Differential Effect of ik3-1/Cables on p53- and p73-induced Cell Death*

Received for publication, September 5, 2001, and in revised form, November 2, 2001
Published, JBC Papers in Press, November 12, 2001, DOI 10.1074/jbc.M108535200

Keitaro Tsuji‡, Kiyohisa Mizumo§, Tadanori Yamochi‡, Ikuo Nishimoto‡, and Masaaki Matsuoka‡¶

From the ‡Department of Pharmacology, KEIO University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan and the §Department of Biochemistry, School of Pharmaceutical Sciences, Kitasato University, Shirokane, Minato-ku, Tokyo 108-8641, Japan

ik3-1/Cables is associated with cdk3 in self-replicating cells. In postmitotic neurons, it may serve as an adaptor molecule, functionally connecting c-abl and cdk5, and supporting neurite growth. Here we report that ik3-1 binds to p53 and p73 in vivo. Ectopically expressed ik3-1 potentiates p53-induced cell death but not p73-induced cell death in U2OS cells. On the contrary, coexpression of ik3-1-/C, an ik3-1 deletion mutant lacking the C-terminal 134 amino acids (corresponding to the cyclin box-homologous region), inhibits p73-induced cell death but not p53-induced cell death. ik3-1-/C-mediated inhibition of p73-induced cell death are partially attenuated by overexpression of ik3-1. These data indicate that ik3-1 is not only a regulator for p53-induced cell death but also an essential regulator for p73-induced cell death, and ik3-1-/C competes with ik3-1 only in p73-induced cell death. Furthermore, functional domains of p53 responsible for its interaction with ik3-1 are partially different from those of p73. In conclusion, we found that ik3-1, a putative component of cell cycle regulation, is functionally connected with p53 and p73, but in distinct fashions.

Mammalian G1 phase progression is regulated by G1 cyclin/cdk complexes. In addition to the well known G1 cyclin/cdks including cyclin D/cdk4 or cdk6, and cyclin E/cdk2, cdk3 is considered to be another putative G1 cdk, but its precise function and physiological cyclin partners have not been identified (1). Overexpression of a dominant-negative cdk3 induces G1 arrest, which is not completely rescued by up-regulation of wild-type cdk2, suggesting that the function of cdk3 is distinct from that of cdk2 and independently essential for the mammalian G1-S transition (2). Cdk3 participates in the G1-S progression at least partially by binding to E2F-1, E2F-2, or E2F-3 through DP-1 and by enhancing their transcriptional activities (3).

To further understand the role of cdk3 in mammalian G1-S transition, we searched for new molecules interacting with cdk3 and cloned ik3-1 (designated ik3-1 from an interactor with cdk3) (4). ik3-1 belongs to the cyclin superfamily as its C-terminal domain composed of 124 amino acids resembles the cyclin box. ik3-1 is expressed in almost all tissues while it is highly expressed in neuronal tissues. ik3-1 is expressed during all cell cycle phases with minor oscillation (4). We also found that ik3-1 is associated with Pctaire2 and Trap (5). Pctaire 2 belongs to a Pctaire family, a cdc2-related kinase family of undetermined functional significance (6), and Trap is its associate (7).

Independently, ik3-1 was also cloned as a putative adaptor molecule connecting cdk5, a neuron-specific kinase, with c-abl in neuronal cells, and named Cables (8). Cables enhances neurite growth by enhancing the c-abl-mediated phosphorylation of tyrosine 15 of cdk5 and up-regulating cdk5 activity. While cdk5 activity is detected only in postmitotic neurons (9), ik3-1 (Cables) is nevertheless expressed in non-neuronal cells (4, 8), suggesting that ik3-1 functions differently in non-neuronal cells in which cdk5 is inactive.

Despite these previous findings, the actual critical function of ik3-1 has been unknown. Unexpectedly, however, we have found that ik3-1 binds to p53 and p73. p53 is a crucial tumor suppressor. Its genetic inactivation is observed very frequently in many tumors. p53 prevents tumor progression by inducing cell cycle arrest and cell death. In response to various cellular stresses, p53 is stabilized and activated. p73 is one of two other members belonging to the p53 family (10–12).

When ectopically overexpressed in cell culture, p73 closely mimics p53 activities including induction of cell cycle arrest and cell death (13). Ectopic p73 transactivates many p53-responsive promoters, although relative efficiency differences on a given promoter are observed (14). Despite its functional elucidation, the biological significance of p73 is as yet unclear.

Functional difference between p53 and p73 was pointed out especially regarding their interaction with various viral oncoproteins. SV40 T antigen, adenovirus E1B 55-kDa protein, and HPV E6, which inactivate p53 during host cell transformation, do not target the p73 protein (15, 16). An oncoprotein mdm2 suppresses p73 transactivation function like p53. However, in contrast to p53, cellular mdm2 does not mediate degradation of exogenous p73 (17).

Here we found that ik3-1 binds to both p53 and p73, and modifies their cell death inducing activities in distinct fashions. Their distinct interaction with ik3-1 may be a clue to know the biological significance of p73 in contrast to tumor suppressor p53. Furthermore, we could expect that as an interactor with cdk3, ik3-1 may be a key molecule harmonizing the cell cycle progression and cell death.

MATERIALS AND METHODS

Cell Culture and Transfection—U2OS cells (p53-intact) and HEK293 cells were obtained from ATCC. COS7 cells and MCF7 cells were kind
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gifts from Dr. T. Yamamoto (Institute of Medical Science, Tokyo University, Japan) and Dr. S. Takahashi (Cancer Institute, Japan). All these cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 2 mM glutamine/ml.

Transient transfection to COS7 cells was performed with LipofectAMINE PLUS™ reagents according to the manufacturer’s instructions (Invitrogen) (4, 5). COS7 cells (80–100% confluency) in 100-mm dishes were incubated for 3 h with pre-complexed DNAs and the LipofectAMINE PLUS™ reagents. Unless specified, 7 µg of each DNA, 20–30 µl of PLUS, and 15–25 µl of LipofectAMINE reagents were used for each dish.

Plaque Assays and Adenoviral Vectors—pMF-ik3-1 (Flag-ik3-1), pCG-N-BL-ik3-1 (HA-ik3-1), and pEBG-ik3-1 (GST-ik3-1) were described (4, 5).

Virus titer of p73 adenovirus determined by the plaque assays was similar to those determined by examination of infective virus number particle number with a spectrophotometer at 260 nm were mostly very similar to those determined by examination of infective virus number particle number with a spectrophotometer at 260 nm (1 absorbance unit at 260 nm is equivalent to 1.1 mg/ml) (19). Using purified virus-derived DNAs as templates (19), reverse, CCT CTT CAT CTT CGT CGT CAC TGGGTGGAA AGCCA (forward, ATG AGA CAT ATT ATC TGC CAC GGAGGTGTT ATTAC; regeneration of the wild-type adenovirus in HEK293 cells were examined (135), primers AAGCTTGGTACCGAGCTCGGATC and ACTCGAGCTCGTCTGGACTGGTA for p73N (amino acids 50–135), primers AAGCTTGGTACCGAGCTCGGATC and ACTCGAGCTCGTCTGGACTGGTA for p73N2 (amino acids 1–135), primers CCAGCTTTTCTCGAGGTAAACGCTACGG and CTGAGACTCGG GTAGTGTCCTCCTACAGC for p73D (amino acids 130–310), and primers AAGCTTGGTACCGAGCTCGGATC and CTGAGACTCGG GTAGTGTCCTCCTACAGC for p73D (amino acids 327–636). To construct p73PC (amino acids 1–186), we subcloned the 700-bp HindIII-Pmll fragment to pcDNA3-HA in-frame. To generate a HA-tagged ik3-1 mutant, ik3-1 (amino acids 106–206), we subcloned a 480-bp MluI-BamHI DNA fragment into pCG-N-BL. Because this mutant binds to ik3-1 (data not shown), it was used as positive control.

The system of a replication-deficient adenoviral vector, described in detail (18), was purchased from TaKaRa (Shiga, Japan). ik3-1-1/3C is an ik3-1 partial DNA in which the C-terminal 134-amino acid region of ik3-1 is deleted. ik3-1, ik3-1-1/3C, p53, and HA-p73 DNAs (blunt) were inserted into the HindIII site of a cosmid adenoviral vector, pAxCAwt. Recombinant adenoviruses were generated and used to immunize rabbits as described previously (23).

Reciprocally, if GST-p53 was pulled down, co-expressed Flag precipitated instead of GST-ik3-1, p53 was not co-precipitated (Fig. 1A). If GST-p53 was pulled down, co-expressed Flag precipitated (Fig. 1A lane 1). However, when GST-ik3-1 was pulled down, co-expressed Flag precipitated endogenous p53 from COS7 cells (data not shown). Phosphorylation of ik3-1 has been found to regulate its association with p53, and it has been suggested that the interaction with p53 may be important for the regulation of the p53 pathway (20, 21). Actual bioactive virus titers were determined with a MODFIT program.

Apotosis was visualized with TUNEL staining using the In Situ Cell Death Detection Kit, Fluorescein (Roche Molecular Diagnostics, Basel, Switzerland), that detected DNA strand breaks by terminal transferase-mediated dUTP nick end labeling. Briefly, U2OS cells (5 × 10⁴), seeded onto six-well plates and infected with indicated viruses were then fixed at 40 h after infection with 4% paraformaldehyde in phosphate-buffered saline for 1 h. Permeabilized cell end were labeled with terminal deoxynucleotidyltransferase and examined with fluorescence microscopy.

Antibodies—Rabbit polyclonal antibodies to p53 (FL-393) and p21 (C-19), mouse monoclonal antibodies to Bax (P-19) and GST (B-14) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal antibodies to p73 (Ab-2) and FLAG (M2) were from Oncogene Science (Cambridge, MA) and Eastman Kodak (Kingsport, TN). An antibody to ik3-1 was described earlier (4). An additional antibody to ik3-1 has been raised with a bacterially generated His-tagged cyclin box-like region of ik3-1, corresponding to the C-terminal 123 amino acids of ik3-1, as immunogens. His-tagged bacterial proteins were purified and used to immunize rabbits as described previously (23).

Pull-down Assays, Immunoprecipitation, and Immunoblotting—Cells were suspended at 5 × 10⁴/ml in Nonidet P-40 lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.5% Nonidet P-40) containing 2.5 µg/ml leupeptin, 5 µg/ml apronin, 0.2 mM phenylmethylsulfonyl fluoride, and 0.1 mM orthovanadate and sonicated at 4 °C. To perform pull-down assays, the cleared cell lysates were incubated with 20 µl of 1:1 slurry of glutathione-Sepharose at 4 °C for 1 h. GST or GST-tagged proteins in the cell lysates were adsorbed onto glutathione-Sepharose as precipitates (5). Immunoprecipitation and co-immunoprecipitation were performed as described previously. Supernatants from cell lysates were preincubated with 10 µl of non-immune rabbit serum and 20 µl of 1:1 slurry of protein G-Sepharose 4 FF (Amersham Bioscience, Inc.) per ml for 1–2 h. The cleared supernatants were then incubated for 2 h with indicated antibodies and precipitated with 1 h with 20 µl of 1:1 slurry of protein G-Sepharose 4 FF per ml at 4 °C. Immunoblotted signals were visualized with an ECL detection kit from Amersham Bioscience AB (Uppsala, Sweden).

Immunocytochemistry—U2OS cells (5 × 10⁴), seeded onto six-well plates and infected with p73 and ik3-1 viruses, were fixed at 24 h after infection with 100% ethanol for 30 min at 20 °C, rinsed for 5 min with phosphate-buffered saline, and then double stained with the anti-p73 antibody and anti-ik3-1 antibody for 60 min at 37 °C. After being washed for 45 min with phosphate-buffered saline, cells were doubly stained with Texas Red-conjugated goat anti-mouse IgG and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Vector Laboratories) for 60 min at 37 °C. Before detection, they were washed for 45 min with phosphate-buffered saline. Fluorescence signals were detected with a laser scanning, confocal microscope LSM (Carl Zeiss, Germany).

RESULTS

ik3-1 Is Associated with p53 and p73 in Vivo—To examine whether ik3-1 is associated with p53 in vivo, we overexpressed both proteins in COS7 cells by transient transfection (Fig. 1A, lane 4). When GST-ik3-1 was pulled down, co-expressed Flag precipitated (Fig. 1A, lane 4). However, when GST was expressed instead of GST-ik3-1, p53 was not co-precipitated (lane 3). In addition, if GST-ik3-1 was overexpressed alone, it co-precipitated endogenous p53 from COS7 cells (data not shown). Reciprocally, if GST-p53 was pulled down, co-expressed Flag

The abbreviations used are: HA, hemagglutinin; m.o.i., multiplicity of infection; GST, glutathione S-transferase; FACS, fluorescence-activated cell sorter.
ik3-1 was co-precipitated (Fig. 1A, lane 8), indicating that ik3-1 specifically binds to p53 in COS7 cells. Likewise, we observed that GST-ik3-1 bound to p73 in COS7 cells with similar pull-down experiments (Fig. 1B). In a reciprocal experiment, GST-p73 also co-precipitated Flag-ik3-1 when pulled down with glutathione beads (data not shown).

In parallel, we examined whether endogenous p53 is co-immunoprecipitated with endogenous ik3-1 (Fig. 1C). To enhance expression of p53, we stabilized it by UV irradiation (UV-C) in MCF7 cells. We recognized p53 only in the ik3-1 immunoprecipitates (bottom panel, lane 6) but not in the precipitates with the preimmune serum (lane 5). Expression of p53 was induced by UV treatment. However, we could not be sure whether expression of ik3-1 was induced by UV treatment (compare lanes 4 and 6 of top panel) because we were unable to see ik3-1 bands by direct lysate immunoblotting (negative data not shown), and the antibodies to ik3-1 might not recognize ik3-1 associated with p53. Unfortunately, we could not detect binding of ik3-1 to p73 using endogenous ik3-1 and p73 (negative data not shown). Apparently, expression of p73 is relatively low as compared with p53, whose expression is enhanced by stabilization treatment. This may be the major reason why we could not detect association of endogenous ik3-1 and p73.

Then we asked whether ik3-1 is co-localized with p73 in the subcellular compartments. U2OS cells, co-infected with p73 and ik3-1 viruses, were doubly stained with the antibody to p73 (Fig. 2A) and the anti-ik3-1 antibody (Fig. 2B), followed by the second double staining with secondary antibodies, Texas Red-conjugated anti-mouse IgG and fluorescein isothiocyanate-conjugated anti-rabbit goat IgG. p73 and ik3-1 look red and green, respectively. In D, both stainings were overlapped.

| Viruses | 200   | 400   | 800   |
|---------|-------|-------|-------|
| LacZ    | 2.3 ± 0.1 | 2.0 ± 0.5 | 3.0 ± 0.3 |
| ik3-1-ΔC| 2.0 ± 0.3 | 2.3 ± 0.0 | 3.9 ± 0.4 |
| ik3-1   | 2.7 ± 0.4 | 3.5 ± 0.3 | 3.7 ± 0.7 |
| p73(10)+LacZ| 5.7 ± 1.5 | 9.7 ± 1.3 | 9.5 ± 1.3 |
| p53(10)+ik3-1-ΔC| 5.0 ± 0.5 | 7.5 ± 0.1 | 11.2 ± 0.6 |
| p53(10)+ik3-1| 8.7 ± 0.2 | 17.9 ± 1.3 | 21.5 ± 2.4 |

### Table I

**ik3-1 potentiates p53-induced cell death and ik3-1-ΔC attenuates p73-induced cell death in a dose-responsive fashion**

U2OS cells (5 × 10⁴), seeded on six-well plates, were infected with indicated moi.s of p53, p73, ik3-1, ik3-1-ΔC, and/or LacZ viruses. Percentages of the subG₁ cells were determined with FACS. Indicated moi.s are for LacZ, ik3-1-ΔC, and ik3-1 viruses while moi.s of p53 (10) and p73 (20) were fixed. Experiments for p53-induced cell death were performed with n = 3.

and ik3-1 viruses, were doubly stained with the antibody to p73 and the antibody to ik3-1 (Fig. 2). As a control, U2OS cells were infected only with ik3-1 viruses (Fig. 2A). The majority of ik3-1 is distributed in the cytoplasm whereas a minority of ik3-1 is also seen in the nucleus in case of single infection (left upper panel) (5). However, when co-expressed with p73, a substantial portion of ik3-1 moved to the periphery of the nucleus (or to the perinuclear region in the cytoplasm) (Fig. 2B) where a majority of p73 was located (Fig. 2, C and D). On the contrary, subcellular distribution of ik3-1 was apparently not modulated in the case of coexpression with p53. In the nucleus, a minority of
ik3-1 was co-localized with p53 (data not shown).

*ik3-1 Activates p53-induced Cell Death*—Next, we addressed how ik3-1 functionally interacts with p53 and p73. Cellular manifestations of p53 and p73 activities are represented by cell cycle arrest and cell death. We therefore examined how cell cycle arrest and cell death induced by p53 and p73 were modified by ectopic expression of ik3-1. To tightly regulate ectopic expression levels of ik3-1, p53, and p73, and to increase ectopic expression efficiency up to 100% of cells, we adopted the adenovirus-mediated expression system. However, we found that as compared with the lipofection-based transient expression (transfection efficiency is 30–50%), expression of ik3-1 using standard m.o.i. of ik3-1 viruses (ranging 25–100) was very low. We therefore needed higher m.o.i. (more than 200) of ik3-1 viruses to express ik3-1 in 100% of U2OS cells, which was approximately 10 times more than those necessary for expression of p53 and p73. Adenovirus-mediated gene expression in mammalian cells at higher m.o.i. may bring about cytotoxicity including apoptosis and G2 arrest (24). However, within 48 h after infection, at least adenovirus-mediated cell death did not occur (data not shown, but see Table I).

Adenovirus-mediated expression of p53 and p73, but not LacZ nor p53 mutant (C135Y), induced both G1 and G2 arrest as well as cell death in U2OS cells (data not shown but see Fig. 3A). To quantitate cell death, we used FACS analysis to determine the percentage of sub-G1 population.

If ik3-1 was co-expressed in addition to p53 in U2OS cells, cell death induced by p53 was substantially potentiated (Fig. 3, A and B). When ik3-1ΔC, a ik3-1 deletion mutant lacking the C-terminal 134 amino acids, was used instead of ik3-1 as a negative control, it was not. When we changed the m.o.i. of ik3-1, LacZ, or ik3-1ΔC from 200 to 800 with the fixed m.o.i. (10) of p53 (Table I), or when we changed the m.o.i. of p53 from 0 to 25 with the fixed m.o.i. (400) of ik3-1, LacZ, or ik3-1ΔC in this assay (data not shown), we consistently observed that p53-induced cell death was enhanced by coexpression of ik3-1 as compared with coexpression of LacZ or ik3-1ΔC. It was supposed that the slight increase in p53-induced cell death by coexpression of LacZ and ik3-1ΔC at higher m.o.i. (Table I) was because of toxicity generated by adenovirus infection to cells. Immunoblot analysis indicated that expression of p53 was basically invariant irrespective of co-infection of ik3-1, ik3-1ΔC, and LacZ viruses, excluding the possibility that ik3-1 enhanced p53-induced cell death by up-regulating expression of p53 (data not shown, but see Fig. 6A).

**ik3-1ΔC Blocks p73-induced Cell Death**—In contrast to p53, p73-induced cell death was not potentiated by coexpression of ik3-1 (Fig. 3, A and C), but unexpectedly, it was markedly inhibited by coexpression of ik3-1ΔC (Fig. 3, A and C). This observation suggested that ik3-1ΔC suppressed p73-induced cell death by competing with endogenous ik3-1 that was an essential component of the p73-mediated cell death pathway. We could also speculate that p73-induced cell death was not augmented by coexpression of ik3-1 because the cell death system mediated by p73 was already saturated with endogenous ik3-1. In U2OS cells, endogenous expression of ik3-1 was really recognized (data not shown). On the contrary, G1 and G2 arrest induced by expression of p73 was not significantly altered by co-expression of ik3-1ΔC.

Then we analyzed the dose-response effect of ik3-1ΔC-mediated inhibition of p73-induced cell death. When we changed the m.o.i. of ik3-1, LacZ, and ik3-1ΔC viruses from 50 to 400 with the fixed m.o.i. (20) of p73 viruses (Table I), we observed that p73-induced cell death was inhibited in a dose responsive fashion by coexpression of ik3-1ΔC as compared with coexpression of LacZ or ik3-1. Furthermore, when we changed the m.o.i. of p73 from 1 to 20 with the fixed m.o.i. (400) of ik3-1, LacZ, and ik3-1ΔC viruses in this assay, we invariably observed that p73-induced cell death was inhibited markedly only by co-infection of ik3-1ΔC (data not shown). Immunoblot analysis indicated that expression of p73 was invariant irrespective of co-infection of ik3-1, ik3-1ΔC, and LacZ viruses (data not shown, but see Fig. 6B), excluding the possibility that ik3-1ΔC reduced p73-induced cell death by down-regulating expression of p73.

In addition to FACS analysis, we used TUNEL assays to examine whether p53- or p73-induced cell death is apoptosis, and furthermore, to see how coexpression of ik3-1 or ik3-1ΔC affects p53- or p73-induced cell death (Fig. 4). Apparently, at least some portion of p53- or p73-induced cell death is apo-
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Fig. 4. ik3-1 activates the p53-induced apoptosis and ik3-1-ΔC inhibits p73-induced apoptosis. U2OS cells (5 × 10⁴), seeded on six-well plates, were infected with indicated m.o.i.s of p53, p73, ik3-1, and/or ik3-1ΔC viruses. At 40 h after infection, cells were analyzed with TUNEL reaction. There are an approximately comparable number of cells for each panel.

Fig. 5. ik3-1-ΔC-mediated inhibition of p73-induced cell death is attenuated by co-infection of ik3-1. U2OS cells (5 × 10⁴), seeded on six-well plates, were infected with p73 viruses at m.o.i. of 20 and indicated m.o.i.s of ik3-1, ik3-1ΔC, and LacZ viruses. A, at 48 h after infection, cells were trypsinized and stained with propidium iodide for FACS analysis. Numbers 1, 4, 8, and 16 indicate m.o.i.s of 100, 400, 800, and 1600. The experiment was performed with n = 3. B, at 24 h after infection, cells were harvested to examine ik3-1 and ik3-1ΔC expression by immunoblotting with the antibody to ik3-1.

Protein Expression of Bax and p21 Are Not Affected by Coexpression of ik3-1 or ik3-1-ΔC—Because p53 and p73 exert their activities by transactivating and transpressing downstream genes, we examined expression of representative downstream gene products by immunoblotting (Fig. 6). Apparently, expression of p21 and Bax was induced by infection of ik3-1 viruses (Fig. 6A and B, p21 and Bax panels, lanes 1 and 2). Co-infection of ik3-1 viruses with p53 viruses did not increase expression of p21 (Fig. 6A, p21 panel, lanes 3–11). Unexpectedly, Bax expression was decreased by co-infection of ik3-1 viruses (Bax panel, lanes 3–5). These findings indicated that they were not targets of ik3-1-mediated potentiation of p53-induced cell death. Likewise, they were not targets of ik3-1-ΔC-mediated inhibition of p73-induced cell death (Fig. 6B).

In addition, we examined how expression of ik3-1 and ik3-1-ΔC modifies p53- and p73-mediated transactivation. Consistent with the immunoblotting results, the co-transfection of ik3-1 or ik3-1-ΔC did not have significant influence on p73-mediated transactivation of the Bax promoter nor the mdm2 promoter (data not shown). Likewise, the co-transfection of ik3-1 or ik3-1-ΔC did not have significant influence on p53-mediated transactivation of the Bax promoter or the mdm2 promoter (data not shown).

ik3-1 Binds to the Different Functional Domains of p53 and p73—To identify the domains of p53 and p73 responsible for their interaction with ik3-1, we constructed several deletion mutants of p53 and p73. The functional domains of p53 have already been well characterized. We can therefore identify functional domains in p73 analogous to the transcriptional activation domain (called N1), the DNA-binding domain (DB), the region containing the tetramerization domain (DD), and the proline-rich domain between AD and DB (called N2) of p53. We asked which domains are responsible for interaction of p53 and p73 with ik3-1. For that purpose, we constructed mammalian expression vectors for HA-tagged N2 and DD of p53, and for HA-tagged N2, DD, and DB of p73 (Fig. 7C). In addition, two vectors were constructed to express the region N1 plus N2 (called here p73N12) as well as the region N1 plus N2 plus 51 amino acids of the N-terminal end of DB of p73 (called here p73PX) (Fig. 7C). GST-ik3-1 and one of the constructed mutants were coexpressed in COS7 cells for pull-down assays with glutathione beads. We recognized that ik3-1 bound to p53DD but not to p53N2 (Fig. 7A) while ik3-1 bound to both p73DD and p73N2 (Fig. 7B). Long exposure indicated that ik3-1 also bound weakly to p73DB. Obviously, it is extraordinary that ik3-1 bound to both p73PX and p73N2, but not p73N12, which contained p73N2 (Fig. 7C). To be certain that this phenomenon was not an artifact of pull-down experimental procedures, Flag-ik3-1 and one of these mutants were coexpressed in COS7 cells, followed by immunoprecipitation of Flag-ik3-1 with anti-ik3-1 antibodies and immunoblotting with the antibodies to HA. Basically, the co-precipitation pattern was the same as the pull-down assays (data not shown). We therefore speculate that p73AD inhibits binding of ik3-1 to p73N2 when p73N2 is separated from p73DB. Here, we concluded that p73 binds to ik3-1 through domains at least partially different from p53 functional domains to which ik3-1 binds.

DISCUSSION

ik3-1 seems to be a multifunctional protein. In neuronal cells, it may regulate neuronal function by connecting c-abl with cdk5 (8), and in non-neuronal cells, it is phosphorylated by Gₛ cyclin/cdkks containing cdk3 and cdk2 (25), supporting the idea that ik3-1 is located downstream of Gₛ cyclin/cdkks as a substrate. Furthermore, we have identified Petaire2 and Trap...
as interactors with ik3-1 (5).

In addition, here we found that ik3-1 affects cell death induced by p53 and p73. Actually, ectopically overexpressed ik3-1 did not augment p73-induced cell death while expression of a deletion mutant of ik3-1, ik3-1-ΔC, suppressed it, suggesting that ik3-1 positively affects p73-induced cell death and ik3-1-ΔC competes with ik3-1 in p73-induced cell death. We speculated that p73-induced cell death was not augmented by coexpression of ik3-1 because the cell death system mediated by p73 was already saturated with endogenous ik3-1. On the contrary, we found that ik3-1 actually potentiates p53-induced cell death but ik3-1-ΔC does not compete with ik3-1 in p53-induced cell death. We have not yet completely assessed how this difference occurred. However, our preliminary experiments suggested that ik3-1-ΔC binds to p73 tightly but binds to p53 relatively weakly as compared with ik3-1.2 In vivo, difference between ik3-1 and ik3-1-ΔC in affinity to p53 and p73 may explain these phenomena.

Furthermore, in vivo binding study indicated that ik3-1 binds to the proline-rich N2 domain of p73, but not to the N2 domain of p53 (Fig. 7), while ik3-1 binds to both p53DD and p73DD. The proline-rich N2 domain of p53 is supposed to contain a region responsible for induction of apoptosis by interacting with Src homology domain 3-containing proteins through its PXXP motif (26–28), although there have been no clear investigations on the function of p73N2. Thus, we conclude that ik3-1 functionally and physically interacts with p53 and p73 in distinct ways.

If we remember that ik3-1/Cables binds to c-abl in neurons (8) and c-abl cooperates with p73 to induce cell death (29–31), the above finding is not so extraordinary. By its binding to and phosphorylating p73, c-abl potentiates p73-mediated apoptosis (29–31). In contrast, by its binding to p53, c-abl up-regulates the function of p53 (32). Without c-abl, p73-induced cell death is markedly compromised (29–31). It is therefore speculated that, in this context, ik3-1 may functionally connect c-abl with p53 and p73. However, it still remains to be clarified how ik3-1 actually interacts with p53 and p73.

Moreover, we must pay attention to the fact that ik3-1 regulates only the activity of p53 and p73 in inducing cell death, but not in inducing cell cycle arrest. In this regard, neither potentiation of p53-mediated transactivation of the mdm2 and Bax promoters by coexpression of ik3-1, nor inhibition of p73-mediated transactivation of the mdm2 and Bax promoters by co-expression of ik3-1ΔC, was found to occur. In agreement, the protein level of p21 was constant irrespective of co-infection of ik3-1 or ik3-1ΔC. Likewise, protein level of Bax was not significantly altered by coexpression of ik3-1 or ik3-1ΔC (Fig. 6). These findings suggest that ik3-1 regulates p53- and p73-induced cell death through transcriptional regulation of un-

2 K. Tsuji and M. Matsuoka, unpublished observations.
known genes responsible for cell death. We remember that the p338A mutant of p73, a p73 mutant not activated by the c-abl-mediated phosphorylation, does not induce cell death while it keeps its transactivation of the mdm2 promoter, indicating that the regions responsible for induction of apoptosis are distinct from those responsible for transactivation of the mdm2 promoter (29). Regarding p53, a lot of evidence has indicated that the regions responsible for induction of apoptosis are distinct from those responsible for cell cycle arrest (26–29). p53 has many transcriptional targets, including ones transcriptionally repressed by p53. p21 is the major mediator of p53-induced cell death. We remember that the critical and universal mediators have not been identified for p53-induced cell death. A lot of candidates include PIG3 (34), p53AIP1 (35), and PUMA (36). Currently, we have not yet identified the genes responsible for ik3-1-mediated modification of p53- and p73-induced cell death.

There are many endogenous p53-binding proteins that modify function of p53, including c-abl (29–32), mdm2 (17), 14-3-3 proteins (37), Ref-1 (38), and SUMO-1 (39). ik3-1 may be added to this group. Previously, it has been shown that ik3-1 may interact with G1 cyclin/cdk5 in self-replicating cells (4) and enhances neuronal function by potentiating neurite growth in cooperation with cdk5 and c-abl in neuronal cells (8). Here we identify ik3-1 function as a positive regulator for p53 and p73-induced cell death. These multifunctional aspects of ik3-1 let us speculate that ik3-1 may function as a putative molecular switch located at the boundaries between cell cycle regulation and cell death, and between cell cycle regulation and cell differentiation.

Acknowledgments—We are indebted to Tomo Yoshida, Kazumi Nishihara, Kouichi Tsuchiya, Fusano Igarashi, and Dovie Wylie for expert technical assistance. We are grateful to Etsuro Ogata and Kyoji Ikeda for helpful discussion and Yoshito Kaziro for scientific suggestion.

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Keitaro Tsuji, Kiyohisa Mizumoto, Tadanori Yamochi, Ikuo Nishimoto and Masaaki Matsuoka

J. Biol. Chem. 2002, 277:2951-2957.
doi: 10.1074/jbc.M108535200 originally published online November 12, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M108535200

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