34: 1395-1402.

Reuther GW and Der CJ (2000) The Ras branch of small GTPases: Ras family members don't fall far from the tree. *Curr Opin Cell Biol* 12: 157-165.

Rodriguez J and Lazebnik Y (1999) Caspase-9 and APAF-1 form an active holoenzyme. *Genes Dev* 13: 3179-3184.

Takai Y, Sasaki T, and Matozaki T (2001) Small GTP-binding proteins. *Physiol Rev* 81: 153-208.

- Ueda Y, Hijikata M, Takagi S, Chiba T, and Shimotohno K (1999) New p73 variants with altered C-terminal structures have varied transcriptional activities. *Oncogene* 18: 4993-4998.
- Vilgelm A, El-Rifai W, and Zaika A (2008) Therapeutic prospects for p73 and p63: rising from the shadow of p53. *Drug Resist Updat* 11: 152-163.
- Zhu J, Jiang J, Zhou W, and Chen X (1998) The potential tumor suppressor p73 differentially regulates cellular p53 target genes. *Cancer Res* 58: 5061-5065.
- Zou H, Li Y, Liu X, and Wang X (1999) An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. *J Biol Chem* 274: 11549-11556.
- Zwahlen D, Tschan MP, Grob TJ, Peters UR, Fink D, Haenggi W, Altermatt HJ, Cajot JF, Tobler A, Fey MF, and Aebi S (2000) Differential expression of p73 splice variants and protein in benign and malignant ovarian tumours. *Int J Cancer* 88: 66-70.

[Received October 27, 2009; accepted December 17, 2009]