THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 13, pp. 8707–8715, March 31, 2006

p19^{ras} Interacts with and Activates p73 by Involving the MDM2 Protein*◆

Received for publication, December 29, 2005 Published, JBC Papers in Press, January 25, 2006, DOI 10.1074/jbc.M513853200

Mi-Hee Jeong[‡1], **Jeehyeon Bae**[§1], **Won-Ho Kim**[¶], **Sang-Mi Yoo**[‡], **Jung-Woong Kim**[‡], **Peter I. Song**[‖], **Kyung-Hee Choi**[‡2]

From the [‡]*Laboratory of Molecular Biology, Department of Biological Science, College of Natural Sciences, Chung-Ang University, Seoul 156-756, Korea, the* [§]*Laboratory of Molecular Omics Therapy, Graduate School of Life Science and Biotechnology, Pochon CHA University, Seongnam 463-836, Korea, the* [¶]*Korea Center for Disease Control and Prevention, Korean National Institute of Health, Seoul 122-701, Korea, and the* [‖]*Department of Dermatology, University of Colorado Health Sciences Center, Denver, Colorado 80045*

p73*β* is a structural and functional homologue of p53, a tumor suppressor gene. In this study, we identified a novel p73*β*-binding protein, p19^{ras}, by the yeast two-hybrid screening method. Alternative splicing of the proto-oncogene H-*ras* pre-mRNA has led to two distinct transcripts, p19^{ras} and p21^{ras}. In both endogenous and overexpressed systems, we confirmed that p19^{ras} binds to full-length p73*β* *in vivo* and *in vitro*. Coexpression of p19^{ras} with p73*β* stimulated the transcriptional activity of p73*β*. Ras proteins are known to be small membrane-localized guanine nucleotide-binding proteins. However, unlike other Ras proteins, p19^{ras} is localized in the nucleus and the cytosol and its interaction with p73*β* occurred exclusively in the nucleus. Oncogenic MDM2 (mouse double minutes *2*) is a known repressor of p73 transcriptional activity. In this study, when p19^{ras} was bound to MDM2, it further inhibited the association of MDM2 to the p73*β* protein. In addition, p19^{ras} abolished MDM2-mediated transcriptional repression of p73*β*. Therefore, this study presents a novel pathway of Ras signaling that occurs in the nucleus, involving p19^{ras} and p73*β*. Furthermore, a p19^{ras}-mediated novel regulatory mechanism of p73 involving the MDM2 protein is described.

More than a decade after the recognition of p53 as a major tumor suppressor, two p53 homologues, p73 and p63, were identified (1). The significant sequence homology between p73 and p53 suggests that the two proteins have similar functions as transcriptional factors. p73 not only activates the transcription of p53 target genes, but it also induces apoptosis and cell cycle arrest, like p53 (2, 3). However, a number of differences between p73 and p53 have been reported. p73 transactivates only certain p53-responsive genes (4, 5). Viral oncoproteins that bind to p53 and inhibit its activity do not interact with p73 (6, 7). Furthermore, the expression level of p73 is not affected by exposure to DNA-damaging agents that increase p53 levels, indicating that the two proteins have distinct cellular functions (8).

MDM2 (mouse double minutes *2*) is an oncoprotein that is overexpressed in many human cancers (9). It is well established that MDM2 inactivates p53 activity through monomeric ubiquitination of lysine residues of p53 (10–12). In contrast, although p73 binds to MDM2 as well, their interaction does not lead to the proteosomal degradation of p73 (13). Nevertheless, the binding of p73 to MDM2 results in a dramatic inhibition of p73 transactivating activity (14). It has been shown that the binding to a cofactor, p300/CREB-binding protein (CBP),[3] is required for the transcriptional activity of p73 and that MDM2 competes with p73 for the p300/CBP (15). In a previous report, we demonstrated that Amphiphysin IIb-1 sequesters nascent p73 proteins in the cytoplasm and thus inhibits its transcriptional activity (16).

Using the yeast two-hybrid screening method, we identified a novel p73*β*-binding protein, p19^{ras}, which is an alternative splicing variant of c-H-*ras* (17). Proto-oncogenic Ras family proteins are GTPases that alternatively bind to GDP or GTP and elicit diverse cellular responses, including cell proliferation, differentiation, growth, arrest, and/or apoptosis (18–21). The classical Ras proteins include three members, N-Ras, H-Ras, and K-Ras, and their pre-mRNAs are regulated by alternative splicing. K-Ras4A and K-Ras4B, which were produced by alternative splicing in the last exon of the k-*ras* gene, displayed differential localization (22, 23). Likewise, alternative splicing of H-*ras* precursor mRNA led to the formation of two transcripts, p19^{ras} and p21^{ras}. The differences in their mRNA are that p19mRNA contains exon IDX (intron D exon) and lacks exon E4A, due to an in-frame stop codon (17, 24).

Recently, stable and abundant expressions of p19^{ras} have been elucidated, but the functional significance of this protein has not been well-studied (25). In contrary to oncogenic p21^{ras}, p19^{ras} lacks transforming potential. For effective Ras signaling, p21^{ras} protein must undergo post-translational modifications that facilitate its attachment to the plasma membrane (22, 23). However, p19^{ras} does not have a Ras C-terminal conserved domain that targets the plasma membrane and is shown to be localized in the cytoplasm and nucleus (25). In addition, p19^{ras} has little GTP binding activity and is devoid of two important GTP-binding sites located in exon E4A of the p21^{ras} (25). Therefore, these findings suggest the presence of fundamental differences in the function and regulation between H-ras splicing variants.

In this study, we describe p19^{ras} as a specific binding protein of p73, in which a direct interaction is important for controlling the transcriptional activity of p73. Furthermore, we demonstrate that p19^{ras} activates p73 by alleviating MDM2-mediated p73 inhibition when p19^{ras} directly binds to MDM2 and disrupts the association of MDM2 to p73. There-

* This work was supported by a grant from the Korea Science and Engineering Foundation (R04-2002-000-20112-0). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
◆ This article was selected as a Paper of the Week.
[1] These authors contributed equally to this work.
[2] To whom correspondence should be addressed: Laboratory of Molecular Biology, Dept. of Biological Science, College of Natural Sciences, Chung-Ang University, Heuksuk Dong, Dongjak Ku, Seoul 156-756, Korea. Tel.: 82-2-820-5209; Fax: 82-2-824-7302; E-mail: khchoi@cau.ac.kr.

[3] The abbreviations used are: CBP, CREB-binding protein (where CREB is cAMP-responsive element-binding protein); GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; DTT, dithiothreitol; GFP, green fluorescent protein; HA, hemagglutinin; siRNA, small interfering RNA; TAD, transactivation domain; DBD, DNA-binding domain; OD, oligomerization domain.

fore, we present p19^{ras} as a new member of a growing family that interacts with the MDM2 protein to regulate p73 activity.

MATERIALS AND METHODS

Bait Construction—Full-length p73β was cloned into the pLexA yeast expression vector (Stratagene, La Jolla, CA), digested with EcoRI, and then ligated. The resulting plasmid containing the C-terminal region of p73β was denoted pLexA-73C (248–499 amino acids) and used as bait for the library screening. To confirm bait expression in the yeast system, yeast proteins were prepared by the Horvath and Riezman method, and the lysate was Western blotted using LexA antibodies (Clontech, Palo Alto, CA).

Yeast Two-hybrid Screening—EGY48 yeast cells were grown in YPD (1% yeast extract, 2% bacto-peptone, 1% glucose) or synthetic dropout medium lacking the appropriate supplements to maintain selections. Yeast transformation was performed with the lithium acetate method. For the transformation of the bait plasmid, several colonies of EGY48 (p8op-LacZ) on the synthetic dropout/Glu/-Ura plate were cultured in YPD medium. Competent cells were transformed with 1 μg of pLexA-73C and 100 μg of salmon sperm DNA. The human brain library in pB42AD (Clontech) was sequentially transformed into the yeast (p8op-*lacZ* + pLexA-73C). Approximately 2 × 10^6 transformants were screened for positive colonies growing on synthetic dropout/Gal/Raf/-Ura/-His/-Trp/-Leu containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (200 μg/ml). To further confirm the presence of interacting proteins, positive transformants were examined for the expression of the *lacZ* reporter gene by using a filter assay or a whole plate assay. Yeast plasmid DNA was extracted using glass beads and pB42AD-positive plasmids were transformed into *Escherichia coli* XL1-Blue (Clontech). Plasmids were isolated and sequenced, and their interactions with p73β were confirmed after the transformation of the plasmids by using a whole plate assay.

In Vitro Binding Assay—A pB42AD yeast-inducible vector fragment was cut and ligated into EcoRI- and XhoI-digested pGEX4T1 prokaryotic expression vectors (Amersham Biosciences). The fusion protein was expressed in BL21 and purified on glutathione-Sepharose 4B beads (Promega, Madison, WI). *In vitro* translated full-length p73β (Promega) was incubated with the GST fusion proteins coupled to the glutathione-Sepharose bead. After 3 h of incubation at 4 °C, the mixtures were washed three times in PBS containing 1 mM DTT (dithiothreitol). The beads were boiled in 5× Laemmli buffer, proteins were resolved by 10% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), and gels were exposed to x-ray film after drying.

Antibody Production—The polyclonal antibody against the p19^{ras} protein was raised from rabbits using the peptide CSRSGSSSSSGTLWDPPG. The sequences were from 152–170 amino acids of the human p19^{ras} with an additional cysteine at the N-terminal end to allow coupling to the keyhole limpet hemocyanin. Preimmune sera from the same rabbits were obtained before immunization. The sera were tested by Western blot analyses from the 293 human embryonic kidney (HEK) cells expressing either the recombinant GFP-p19^{ras} or GFP-p21^{ras} proteins as antigens. The specificity of the purified antibody was further analyzed by a peptide enzyme-linked immunosorbent assay and Western blot analysis.

Cell Culture and Transfection—HEK293 and HeLa cells were obtained from ATCC (American type culture collection) (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen) and penicillin-streptomycin (50 units/ml). H1299 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum. Transient transfection was performed by Lipofectamine (Invitrogen) with different plasmid DNA according to manufacturer's instructions.

In Vivo Binding Assay—The 293 cells were seeded in 100-mm plates at an initial density of 2 × 10^6 cells and allowed to grow for 24 h. The cells were transfected with the respective plasmids, further incubated for 42 h, and lysed by sonication. For the confirmation of endogenous interaction of p19^{ras} with p73, 293 cells were treated with 2 M doxorubicin for 24 h to stimulate p73 expression. For immunoprecipitation assays, cell lysates were incubated with an appropriate antibody (0.8 μg) in PBS for 3 h at 4 °C. Following the addition of protein A/G-agarose beads, the reaction was incubated overnight at 4 °C with rotation. The beads were washed three times in PBS containing 1 mM DTT, resuspended in SDS sample buffer, and boiled for 5 min. Samples were analyzed by Western blotting, using the appropriate antibodies to detect protein expressions. The polyclonal antibody against the p73 protein generated from GST-p73β was previously raised in the rabbit (26). The monoclonal GFP and FLAG antibodies were purchased from Roche Applied Science and Sigma, respectively. The polyclonal HA, MDM2, p21^{ras}, α-tubulin, and retinoblastoma (Rb) antibodies were from Santa Cruz Biotechnology.

Luciferase Assay—The 293 cells were cultured in 60-mm dishes and transfected with the firefly luciferase p21 reporter gene (500 ng) and pCMVβ (500 ng) (Promega), together with 500 ng of pcDNA-HA73β, pcDNA3-MDM2, and/or pEGFP-p19^{ras} by using the appropriate luminometer tubes. After 38 h of transfection, cells were lysed in reporter lysis buffer (Promega). Cell extracts were analyzed with the luciferase reporter assay system using a Lumat LB 9501 Berthold Luminometer. Luciferase activities of the p21-luciferase vector were normalized based on β-galactosidase activity of the cotransfected vector.

Immunofluorescence Staining—COS-7 cells were grown on a sterile coverslip in 60-mm dishes and transfected with indicated expression vectors using Lipofectamine. Two days after transfection, cells were fixed with 80% acetone and incubated with mouse anti-FLAG IgG antibody (1:500) (Santa Cruz Biotechnology), followed by Cy3-conjugated goat anti-mouse IgG (Amersham Biosciences). Cells were simultaneously incubated with 4′,6-diamidino-2-phenylindole (Sigma) for 30 min. Plates were washed three times in PBS and the fluorescence was photographed using a Nikon microscope (Nikon, Tokyo, Japan).

Preparation of Subcellular Fractions—HeLa cells were washed with ice-cold PBS, harvested by centrifugation, and lysed in Buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride) for 15 min. For cell lysis, 10% of Nonidet P-40 was added and vortexed for 10 s. After centrifugation at 5,000 rpm for 30 s, the supernatant (cytosolic extracts) was transferred to a new tube. The pellet was added to ice-cold Buffer C (20 mM HEPES (pH 7.9), 0.4 M NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride), incubated for 15 min at 4 °C, and centrifuged at 14,000 rpm for 5 min. The supernatant (nuclear extracts) was transferred to new tubes and kept frozen at −70 °C until use.

RNA Interference; siRNA of p19^{ras}—PCR primers (5′-ACCAAGTCTTTTGAGGACATCCAC-3′ and 5′-TCACATGGGTCCCGGGGGGTCCCA-3′) were designed to amplify a 256-bp fragment from the 3′ end of the *p19^{ras}* gene. The PCR products were two separate clones containing the same target region, but in different orientations. The resulting plasmid templates were used in the silencer siRNA mixture kit (Ambion, Austin, TX) to prepare siRNA mixtures according to the manufacturer's protocol. The templates were used in an *in vitro* transcription reaction to generate double-stranded RNA. After a brief purifi-

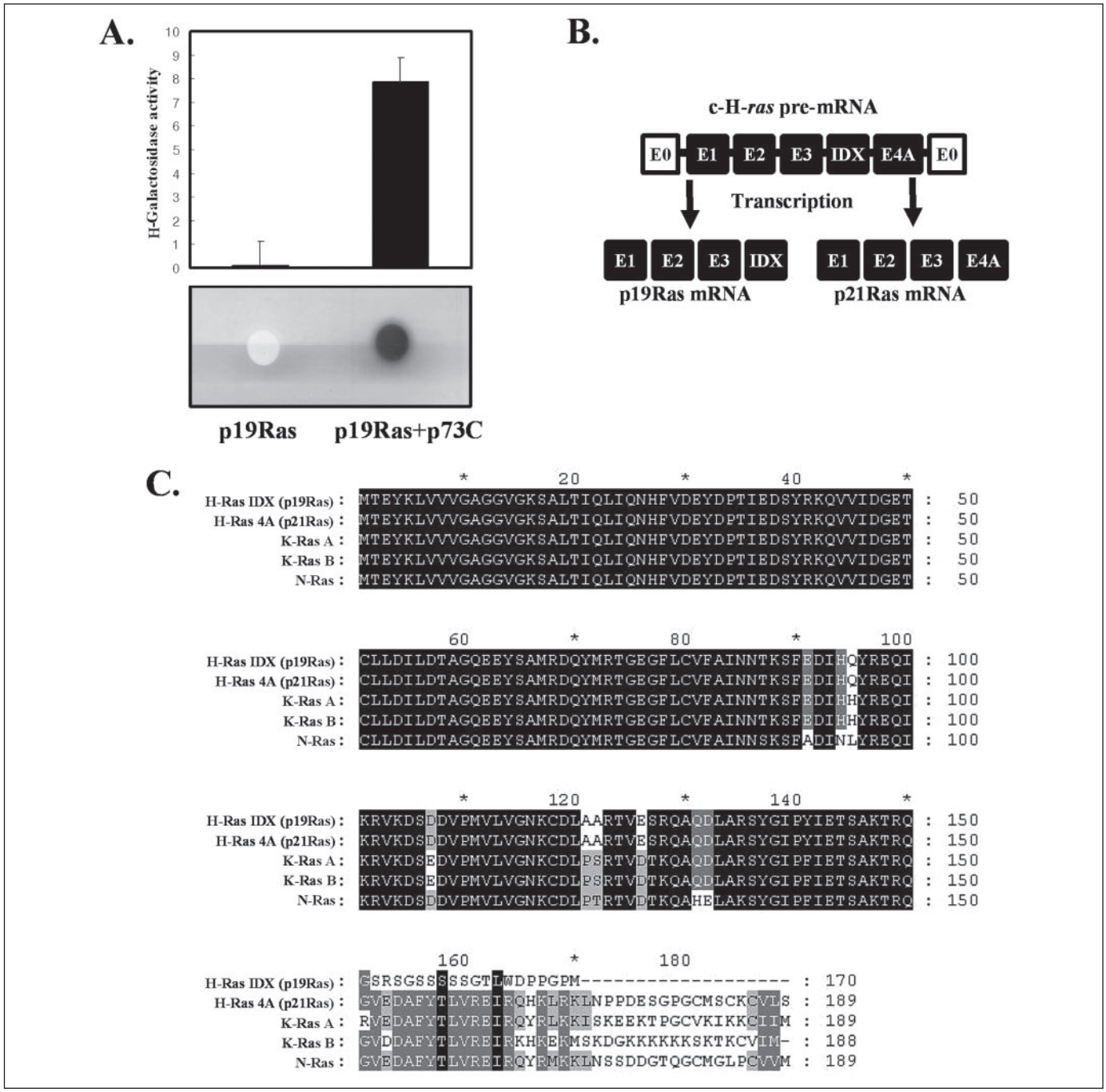

FIGURE 1. **Identification of p19^{ras} as a p73-binding protein, domain arrangement, and the deduced amino acid sequence of p19^{ras}.** *A*, p19^{ras} was identified after screening a human brain cDNA library using the C terminus region of p73 (p73C). The interaction was confirmed by measuring β-galactosidase activity in a whole plate assay with 5-bromo-4-chloro-3-indolyl-β-D-galactoside. Actual staining (*lower panel*) and its quantification are shown (*upper panel*). *B*, H-*ras* pre-mRNA consists of five exons (E1, E2, E3, IDX, and E4A). Alternative splicing leads to the formation of both p19^{ras} and p21^{ras} mRNAs. p19^{ras} is joined by exon E1 to E3 and IDX, whereas the p21^{ras} transcript was devoid of exon IDX but contained E4A. *C*, alignment of deduced amino acid sequences of p19^{ras} with other Ras proteins (p21^{ras} and K- and N-Ras) is shown. Identical residues are *shaded* with a *darker color*. The open reading frame of p19^{ras} predicts a protein with 170 amino acids where only the last 19 amino acid residues are different from another variant, p21^{ras}.

cation step, 15 μg of the resulting double-stranded RNA was digested with Dicer (Ambion) at 37 °C for 16 h. The digestion products were then purified with the siRNA purification units (Ambion) to remove any undigested double-stranded RNA. The resulting siRNA population was quantitated using a spectrophotometer and visualized on a 20% nondenaturing acrylamide gel. 293 cells were transfected with the p19^{ras} siRNA using Lipofectamin 2000 (Invitrogen). p73 siRNA was purchased from Santa Cruz Biotechnology, and the siRNA were transfected to HEK293 or SK-OV-3 using transfection reagent provided by the same manufacturer or Lipofectamin 2000 (Invitrogen), respectively, according to provided protocols.

RESULTS

Identification of p19^{ras} as a p73-interacting Protein—To identify p73-interacting proteins, we screened a human brain cDNA library with the C-terminal (amino acids 248–499) region of p73 (p73C) using the yeast two-hybrid system. Screening of 2×10^6 yeast transformants led to the

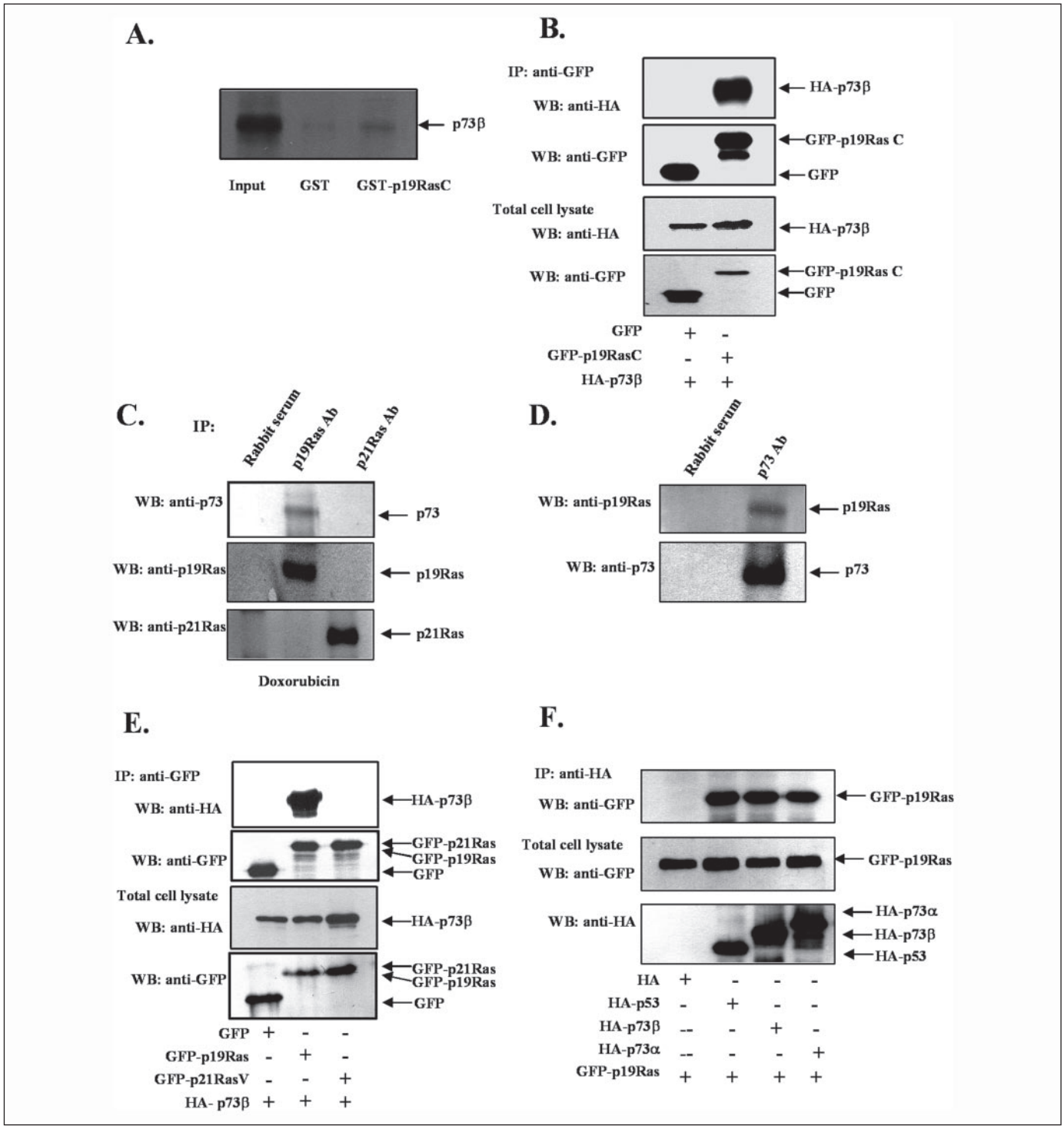

FIGURE 2. ***In vitro* and *in vivo* interactions of p73β with p19^{ras} protein.** *A*, GST (5 μg), or GST-p19^{ras}, was mixed with an equal amount of [^{35}S]methionine-labeled full-length p73β. The input represents one-fourth of the [^{35}S]methionine-labeled full-length p73β used for GST pull-down assays. *B*, the HEK293 cells were transfected with plasmids expressing HA-tagged p73β (3 μg) together with either GFP (1 μg) or GFP-p19^{ras}C (3 μg). Total cell lysates were prepared and immunoprecipitated with anti-GFP antibody. Proteins were detected by Western blotting, using appropriate antibodies as indicated in the figure. *C* and *D*, *in vivo* interaction of endogenous p19^{ras} with p73β proteins was determined in the HEK293 cells stimulated with doxorubicin. Using anti-p19^{ras} or anti-p73 antibodies, we performed immunoprecipitation experiments in both directions to confirm the association of p19^{ras} with p73β. *E*, GFP-fused plasmids expressing either p19^{ras} or p21^{ras}V, the constitutively active form of p21^{ras}, were transfected to the HEK293 cells in the presence of HA-tagged p73. The lysates were immunoprecipitated with anti-GFP antibody, and the bound p73 was detected using anti-HA antibody. *F*, the HEK293 cells were transfected with either HA-tagged p53, p73α, and p73β together with GFP-fused p19^{ras}. Total cell lysates were immunoprecipitated (*IP*) with anti-HA antibody and Western blotted (*WB*) using anti-GFP antibody.

isolation of several positive clones. Nucleotide sequence analysis revealed that cDNA of one clone was identical to the p19^{ras} identified in 2001 (GenBank^{TM} accession number BC006499). The clone contained partial coding sequences (56–170 amino acids) of p19, as we denoted p19^{ras}C in subsequent data. We also confirmed the interaction of p19 with the p73C in yeast by measuring β-galactosidase activity (Fig. 1*A*).

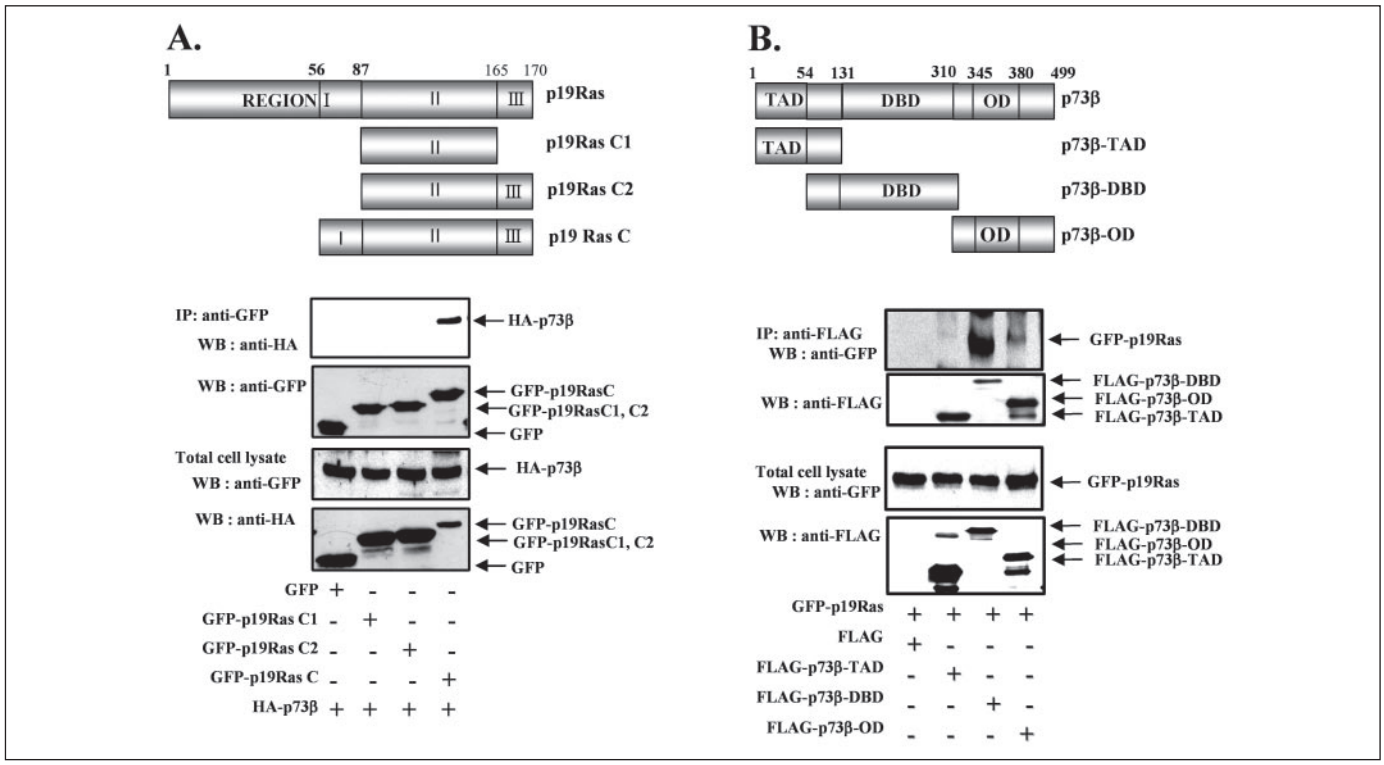

FIGURE 3. **Determination of specific binding regions of p19^{ras} and p73β.** *A*, the p19^{ras} protein was divided into three regions (regions I, II, and III) based on its sequence alignment with other Ras proteins. Each of the differently truncated forms of GFP-fused p19^{ras} was transfected with p73β into the 293 cells. Total cell lysates were immunoprecipitated (*IP*) with anti-GFP antibody, and p73 was detected by Western blot (*WB*) analysis using an anti-HA antibody. Expression of the respective mutants was confirmed by immunoblotting. *B*, according to the domains (TAD, DBD, and OD) of the p73β, mutants containing each of the domains were constructed. After transfecting each of the FLAG-tagged truncated forms of p73 together with p19^{ras} into the 293 cells, the cell lysates were immunoprecipitated with anti-FLAG antibody, and the samples were assessed by immunoblot analysis, using anti-GFP antibody (*upper panel*).

p19^{ras} consisted of an open reading frame of 513 bp that encoded 170 amino acids. H-*ras* pre-mRNA underwent alternative splicing of the last encoding exon, rendering two mRNAs, one with a stop codon on exon 4A and the other with a stop codon on exon IDX (Fig. 1*B*). These messages translated into p21 and p19 (H-Ras IDX) proteins, respectively (17). Amino acid sequence alignment of p19 H-Ras IDX with other Ras proteins (H-Ras, K-Ras, and N-Ras) are shown in Fig. 1*C*, and p19 only lacks the C terminus sequences of p21 ranging from amino acids 152 to 189.

Physical Interaction of p73 with p19^{ras}—To determine the interaction of p19^{ras} to the full-length p73β, GST-fused p19^{ras}C (56–170 amino acids) was produced and used in an *in vitro* binding assay with [^{35}S]methionine-labeled full-length p73β. Consistent with the interaction in yeast, p73β was bound to GST-p19^{ras}C, but not with GST alone (Fig. 2*A*). To further validate this interaction *in vivo*, we examined the co-precipitation of full-length HA-p73β and GFP-p19^{ras}C from transiently transfected HEK293 cells. Cell lysates were incubated with anti-GFP antibody, followed by Western blotting with anti-HA antibody. The HA-73β protein was coimmunoprecipitated with GFP-p19^{ras}C (Fig. 2*B*). Thus, these results confirmed that the full-length p73β interacts with p19^{ras}C both *in vivo* and *in vitro*.

The expression of endogenous p19^{ras} cDNA was abundant in HeLa, HEK293, and SH-SY5Y cells (data not shown). Under the conditions that stimulate p73 expression using doxorubicin, p19^{ras} immunoprecipitated endogenous p73 from the 293 cells, but p21^{ras} did not (Fig. 2*C*). Likewise, p73 also immunoprecipitated endogenous p19^{ras} from the 293 cells (Fig. 2*D*).

Furthermore, the specificity of p19 binding to p73 was assessed by determining the interaction of p21^{ras}V (Val-12), a constitutively active form of p21^{ras} (27), with p73. As shown in Fig. 2*E*, p21^{ras}V did not interact with p73β under the same conditions as p19^{ras} did. In addition, we examined the *in vivo* binding of p19^{ras} to p53 family, p53, p73α, and p73β. Results demonstrated that p19^{ras} interacts with both p53 and the p73 isoforms (Fig. 2*F*).

Determination of the Binding Region of p19^{ras} to p73—The next step was to determine the p73β-binding domain of p19^{ras}. Since p19^{ras} can be grouped into regions I, II, and III based on the alignment of other Ras proteins (H-Ras, K-Ras, and N-Ras), we constructed truncated mutants of p19^{ras}. After transfection of the p19^{ras} (p19^{ras}C1, -C2, and -C) mutants and p73β into the HEK293 cells, overexpressed GFP-p19^{ras} was immunoprecipitated with anti-GFP antibody, and the immunoprecipitates were Western blotted using anti-HA antibody to detect p73β. As shown in Fig. 3*A*, neither region II (p19^{ras}C1) nor region III (p19^{ras}C2) bounded to p73β. Instead, the p19^{ras}C mutant containing the amino acids 56–87 in region I interacted with p73β, suggesting that this is the binding region responsible for the interaction with p73β.

Assessment of p19^{ras}-binding Region of p73—To define the region of p73β that is required for the interaction with p19^{ras}, we performed *in vivo* binding assays with p19^{ras} and a series of truncated proteins that contained distinct domains of p73β. Although the transactivation domain (TAD)-containing region (amino acids 1–131) of p73 did not appear to interact with p19^{ras}, the p73 DNA-binding domain (DBD)-containing region (amino acids 54–310) strongly interacted with p19^{ras}. The p19^{ras} oligomerization domain (OD)-containing region (amino acids 310–499) also interacted with p19^{ras}, but the binding was weaker than with the DBD-containing region as more of GFP-tagged p19^{ras} protein was immunoprecipitated with less amount of p73β-DBD peptide precipitated (Fig. 3*B*). Therefore, this showed that p19^{ras} interacts

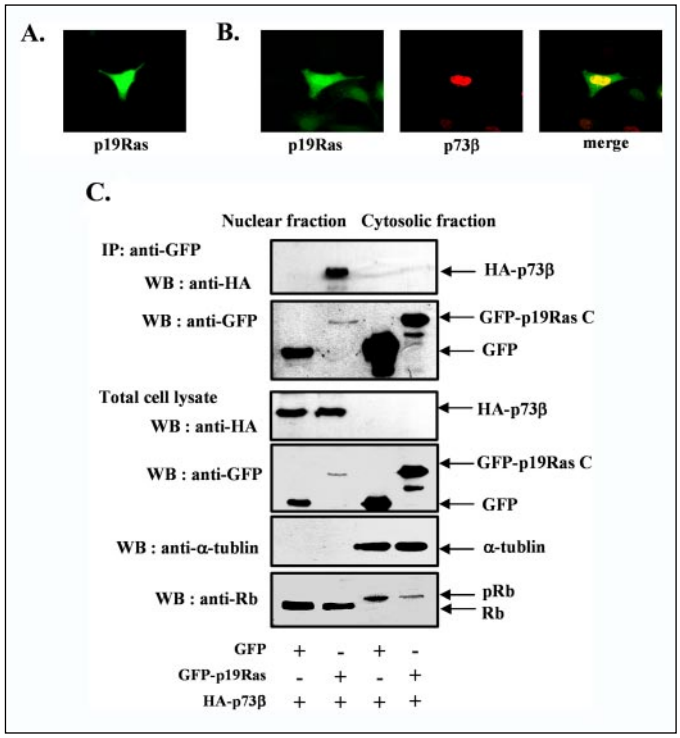

FIGURE 4. **Subcellular localization of p19^{ras}.** *A*, HeLa cells were transfected with a plasmid expressing GFP-fused p19^{ras} and subjected to immunofluorescence with anti-GFP antibody. *B*, both GFP-p19^{ras}- and HA-73β-expressing plasmids were transfected into HeLa cells. GFP-p19^{ras} and HA-73 were immunostained and visualized with green or red dye, respectively. *C*, H1299 cells were transfected with GFP-p19^{ras} and HA-73β, and their nuclear and cytosolic fractions were separated. Each subcellular fraction was immunoprecipitated (*IP*) with anti-GFP antibody, and proteins were detected by Western blotting (*WB*), using anti-HA antibody. Total lysates from either cytosolic or nuclear fraction were immunoblotted with anti-α-tubulin or anti-RB antibody, respectively.

with both the DBD and OD of p73β, but its binding is more prominent and sufficient with the DBD.

Colocalization and Interaction of p19^{ras} and p73β in the Nucleus—Since p73 predominantly locates in the nucleus, we determined whether p19^{ras} colocalizes with p73 in cultured mammalian cells using immunocytochemistry. The p19^{ras} protein was localized in both the nucleus and cytoplasm, as detected by anti-GFP antibody (Fig. 4*A*). Colocalization of p19^{ras} and p73 in the same cell was assessed using double immunofluorescence. HeLa cells were transfected with GFP-p19^{ras} and HA-73β. GFP-p19^{ras} was immunostained with green dye, and HA-73β was visualized by red fluorescence. As shown in Fig. 4*B*, p19^{ras} and p73 colocalize in the nucleus. To further confirm the colocalization and interaction of p19^{ras} and p73 in the nucleus, H1299 cells were transfected with GFP-p19^{ras} and HA-73β and nuclear, and cytosolic fractions were separated. The extracts were immunoprecipitated with anti-GFP antibody and detected by Western blot analysis using the anti-HA antibody. To ensure the complete separation of subcellular fractions, marker proteins (α-tubulin and Rb) were immunoblotted. Therefore, consistent with immunocytochemistry data, p19^{ras} specifically interacts with p73β in the nucleus (Fig. 4*C*).

Increased Transcriptional Activation of p73 by p19^{ras}—To examine whether p19^{ras} could affect p73β-dependent transcriptional activation, HeLa cells were transiently transfected with the p73β expression plasmid and a luciferase reporter plasmid containing the p73-responsive element from p21 promoter, together with or without the p19^{ras} expression plasmid. As shown in Fig. 5*A*, p19^{ras} alone did not induce the luciferase reporter activation and coexpression of p19^{ras} with p73β resulted in the increase of p21-luciferase activity, which was induced by p73 in a concentration-dependent manner. In addition, we measured the transcriptional activity of p73β in the presence of p19^{ras}C1, a mutant that does not bind to p73β, to test the necessity of p19^{ras} interaction in the functional activation of p73β. The mutant (p19^{ras}C1) was unable to activate the transcriptional function of p73β (Fig. 5*A*), indicating that the additional activation of p73β required its physical association with p19^{ras}. Transfection of p19^{ras} siRNA into HEK293 cells effectively decreased both overexpressed GFP-p19^{ras} and endogenous p19^{ras} expression (Fig. 5*B*, *top panel*). The silencing of p19^{ras} itself reduced transcriptional activity of p73β in some degrees and further prevented overexpressed p19^{ras}-mediated additional activation of p73 transactivity (Fig. 5*B*, *top panel*). Thus, these data coherently demonstrate that p19^{ras} is a positive regulatory protein of p73β.

A Direct Interaction of p19^{ras} to MDM2—The binding of MDM2 to p73 does not target p73 for degradation but suppresses the transcriptional function of p73 (13, 15). We investigated whether the p73β activation by p19^{ras} could be the result of inhibiting p73β-MDM2 interaction. First, we examined the amount of p73-MDM2 complexes in the presence of a p19^{ras} in H1299 cells. Expression of p19^{ras} remarkably inhibited the p73β-MDM2 interaction (Fig. 6*A*). Interestingly, coprecipitated p19^{ras} was detected in the anti-MDM2 immunoprecipitates (Fig. 6*A*), raising the possibility that p19^{ras} could be associated with p73β and/or MDM2. To examine whether p19^{ras} could interact with MDM2, we performed coprecipitation experiments using HEK293 cells transfected with MDM2 and p19^{ras} expression plasmids. As shown in Fig. 6*B*, p19^{ras} clearly immunoprecipitated with MDM2 in the absence of p73, confirming that MDM2 interacts with the p19^{ras} protein. The interaction of p19^{ras} with MDM2 in Fig. 6*B* could be mediated by endogenous p73. Thus, we silenced the expression of endogenous p73 by transfecting siRNA of p73 and performed immunoprecipitation experiments in HEK293 (Fig. 6*C*) and p53-null SK-OV-3 cells (Fig. 6*D*). Regardless of the presence of p73, p19^{ras} directly interacts with MDM2 in both cells. Under the conditions that stimulate p73 expression using doxorubicin, endogenous p19 doxorubicin was still interacted with endogenous MDM2 in the p73 siRNA-transfected HEK293 cells (Fig. 6*C*). Furthermore, we examined the interaction with MDM2 using p73 siRNA-transfected SK-OV-3 cells, which are deficient in p53. We also identified that the p19^{ras} could be directly interacted with MDM2 in the absence of p53 protein (Fig. 6*D*). Therefore, we demonstrate that the p19^{ras} is directly interacted with MDM2 in the absence of p73, p53, or both proteins.

Abrogation of MDM2-mediated p73 Inhibition by p19^{ras}—These results raise the possibility that p19^{ras} may abrogate the inhibitory effect of MDM2 on p73β through its interaction with MDM2. To test the possibility that p19^{ras} blocks MDM2-mediated inhibition of the transcriptional activity of p73β, we first determined the binding region of p19^{ras} to MDM2. As shown in Fig. 6*E*, p19^{ras}C1 and -C2 lost their association with MDM2 as with p73β, and an interaction was still detected by p19^{ras}C mutant protein. Subsequently, we performed a luciferase assay with MDM2, p73, and p19^{ras} in H1299 cells. Consistent with previous studies, MDM2 significantly reduced the level of p73β-mediated luciferase activity (Fig. 6*F*). Strikingly, p19^{ras} completely abolished the transcriptional inhibition caused by MDM2 (Fig. 6*F*). In addition, a p19^{ras}C1 mutant that lacks the interaction with both p73 and MDM2 (Fig. 6*E*) was not effective in blocking MDM2-induced transcriptional repression of p73 (Fig. 6*F*), suggesting that p19^{ras}-mediated blockage of the inhibitory effect of MDM2 on p73 requires the association of p19^{ras} to p73, MDM2, or both (Fig. 6*F*). Since the binding region of p19^{ras} to p73β and MDM2 overlaps, any further conclusion requires more studies.

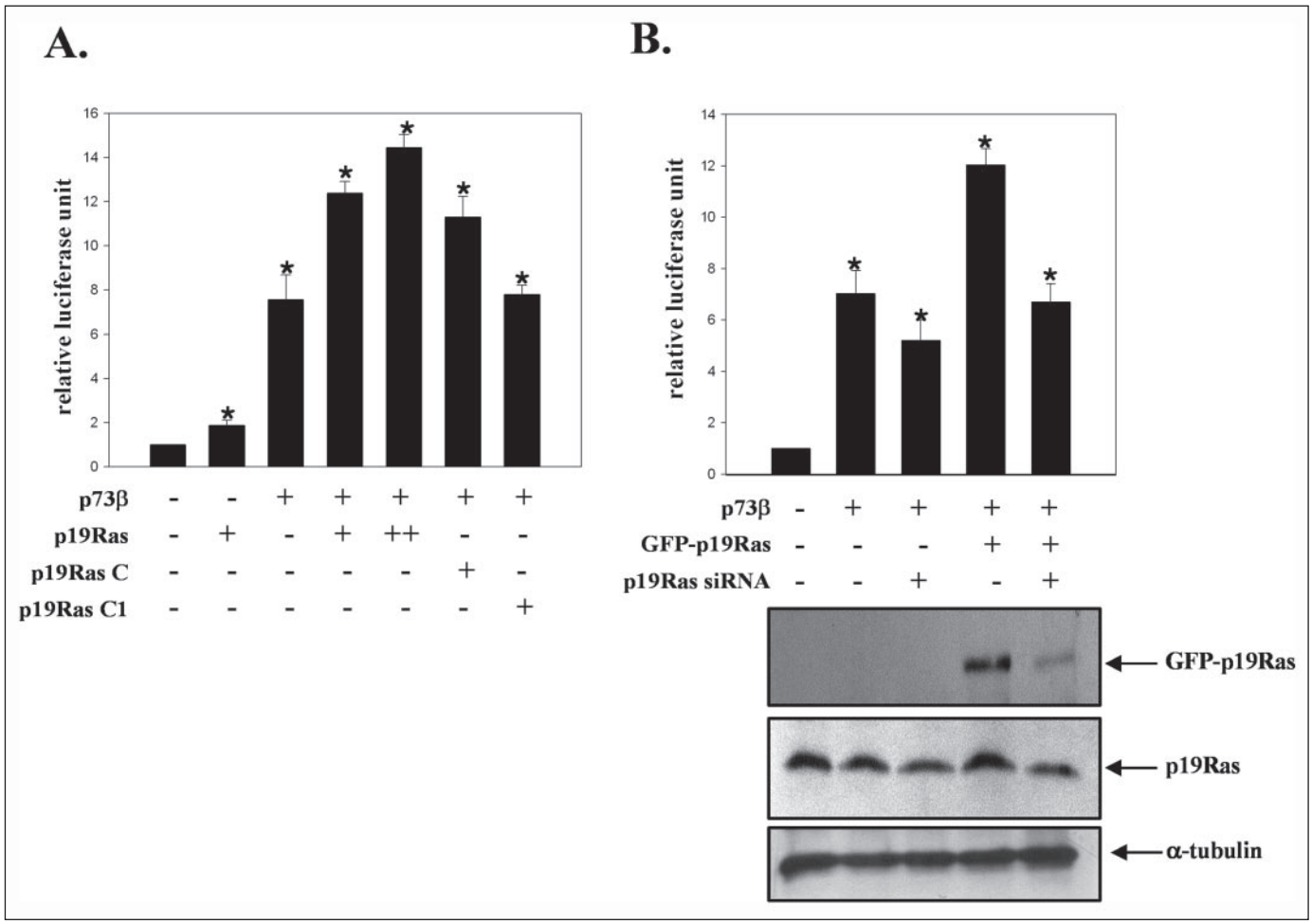

FIGURE 5. **Additional activation of p73β-mediated transcriptional activity by p19^{ras}.** HeLa cells were co-transfected with p21-Luc and pCMV-β, together with various sets of indicated plasmids. At 38 h after transfection, luciferase activity was measured. All data were normalized using β-galactosidase activity. Data are expressed in fold of relative luciferase unit normalized by control. All data are representatives of 3 independent experiments and all values are expressed as mean ± S.D. Statistically significance in luciferase activities are determined by Turkey's post hoc test, indicated by asterisks ($p < 0.05$). *A*, p73-expressing plasmid (0.5 μg) was transfected together with either p19^{ras} full-length (0.5 μg), increasing amounts (0.5–1 μg) of the plasmid encoding p19^{ras} or truncated p19^{ras} mutant (C and C1) DNA, as described in Fig. 3*A*. *B*, cells were transfected with p73-encoding plasmid in the presence or the absence of p19^{ras}, siRNA of p19^{ras}, or both, and luciferase activities were measured (*top panel*). In a reciprocal experiment, the expression levels of respective proteins were assessed by immunoblotting using anti-GFP and anti-p19^{ras} antibodies (*bottom panels*). Expression of α-tubulin was included as a loading control.

DISCUSSION

In this study, we report that p19^{ras}, an alternative splicing variant of H-*ras*, functioned as a direct interacting partner of p73β. Moreover, p19^{ras} was also able to bind to p53 and p73α indicating that the binding may be an essential and common feature of the p53 family proteins. We also observed that p19^{ras} is capable of activating p73β-dependent transcriptional activity of a reporter containing the p21^{ras} promoter. Furthermore, we demonstrated that p19^{ras} directly interacts with MDM2, a repressor of p73, which may results in the inhibition of MDM2 binding to p73. Consequently, p19^{ras} blocks MDM2-mediated transcriptional repression of p73 and leads to the activation of p73.

Recently, it has been shown that the heterogeneous nuclear ribonucleoprotein A1, SR proteins, and the RNA-dependent helicase p68 regulate alternative splicing events of c-H-*ras* (24). Alternative splicing of the c-H-*ras* precursor mRNA produced two transcripts: p19^{ras} (H-Ras IDX) and p21^{ras} (H-Ras4A). The difference in their sequences exists only at the C terminus region, constituting a short stretch of amino acids. The mutant study of p19^{ras} suggests that the sequences between amino acids 56 and 87 are required for the binding with p73 (Fig. 3*A*) and MDM2 (Fig. 6*E*). Although p21^{ras} and its active mutant form, p21^{ras}V, retain this region, they were not able to interact with p73 (Fig. 2), suggesting that the C-terminal end sequences are also important for association with p73. Current data indicate that the sequences in the C terminus end may significantly affect the conformation of Ras proteins in the selective and proper association with binding partners. However, further studies are needed to reach a conclusion.

Binding of GTP to Ras is accompanied by a conformational change in Ras, allowing it to bind to various effector proteins, including c-Raf, a kinase involved in triggering the mitogen-activated protein kinase pathway (28, 29). Unlike p21^{ras}, p19^{ras} has little GTP binding activity, as it lacks two important GTP-binding sites located in the C terminus end (E4A) of the p21^{ras} (25). This difference in p19^{ras} and p21^{ras} may affect their localizations and functions, but the function of the p19^{ras} protein has not been described previously. For effective Ras signaling, p21^{ras} proteins must undergo post-translational modifications that facilitate their attachment to the plasma membrane (22, 23). Our data and a previous report show that p19^{ras} is localized in the cytoplasm and the nucleus, while p21^{ras} resides in the plasma membrane where it associates with GTP for further signaling (25), and p73 resides exclusively in the nucleus where it interacts with p19^{ras} (Fig. 4). It has been reported that p21^{ras}V is localized in the nucleus and cytoplasm (27). Although the mutant p21^{ras}V was present in the nucleus, it did not result in binding to

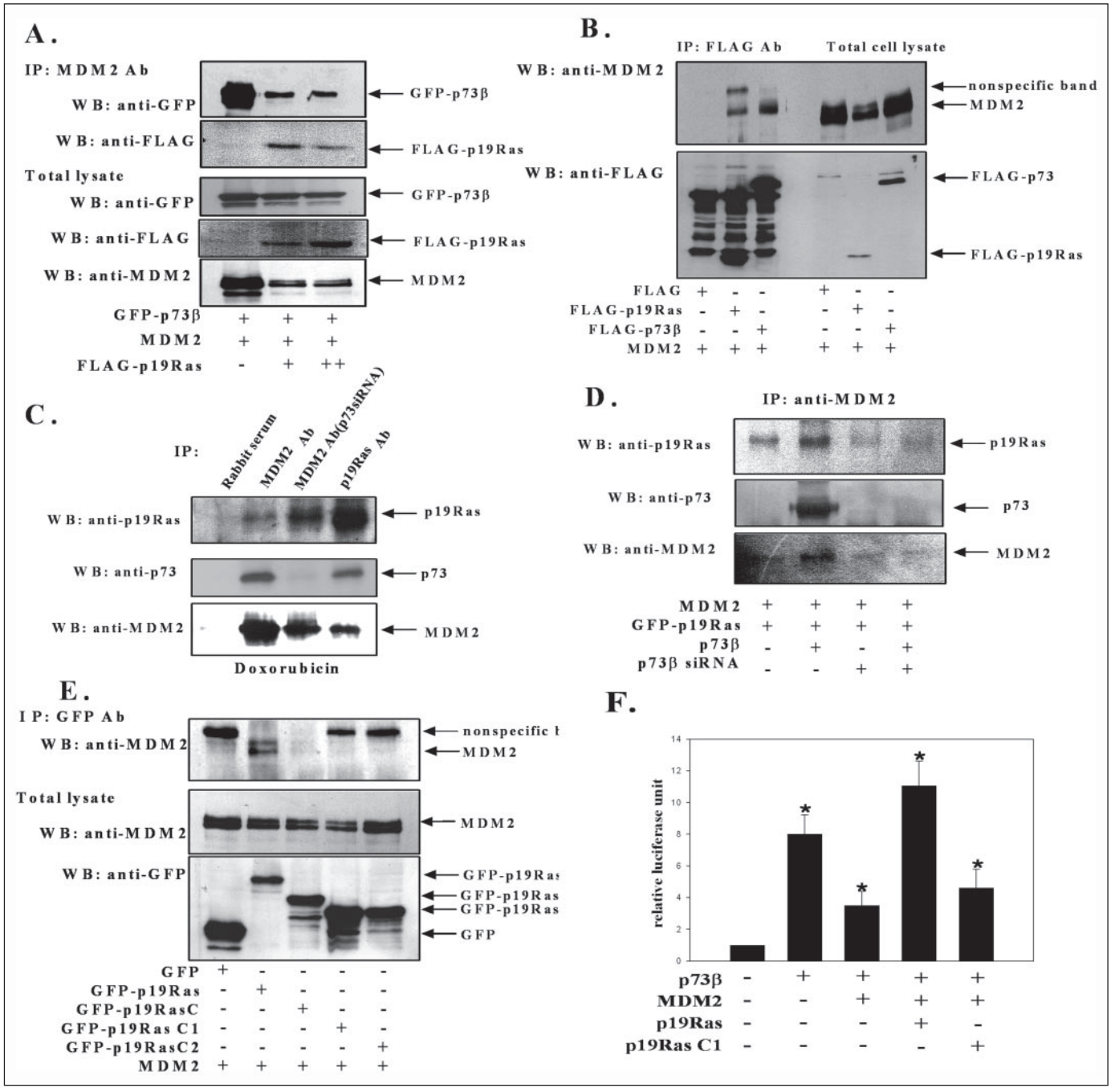

FIGURE 6. **Binding of p19^{ras} to MDM2 and p19^{ras}-mediated inhibition of MDM2-binding p73 and blockage of repression of p73 activity by MDM2.** *A*, H1299 cells were transiently transfected with the expression plasmids for both GFP-p73 (1 μg) and MDM2 (1 μg) with increasing amounts of the p19^{ras} expression plasmid (0, 1, or 2 μg). Whole-cell lysates were immunoprecipitated (*IP*) with the anti-MDM2 antibody, followed by Western blotting (*WB*) using the anti-GFP and M2 antibody. Total lysates were immunoblotted with the anti-GFP and anti-MDM2 antibodies. *B*, HEK293 cells were transfected with indicated plasmids. Total lysates were immunoprecipitated using FLAG M2 antibody and detected by the anti-MDM2 antibody. *C*, under the conditions that stimulate p73 expression using doxorubicin, *in vivo* interaction of endogenous p19^{ras} with MDM2 proteins was determined in the p73 siRNA-transfected HEK293 cells. Using anti-MDM2 or anti-p19^{ras} antibodies, the lysates were coprecipitated and immunoblotted with anti-p19^{ras}, anti-p73, and anti-MDM2 antibodies. *D*, SK-OV-3 cells were transfected with indicated plasmids and p73 siRNA. Total lysates were immunoprecipitated using MDM2 antibody and detected by the anti-p19^{ras}, anti-MDM2, and anti-p73 antibodies. *E*, each of the differently truncated forms of GFP-fused p19^{ras} plasmids was transfected with a plasmid coding MDM2 into the 293 cells. Total cell lysates were immunoprecipitated with anti-GFP antibody, and MDM2 was detected by Western blot analysis using an anti-MDM2 antibody. Expression of the respective mutants and MDM2 was confirmed by immunoblotting. *F*, H1299 cells were cotransfected with the indicated expression plasmids together and the reporter plasmid containing the p21 promoter driving luciferase expression. At 48 h after transfection, cells were lysed and subjected to luciferase assays. p19^{ras}C1 included as a negative control. Data (mean $\pm$ S.D.) were normalized using β-galactosidase activity and expressed in fold of relative luciferase unit normalized by control. All data are representatives of three independent experiments. Statistically significant differences in relative units of luciferase activities are determined by Turkey's *post hoc* test, indicated by *asterisks* ($p < 0.05$).

p73, suggesting that the lack of interaction between p21^{ras} and p73 may not be due to different localization but to differences in sequences among Ras proteins.

Until now, several isoforms of p73 (α, β, γ, δ, ϵ, θ, ζ, and ΔN-p73) have been cloned from various species (30). Many of these isoforms differ in their C-terminal sequences and transactivating activities (31). According to our data, p19^{ras} binding to β isoform of p73 was mediated mainly by the DNA-binding domain of p73 (Fig. 3*B*). Therefore, p19^{ras} would be a likely and common regulator for other splicing variants of p73, since they all possess the DNA-binding motif. However, the binding ability of

p19^{ras} to diverse p73 proteins and the elucidation of the functional roles of each interaction require additional studies.

Furthermore, we tested the physiological significance of binding between p19^{ras} and p73 by assessing modulations in the transcriptional activity of p73. p19^{ras} significantly increased the activity in a concentration-dependent manner, in which the stimulatory effect was mediated by the direct binding of p19^{ras} to p73 (Fig. 5). It has been shown that the p73 protein is regulated by diverse factors, including MDM2, MDMX, p300/CBP, Amphiphysin IIb-1, ASPP (apoptosis-stimulated proteins of p53) 1 and 2, c-Abl (cellular Abelson oncogene), c-Myc, CTF2 (CCAAT-binding transcription factor 2), Cyclin G, HCV (hepatitis C virus protein), MM1, PMS2 (mismatch-repair protein s2), WT1 (Wilms tumor protein), and YAP (Yes-associated protein) (15, 16, 32–43). Among these regulators, MDM2 is a well known repressor of p73 and p53, but its inhibitory effect on p73 does not involve degradation. Thus, we further tested the possibility that p19^{ras}-mediated stimulatory effects on p73 involved the blockage of the repression of MDM2. Surprisingly, p19^{ras} also interacted with MDM2 directly and completely abolished MDM2-mediated inhibition of p73 activity (Fig. 6). The association of p63 to p19^{ras} and its ability to modulate p63 activity would be interesting to investigate, since MDM2 activates the transcriptional effect of p63, whereas it represses the transcriptional effects of p73 and p53 (44).

In addition, we found that p19^{ras} significantly decreases the physical contact between MDM2 and p73β (Fig. 6*A*). The following are possible speculations that may lead to the inhibitory effect of p19^{ras}: 1) p19^{ras} binding to p73 prevents the association of MDM2 to p73; 2) p19^{ras} binds to MDM2, which prohibits the interaction of MDM2 to p73; 3) the direct contact of p19^{ras} to both p73 and MDM2 simultaneously affects conformation of both proteins; and/or 4) the interaction of p19^{ras} to MDM2 increases the degradation of the MDM2 protein. Regardless, our findings provide a solid molecular and functional linkage between p73, MDM2, and p19^{ras} in cell signaling.

Also, we observed that p19^{ras} reduced the expression of MDM2 protein in H1299 cells deficient of p53 (Fig. 6). p19^{ras} may act as a negative regulator of p21^{ras}, which induces MDM2 via the Raf-MEK-ERK-ARF pathway, but this is unlikely, since p19^{ras} does not interact with Raf (25, 45, 46). Another possibility is that p19^{ras} may promote the degradation of MDM2. MDM2 possesses the activity of an E3 ubiquitin ligase, which autoubiquitinates as well as ubiquitinates other proteins. The balance between autoubiquitination and substrate ubiquitination of MDM2 is modulated by post-translational modifications, including sumoylation and phosphorylation. Thus, protein-protein interactions may affect MDM2 E3 ligase activity. At the same time, it is possible that p19^{ras} acts in the same manner as ARF and L11, which sequester MDM2 in the nucleus. Although the underlying mechanism needs further study, our current data suggest that p19^{ras} is a novel inhibitor of MDM2.

Despite more than 2 decades of intensive study, the effects of Ras on cell physiology remain to be fully elucidated. In this respect, it is important to note that our findings contribute to a functional linkage between p19^{ras} and p73β in the biological network, showing that p19^{ras} associates with and regulates p73β by involving its negative regulator, the MDM2 protein.

REFERENCES

1. Kaghad, M., Bonnet, H., Yang, A., Creancier, L., Biscan, J. C., Valent, A., Minty, A., Chalon, P., Lelias, J. M., Dumont, X., Ferrara, P., McKeon, F., and Caput, D. (1997) *Cell* **90,** 809–819
2. Jost, C. A., Marin, M. C., and Kaelin, W. G., Jr. (1997) *Nature* **389,** 191–194
3. Kaelin, W. G., Jr. (1999) *J. Natl. Cancer Inst.* **91,** 594–598
4. Lee, C. W., and La Thangue, N. B. (1999) *Oncogene* **18,** 4171–4181
5. Zhu, J., Jiang, J., Zhou, W., and Chen, X. (1998) *Cancer Res.* **58,** 5061–5065
6. Marin, M. C., Jost, C. A., Irwin, M. S., DeCaprio, J. A., Caput, D., and Kaelin, W. G., Jr. (1998) *Mol. Cell Biol.* **18,** 6316–6324
7. Roth, J., and Dobbelstein, M. (1999) *J. Gen. Virol.* **80,** 3251–3255
8. Vikhanskaya, F., Marchini, S., Marabese, M., Galliera, E., and Broggini, M. (2001) *Cancer Res.* **61,** 935–938
9. Jones, S. N., Hancock, A. R., Vogel, H., Donehower, L. A., and Bradley, A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 15608–15612
10. Kawai, H., Wiederschain, D., and Yuan, Z. M. (2003) *Mol. Cell Biol.* **23,** 4939–4947
11. Li, M., Brooks, C. L., Wu-Baer, F., Chen, D., Baer, R., and Gu, W. (2003) *Science* **302,** 1972–1975
12. Shmueli, A., and Oren, M. (2004) *Mol. Cell* **13,** 4–5
13. Balint, E., Bates, S., and Vousden, K. H. (1999) *Oncogene* **18,** 3923–3929
14. Maisse, C., Guerrieri, P., and Melino, G. (2003) *Biochem. Pharmacol.* **66,** 1555–1561
15. Zeng, X., Chen, L., Jost, C. A., Maya, R., Keller, D., Wang, X., Kaelin, W. G., Jr., Oren, M., Chen, J., and Lu, H. (1999) *Mol. Cell. Biol.* **19,** 3257–3266
16. Kim, K. C., Kim, T. S., Kang, K. H., and Choi, K. H. (2001) *Oncogene* **20,** 6689–6699
17. Cohen, J. B., Broz, S. D., and Levinson, A. D. (1989) *Cell* **58,** 461–472
18. Chen, C. Y., and Faller, D. V. (1995) *Oncogene* **11,** 1487–1498
19. Downward, J. (1998) *Curr. Opin. Genet. Dev.* **8,** 49–54
20. Macaluso, M., Russo, G., Cinti, C., Bazan, V., Gebbia, N., and Russo, A. (2002) *J. Cell. Physiol.* **192,** 125–130
21. Malumbres, M., and Pellicer, A. (1998) *Front. Biosci.* **3,** 887–912
22. Reuther, G. W., and Der, C. J. (2000) *Curr. Opin. Cell Biol.* **12,** 157–165
23. Takai, Y., Sasaki, T., and Matozaki, T. (2001) *Physiol. Rev.* **81,** 153–208
24. Guil, S., Gattoni, R., Carrascal, M., Abián, J., Stévenin, J., and Bach-Elias, M. (2003) *Mol. Cell. Biol.* **23,** 2927–2941
25. Guil, S., de La Iglesia, N., Fernandez-Larrea, J., Cifuentes, D., Ferrer, J. C., Guinovart, J. J., and Bach-Elias, M. (2003) *Cancer Res.* **63,** 5178–5187
26. Kang, K. H., Lee, J. H., Kim, K. C., Ham, S. W., Kim, M. Y., and Choi, K. H. (2002) *FEBS Lett.* **522,** 161–167
27. Wurzer, G., Mosgoeller, W., Chabicovsky, M., Cerni, C., and Wesierska-Gadek, J. (2001) *J. Cell. Biochem.* **81,** 1–11
28. Fujita-Yoshigaki, J., Shirouzu, M., Ito, Y., Hattori, S., Furuyama, S., Nishimura, S., and Yokoyama, S. (1995) *J. Biol. Chem.* **270,** 4661–4667
29. Moodie, S. A., Paris, M., Villafranca, E., Kirshmeier, P., Willumsen, B. M., and Wolfman, A. (1995) *Oncogene* **11,** 447–454
30. Zwahlen, D., Tschan, M. P., Grob, T. J., Peters, U. R., Fink, D., Haenggi, W., Altermatt, H. J., Cajot, J. F., Tobler, A., Fey, M. F., and Aebi, S. (2000) *Int. J. Cancer* **88,** 66–70
31. Ueda, Y., Hijikata, M., Takagi, S., Chiba, T., and Shimotohno, K. (1999) *Oncogene* **18,** 4993–4998
32. Ongkeko, W. M., Wang, X. Q., Siu, W. Y., Lau, A. W., Yamashita, K., Harris, A. L., Cox, L. S., and Poon, R. Y. (1999) *Curr. Biol.* **9,** 829–832
33. Zeng, X., Li, X., Miller, A., Yuan, Z., Yuan, W., Kwok, R. P., Goodman, R., and Lu, H. (2000) *Mol. Cell. Biol.* **20,** 1299–1310
34. Bergamaschi, D., Samuels, Y., Jin, B., Duraisingham, S., Crook, T., and Lu, X. (2004) *Mol. Cell. Biol.* **24,** 1341–1350
35. Agami, R., Blandino, G., Oren, M., and Shaul, Y. (1999) *Nature* **399,** 809–813
36. Uramoto, H., Izumi, H., Ise, T., Tada, M., Uchiumi, T., Kuwano, M., Yasumoto, K., Funa, K., and Kohno, K. (2002) *J. Biol. Chem.* **277,** 31694–31702
37. Uramoto, H., Izumi, H., Nagatani, G., Ohmori, H., Nagasue, N., Ise, T., Yoshida, T., Yasumoto, K., and Kohno, K. (2003) *Biochem. J.* **371,** 301–310
38. Ohtsuka, T., Ryu, H., Minamishima, Y. A., Ryo, A., and Lee, S. W. (2003) *Oncogene* **22,** 1678–1687
39. Alisi, A., Giambartolomei, S., Cupelli, F., Merlo, P., Fontemaggi, G., Spaziani, A., and Balsano, C. (2003) *Oncogene* **22,** 2573–2580
40. Watanabe, K., Ozaki, T., Nakagawa, T., Miyazaki, K., Takahashi, M., Hosoda, M., Hayashi, S., Todo, S., and Nakagawara, A. (2002) *J. Biol. Chem.* **277,** 15113–15123
41. Shimodaira, H., Yoshioka-Yamashita, A., Kolodner, R. D., and Wang, J. Y. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 2420–2425
42. Scharnhorst, V., Dekker, P., van der Eb, A. J., and Jochemsen, A. G. (2000) *J. Biol. Chem.* **275,** 10202–10211
43. Strano, S., Munarriz, E., Rossi, M., Castagnoli, L., Shaul, Y., Sacchi, A., Oren, M., Sudol, M., Cesareni, G., and Blandino, G. (2001) *J. Biol. Chem.* **276,** 15164–15173
44. Calabro, V., Mansueto, G., Parisi, T., Vivo, M., Calogero, R. A., and La Mantia, G. (2002) *J. Biol. Chem.* **277,** 2674–2681
45. Ries, S., Biederer, C., Woods, D., Shifman, O., Shirasawa, S., Sasazuki, T., McMahon, M., Oren, M., and McCormick, F. (2000) *Cell* **103,** 321–330
46. Halaschek-Wiener, J., Wacheck, V., Kloog, Y., and Jansen, B. (2004) *Cell. Signal.* **16,** 1319–1327
47. Horvath, A., and Riezman, H. (1994) *Yeast* **10,** 1305–1310