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Amphiphysin IIb-1, a novel splicing variant of amphiphysin II, regulates p73β function through protein-protein interactions

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p73 is a nuclear protein that is similar in structure and function to p53. Notably, the C-terminal region of p73 has a regulatory function, through interactions with a positive or negative regulator. In this study, we use the yeast two-hybrid technique to identify a novel p73β binding protein, designated amphiphysin IIb-1. Amphiphysin IIb-1 is one of the splicing variants of amphiphysin II, and has a shorter protein product than amphiphysin IIb, which has been previously reported. We confirmed that amphiphysin IIb-1 binds full-length p73β, both *in vitro* and *in vivo*. This association is mediated via the SH3 domain of amphiphysin IIb-1 and C-terminal amino acids 321–376 of p73β. Double immunofluorescence patterns revealed that p73β is relocalized to the cytoplasm in the presence of amphiphysin IIb-1. Overexpression of amphiphysin IIb-1 was found to significantly inhibit the transcriptional activity of p73β in a dose-dependent manner. In addition, the cell death function of p73β was inhibited by amphiphysin IIb-1. These findings offer a new insight into the regulation mechanism of p73β, and suggest that amphiphysin IIb-1 modulates p73β function by direct binding. *Oncogene* (2001) **20,** 6689–6699.

Keywords: p73; amphiphysin IIb-1; SH3 domain; NLS; yeast two-hybrid; splicing variants

Introduction

Protein-protein interaction is one of the central events in cellular biological response. Protein interactions are often mediated by non-catalytic conserved domains in their sequences. Yeast two-hybrid systems have been developed as a powerful genetic tool to rapidly select uncharacterized proteins that specifically interact with a defined protein partner from a library (Field and Song, 1989; Le Douarin *et al*., 1995; Licitra and Liu, 1996; Tirode *et al*., 1997).

Recently, several investigators identified the nuclear proteins p73, which exist as multiple isoforms generated by alternative splicing (Kaghad *et al*., 1997; De Laurenzi *et al*., 1998; Ueda *et al*., 1999). These proteins exhibit sequence similarity with the transactivation, DNA-binding, and oligomerization domains of p53. In this regard, overexpression of p73 not only activates several p53 target genes, but also induces apoptosis in cells (Kaghad *et al*., 1997; Jost *et al*., 1997). However, a number of studies have demonstrated that p53 and p73 proteins are regulated differentially. Unlike p53, the p73 protein is not induced by DNA-damaging agents like actinomycin D or UV (Kaghad *et al*., 1997). Moreover, steady-state levels of p73 are not altered by interactions with mdm2, which induces proteasome-mediated degradation of p53 (Haupt *et al*., 1997; Zeng *et al*., 1999; Dobbelstein *et al*., 1999). Equally, multiple adenoviral proteins that bind and inhibit the activity of p53 do not interact with p73 (Martin *et al*., 1998; Roth *et al*., 1998; Steegenga *et al*., 1999; Wienzek *et al*., 2000). These observations raise a possibility that distinct processes contribute to the biological activities of p73 and p53.

Interestingly, it was recently reported that infrequent mutations in the C-terminal region of p73 result in loss of protein function (Ozaki *et al*., 1999; Ichimiya *et al*., 1999). Infrequent mutation of p73 has been known in C-terminal region of p73, whereas point mutation of p53 is mostly concentrated in the central DNA binding region (Kleihues *et al*., 1997; Ichimiya *et al*., 1999). C-terminal mutants of p73 reduce transactivation capacity *in vitro*, implying that this region is functionally significant (Takada *et al*., 1999). C-terminal region of p73 contains several binding motifs, oligomerization domains and the conserved PxxP motif, which is a potential site of interaction with the SH3 domain (Kaghad *et al*., 1997; Yuan *et al*., 1999). The oligomerization domain of p73 is associated with the p53 family. Excess amounts of p73 or p53 mutant can efficiently inhibit transcriptional transactivation by p73, indicating that p73 is functionally regulated via homo- or heterotypic oligomerization (Davison *et al*., 1999; Di Como *et al*., 1999; Strano *et al*., 2000). Moreover, the p73 protein is activated by c-abl tyrosine kinase-mediated phosphorylation in the pro-apoptotic signaling pathway (Agami *et al*., 1999; Gong *et al*., 1999; Yuan *et al*., 1999). The C-terminal PxxP motif of p73 interacts with the SH3 domain of c-abl tyrosine kinase, suggesting that this sequence may have a regulatory

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function in p73 activity in stimulated cells. The SH3 domain is present in several different proteins, and mediates protein-protein interactions that modulate signal transduction. Based on these observations, it is proposed that the PxxP motif may be important for p73 function, through interactions with SH3 domain-containing proteins. Earlier data, therefore, collectively indicate that p73 activity is mediated by its C-terminal region.

For our experiments, we constructed pLexA-73C, containing the C-terminal region of p73β, to identify proteins that bind p73β, using the yeast two-hybrid system. Novel amphiphysin IIb-1 was obtained as a p73β-interacting protein. In addition, co-expression of amphiphysin IIb-1 and p73β resulted in the inhibition of transactivation and cell death function of p73β. Therefore, we suggest that amphiphysin IIb-1 regulates the function of p73β under physiological conditions.

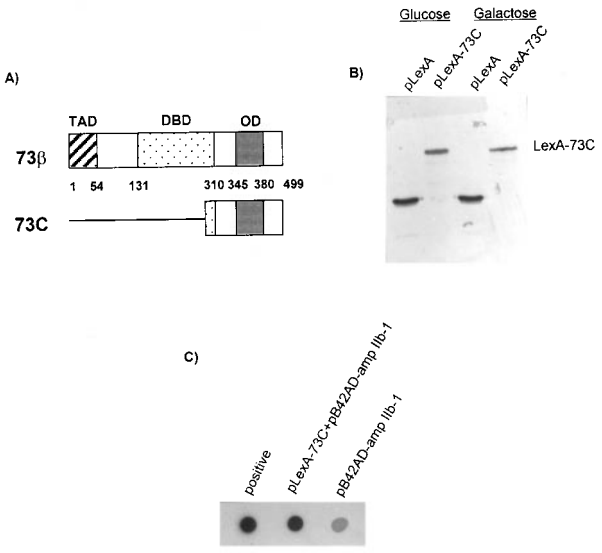

Figure 1 Bait construction and identification of novel amphiphysin IIb-1 from yeast two-hybrid assay. (**a**) C-terminal bait was cloned from pLexA-73β vector with an N-terminal deletion. pLexA-73C contained amino acids 248–499 of full-length p73β. (**b**) Bait plasmid was transformed into EGY48 [p8op-LacZ], and cultured until OD_{600} of 0.6 in SD/Glu/ -Ura/-His or SD/Gal/Raf/-Ura/-His selection medium. Yeast protein was isolated with lysis buffer and Western blotting was performed, using standard protocols. (**c**) Novel amphiphysin IIb-1 was identified after library transformation and tested for β-galactosidase activity in a whole plate assay with 5-bromo-4-chloro-3-indolyl β-D-galactoside. A combination of SNF-1 and SNF-4 was used as a positive control

Results

Bait construction and characterization of novel amphiphysin IIb-1

Point mutations have been reported in the C-terminal region of p73, which resulted in the inhibition of transactivation of p73 *in vitro* (Ichimiya *et al*., 1999; Takada *et al*., 1999). This suggests that p73 activity is regulated via interactions between unknown protein(s) and the C-terminal region of p73. In order to identify binding protein(s) with this region using the yeast two-hybrid, we constructed the C-terminal region of p73β fused to the DNA binding domain of LexA (pLexA-73C) as bait (Figure 1a). Upon introduction of the pLexA-73C vector into yeast strain EGY48, the bait protein was highly expressed in conditioned medium containing glucose and galactose, respectively (Figure 1b). Moreover, pLexA-73C alone did not exhibit intrinsic β-galactosidase activity, which was useful in detecting interactions between bait and prey within yeast cells (data not shown). Using pLexA-73C, we screened a human brain cDNA library expressing proteins fused to the LexA transcriptional activation domain. Screening of 2×10^6 yeast transformants led to the isolation of one positive clone (Figure 1c). The clone was comprised of partial cDNA (1582 bp) with 3′ non-coding regions, in addition to a coding sequence (Figure 2a, underlined). A putative polyadenylation signal (ATTAAA) was found upstream of the poly(A) tail (Figure 2a, boxed) in the 3′ untranslated. Nucleotide sequence analysis revealed that the clone was identical to amphiphysin IIb, one of amphiphysin II splicing variants, described by Ramjaun and McPherson (1997) (accession No. AF043898), except for a sequence variation in the 3′-region (Figure 2a, bold). Therefore, we concluded that the clone comprised a 1473 bp open reading frame encoding 490 amino acids, and is a variant of amphiphysin II. The protein was designated amphiphysin IIb-1, based on its sequence similarity to amphiphysin IIb. A termination codon (TGA) at positions 1471–1473 of amphiphysin IIb-1 revealed that the variant was a protein with 28 less amino acids at the C-terminus than amphiphysin IIb (Figure 2b). Western blot analyses demonstrated a higher molecular size (70 kDa) than predicted (55.1 kDa) from the number of amino acids, suggesting modifications at the post-translational level (data not shown).

Physical interaction of p73 with amphiphysin IIb-1

To determine whether amphiphysin IIb-1 interacts with full-length p73β, GST-fused amphiphysin IIb-1 was produced, and used in an *in vitro* binding assay with ^{35}S-methionine-labeled full-length p73β (Figure 3a, left panel). Full-length p73β interacted with GST-amphiphysin IIb-1, but not with GST alone (Figure 3a, right panel). To further validate this interaction *in vivo*, we examined the co-precipitation of full-length HA-p73β and FLAG-amphiphysin IIb-1 from transiently transfected 293 human embryonic kidney cells. Western blot analyses confirmed expression of HA-73β and FLAG-amphiphysin IIb-1 in each transfected group (Figure 3b, lower panels). Cell lysates were incubated with anti-FLAG antibody, followed by Western blotting with anti-HA antibody. The HA-73β protein was detected, along with FLAG-amphiphysin IIb-1. Reciprocally, FLAG-amphiphysin IIb-1 co-immunoprecipitated with anti-HA antibody (Figure 3b, upper panels). Therefore,

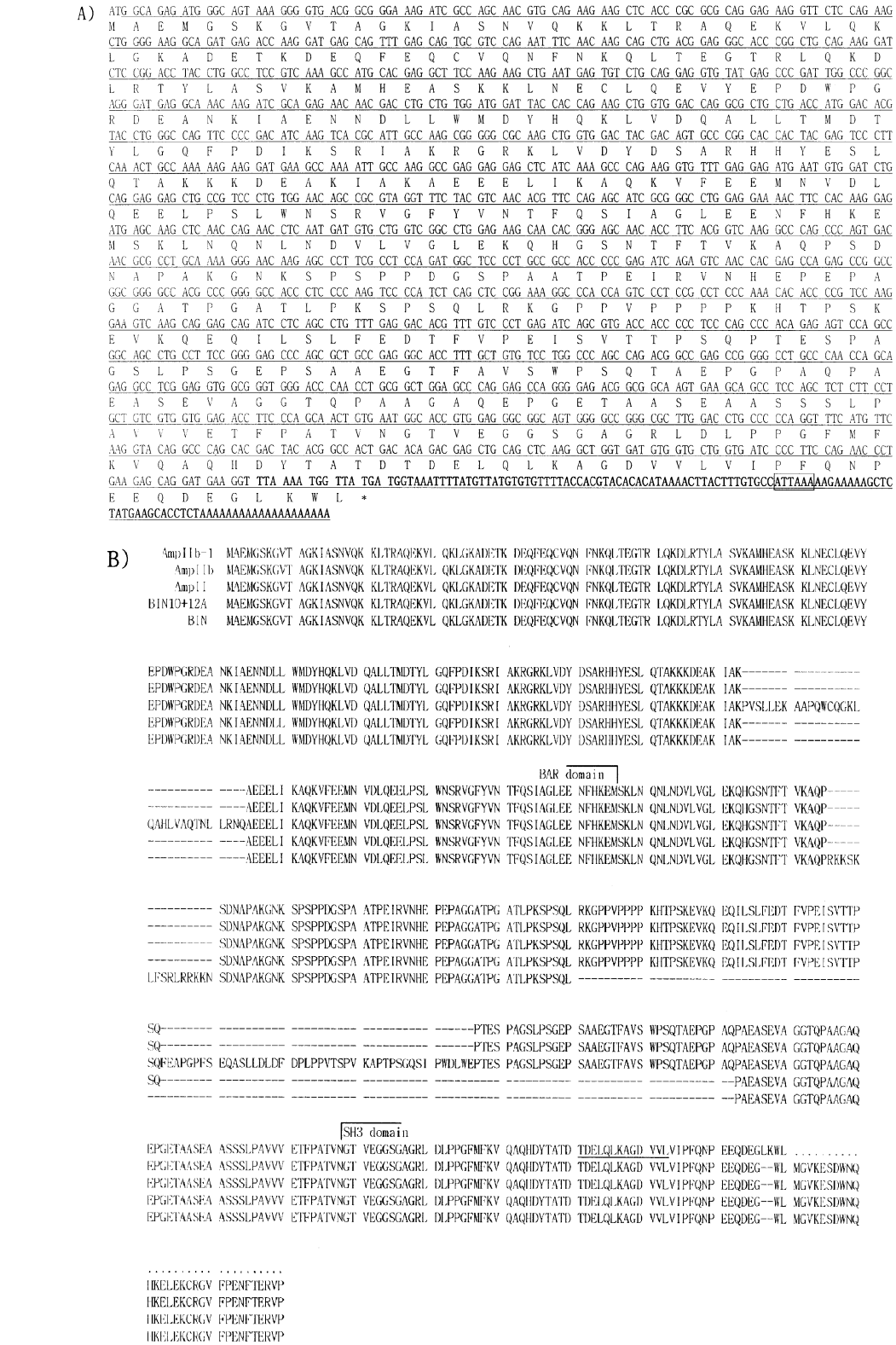

Figure 2 cDNA sequence and amino acid alignment of novel amphiphysin IIb-1. (**a**) DNA sequencing was performed with two sets of sequencing primers from pB42AD vector (5′-CCAGCCTCTTGCTGAGTGGAGATG-3′, 5′-ATTGGAGACTTGACCAAACCTC-3′), and internal primer from amphiphysin IIb-1 (5′-AGATGAGCAAGCTCAACCA-3′). The sequence depicted represents full-length amphiphysin IIb-1. (**b**) Sequences of other amphiphysin II–related proteins are shown. Amphiphysin IIb-1 is identical to amphiphysin IIb except for its truncated C-terminus. BAR domain and SH3 domains are strictly conserved in all amphiphysin II proteins

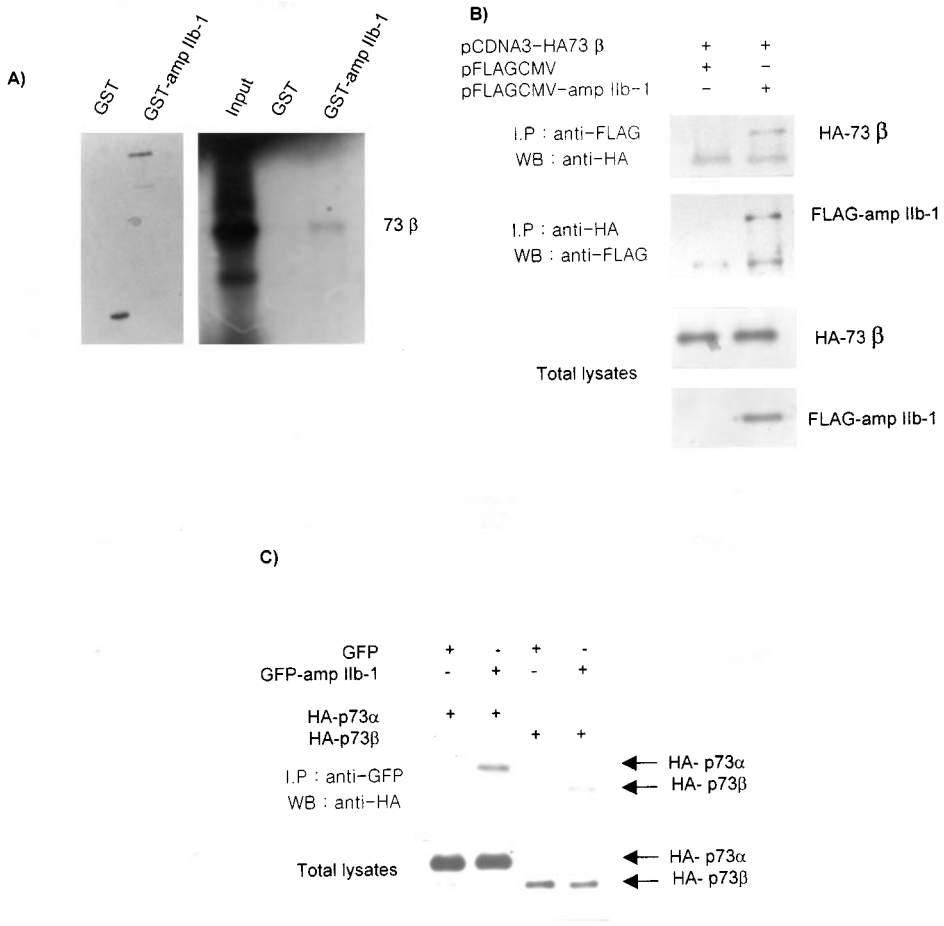

Figure 3 *In vitro* and *in vivo* interactions of p73-amphiphysin IIb-1. (**a**) Left panel; Amphiphysin IIb-1 was cloned into pGEX4T1 prokaryotic expression vector and protein induced by IPTG treatment. An aberrant protein (100 kDa) was detected from Western blot analysis with an anti-GST antibody. Right panel; GST (5 μg), or GST-amphiphysin IIb-1 was mixed with equal amounts 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**) Upper panels; 293 cells were transfected with respective plasmids (6 μg). Total cell lysates were prepared by sonication, and immunoprecipitated with indicated antibody for 3 h at 4°C, with gentle agitation. After washing three times with PBS containing 1 mM DTT, samples were detected by Western blotting, using the appropriate antibody. Lower panels; Expression of transfected plasmids are shown for comparison. (**c**) 293 cells were additionally transfected with HA-tagged p73α and GFP-amphiphysin IIb-1

these results confirmed that full-length p73β interacts with amphiphysin IIb-1, both *in vitro* and *in vivo*. We additionally examined the *in vivo* binding of p73α and amphiphysin IIb-1. Expression of GFP-amphiphysin IIb-1 and HA-tagged proteins were confirmed by green fluorescence microscopy and Western blotting, respectively (data not shown and Figure 3c, lower panel). Results established that amphiphysin IIb-1 interacts with both p73α and p73β (Figure 3c, upper panel). The next step was to determine the p73β-binding domain of amphiphysin IIb-1. Since amphiphysin IIb-1 can be grouped into several functional domains, deletion fragments of the protein were constructed in a pB42AD yeast expression vector (Figure 4a). Fragment expression was validated by Western blotting with HA antibody in transformed yeast cells (data not shown). Following the introduction of these fragments into yeast cells expressing the C-terminal region of p73β, β-galactosidase activity was quantitated (Figure 4b). The reporter gene was activated upon co-expression of the C-terminus of p73β and either the SH3 domain or full-length amphiphysin IIb-1 only. Moreover, a single mutant (P338A) of the PxxP motif and deletion fragments of p73β were also generated to determine the binding site for amphiphysin IIb-1 (Figure 4c). Co-transformation of the mutant form of p73C with amphiphysin IIb-1 activates the reporter gene (data not shown). Furthermore, co-transformation of 73C2 or 73C3 but not 73C1 with amphiphysin IIb-1, induces β-galactosidase activity. Therefore, progressive deletion of the C-terminal region of p73β revealed a fragment (comprising amino acids 321–376) essential for this interaction. Collectively, our data confirm that amphiphysin IIb-1 interacts with full-length p73β under physiological conditions, and that binding is specifically dependent on the SH3

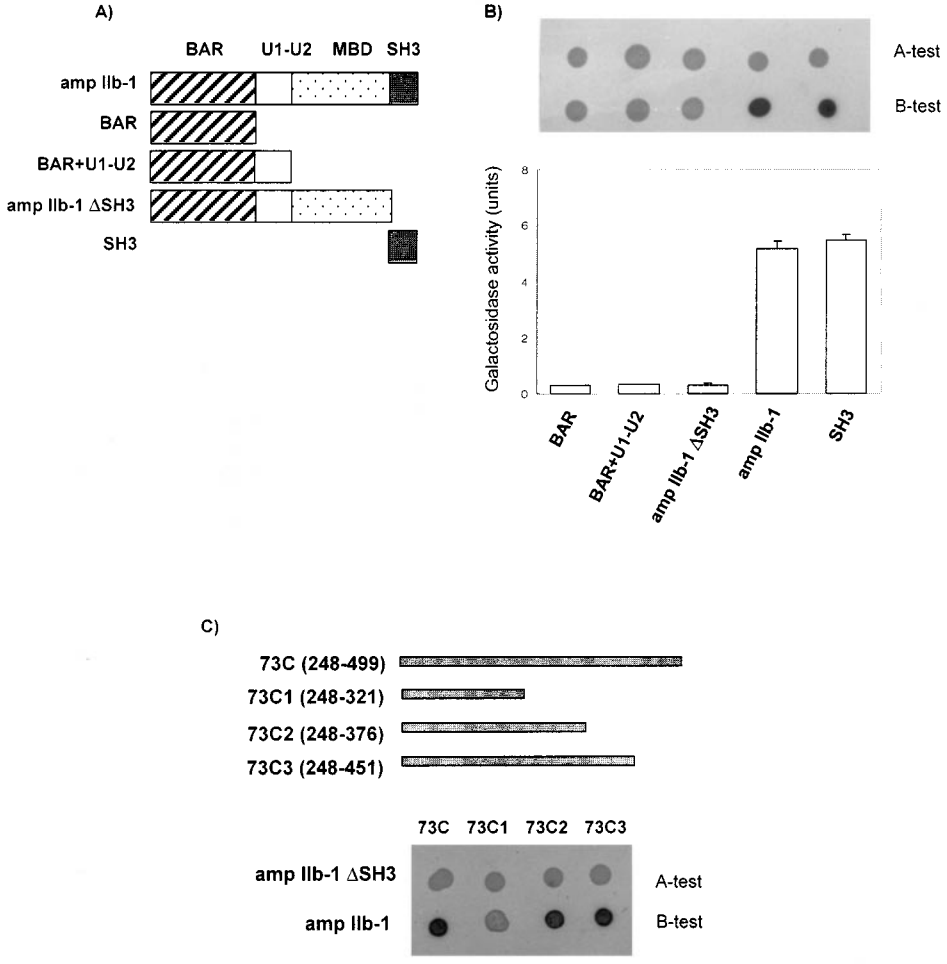

Figure 4 Determination of the specific binding sequences of amphiphysin IIb-1 and p73β. (**a**) Amphiphysin IIb-1 is divided into four regions containing BAR and SH3 domains, according to homology BLAST searches. Deletion fragments of amphiphysin IIb-1 were amplified and cloned into pB42AD yeast expression vector, as described in Materials and methods. (**b**) Upper panel; Each plasmid was transformed into EGY48 [p8op-LacZ] for A-test (autoactivation), or EGY48 [p8op-LacZ + pLexA-73-C] for B-test (binding) in appropriate selection media. After induction of pB42AD vectors in medium containing galactose and raffinose as carbon sources, yeast cells were grown on selection plates containing X-gal (200 μg/ml). Lower panel; Cultured yeast transformants were lysed by freeze-thawing, to measure β-galactosidase activity. Cell lysate (10 μl) was mixed with Z-buffer containing 4 mg/ml ONPG (o-nitrophenyl-β-D-galactopyranoside), and incubated for 1 h at room temperature. The reaction was stopped by adding equal volumes of 1 M sodium bicarbonate, following which β-galactosidase activity was measured at OD_{420}. (**c**) The C-terminal region of p73β is divided into four areas. Deletion fragments of p73C were amplified and cloned into the pLexA vector. Each plasmid was transformed with pB42AD-amphiphysin IIb-1, and β-galactosidase activity was measured at OD_{420}

domain of amphiphysin IIb-1 and amino acids 321–376 of p73β.

Cytoplasmic localization of amphiphysin IIb-1

Most amphiphysin II isotypes are localized in the cytoplasm (Butler *et al*., 1997). However, murine ALP-1 (*a*mphiphysin-*l*ike *p*rotein 1) exhibits primary nuclear localization, despite the lack of NLS (nuclear localization signal) (Kadlec and Prendergast, 1997). Using immunocytochemistry, we investigated the subcellular localization of amphiphysin IIb-1. FLAG-amphiphysin IIb-1 was transiently transfected into COS-7 cells. After 40 h, the FLAG protein was detected by immunostaining with anti-FLAG antibody, followed by incubation with fluorescein-conjugated secondary antibody. Nuclei were simultaneously visualized using DAPI staining. Cells expressing transfected amphiphysin IIb-1 exhibited cytoplasmic staining (Figure 5), indicating localization of the protein in the cytoplasm.

Co-localization of amphiphysin IIb-1 and p73β in the cytoplasmic region

Earlier immunolocalization studies on p73β revealed a pattern of small, punctate dots in the nucleus in an asynchronous population (Kaghad *et al*., 1997). Since amphiphysin IIb-1 is localized in the cytoplasm, it is of interest to clarify whether p73β is relocalized to the cytoplasm by amphiphysin IIb-1, or whether the

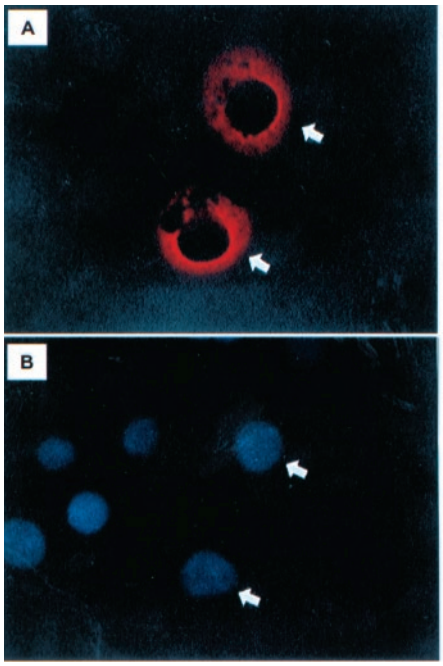

Figure 5 Determination of subcellular localization of amphiphysin IIb-1. COS-7 cells were transfected with the mammalian expression plasmid, pFLAGCMV-amphiphysin IIb-1, and subjected to immunofluorescence (**a**). Cells were also stained with DAPI to locate the nucleus (**b**). Prominent density of amphiphysin IIb-1 is observed in the cytoplasm

variant protein is transported by p73β to the nucleus. To investigate the interaction between amphiphysin IIb-1 and p73β at the subcellular compartment level, we examined the localization of these proteins when coexpressed. 293 cells were co-transfected with FLAG-amphiphysin IIb-1 and a GFP-73β plasmid. FLAG-amphiphysin IIb-1 was immunostained with red dye, while green fluorescence was used to visualize GFP-73β. Double immunofluorescence patterns revealed that in the presence of amphiphysin IIb-1, the p73β protein was present in the nucleus and additionally relocalized to the cytoplasm (Figure 6). Therefore, cytoplasmic relocalization of p73β by interactions with amphiphysin IIb-1 is a feasible process.

Functional inhibition of p73β by amphiphysin IIb-1

We have already suggested that binding of amphiphysin IIb-1 might be significant for the regulation of p73β activity. To elucidate the functional link between the two proteins, we investigated whether amphiphysin IIb-1 affects p73-dependent p21 activation in cells. 293 cells were transfected with equal amounts (1 μg) of p73β expression vector, and 1 and 2 μg amphiphysin IIb-1 expression vector, in the presence of a luciferase reporter plasmid containing the p21 promoter region (p21-Luc). As expected, expression of exogenous p73β resulted in the marked activation of luciferase activity (Figure 7a, lane 3). Transfection of amphiphysin IIb-1 in the absence of p73β had little effect on p21 promoter activation (Figure 7a, lane 2). However, dose-dependent inhibition of p73β-mediated transactivation was observed in amphiphysin IIb-1-coexpressed groups (Figure 7a, lanes 4,5). Since 293 cells contain p53 complexed with adenovirus E1B, which may be reactivated by transfection, we additionally performed a luciferase assay in H1299 cells lacking p53. Amphiphysin IIb-1 consistently inhibited p21 transactivation by p73β in H1299 cells, suggesting that the repression effect of amphiphysin IIb-1 is specific for p73β (Figure 7b). Since the SH-3 domain is required for interactions with p73β, we examined several amphiphysin IIb-1-derived deletion mutants, using the luciferase assay. Coexpression with SH3 but not other domains, inhibited the transactivation function of p73β (Figure 7c). The results strongly imply that amphiphysin IIb-1 has a regulatory function on the transcriptional activity of p73β.

As p73-dependent cell death is a significant molecular process of considerable interest, we tested the effect of amphiphysin IIb-1 on p73β-induced cell death. 293 and neuroblastoma cell lines were transfected with the HA-73β construct and FLAG-amphiphysin IIb-1. FLAG plasmid-transfected cells were not selected in G418-containing medium, consistent with the observation that the FLAG plasmid does not contain a selection marker (data not shown). After plates were maintained for 4 weeks in G418-containing medium, drug-resistant colonies were stained with methylene blue. While only a few colonies were observed in the p73β-transfected group, a large number of colonies were observed in amphiphysin IIb-1 co-transfected groups (Figure 8). Our findings establish that amphiphysin IIb-1 regulates the cell death function of p73β under physiological conditions.

Discussion

The C-terminal region of p73 mediates the regulatory function of the protein through association with a positive or negative regulator, suggesting that protein-protein interactions in this region are closely involved in functional modulation (Davison *et al*., 1999; Agami *et al*., 1999; Gong *et al*., 1999; Yuan *et al*., 1999; Strano *et al*., 2000). Using the yeast two-hybrid assay on the C-terminal region of p73β, we identified cDNA encoding novel amphiphysin IIb-1, one of the splicing variants of amphiphysin II. This protein is identical to amphiphysin IIb, except for a sequence variation at the 3′-region (Ramjaun and McPherson, 1997). The most striking feature of amphiphysin II is the presence of multiple isotypes, due to extensive alternative splicing. Recent studies show that amphyphisin II may have an adaptor-like function in endocytosis (David *et al*., 1996; Ramjaun and McPherson, 1997). BIN 1, another amphiphysin II splicing variant, interacts with the N-terminus of myc and suppresses the cell-transforming activity of the c-myc oncoprotein (Sakamuro *et al*., 1996). Moreover, ALP1 (*a*mphiphysin-*l*ike *p*rotein 1) binds c-abl tyrosine kinase, which transforms normal fibroblasts in c-abl-dependent manner (Kadlec and

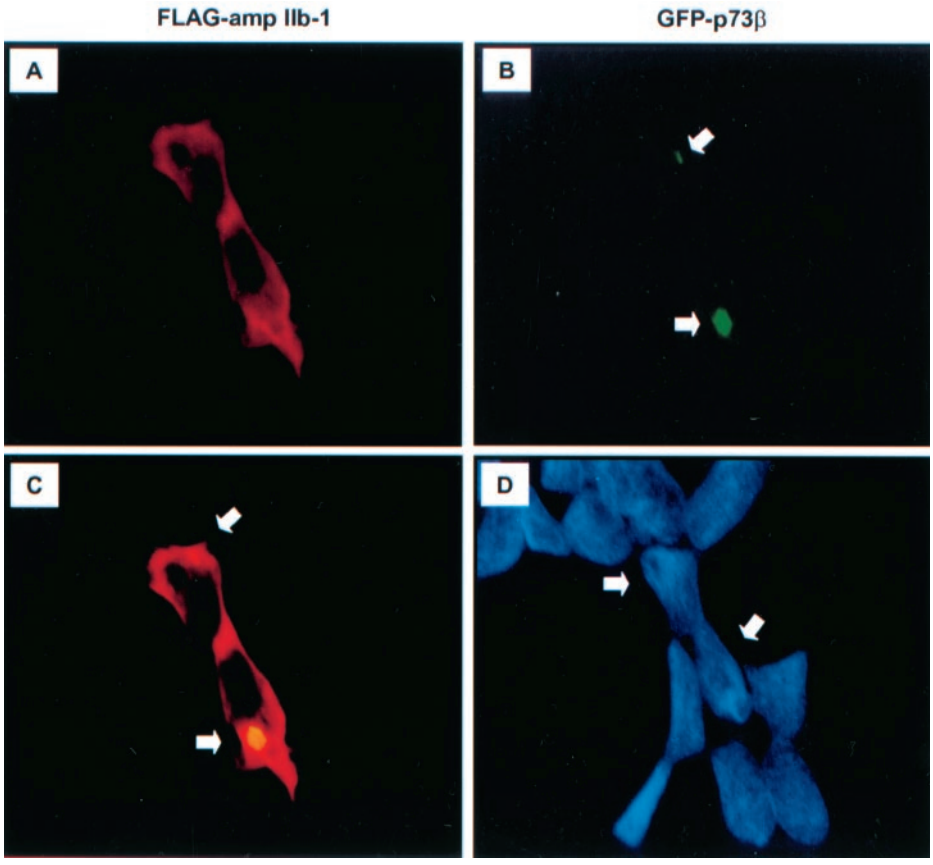

Figure 6 Co-localization of p73β and amphiphysin IIb-1 in the cytoplasmic region. pFLAGCMV-amp IIb-1 and pGFP-73β vectors were transfected into 293 cells. Cells were subjected to double immunofluorescence. (**a**) Amphiphysin IIb-1 (**b**) p73β (**c**) Merged (**d**) Cells were also stained with DAPI. Amphiphysin IIb-1 was present in the cytoplasm, while p73 was detected in the cytoplasm, only in the presence of amphiphysin IIb-1

Prendergast, 1997). Data from our analyses suggest that amphiphysin IIb-1 interacts with full-length p73β, *in vitro* and *in vivo*. The binding of amphiphysin IIb-1 to full-length p73β is direct, as confirmed by *in vitro* translated full-length p73β binding purified amphiphysin IIb-1 in a GST pull-down assay. Therefore, spliced variants of amphiphysin II may function in synaptic vesicle endocytosis or other processes, depending on specific binding partners.

Amphiphysin IIb-1 is composed of a BAR domain (BIN1/amphiphysin/RVS167-related), a central region, and an SH3 domain (Elliott *et al*., 1999). The BAR domain is characterized by homology to yeast proteins (RVS167 and RVS161), and is highly conserved in amphiphysin II splicing variants (Bauer *et al*., 1993; David *et al*., 1996). It was recently reported that BAR domain of a certain splicing variant might have specific heterotypic oligomerization potential, and may therefore be responsible for functional variation via combinatorial interactions (Ge and Prendergast, 2000). However, the BAR domain of amphiphysin IIb-1 is not essential for interactions with the C-terminal region of p73β. The SH3 domain of amphiphysin II binds synaptojanin and dynamin, which function in synaptic vesicle endocytosis (David *et al*., 1996; Ramjaun and McPherson, 1997). Significantly, although this domain of amphiphysin IIb-1 is 28 amino acids shorter than that of amphiphysin IIb and other splicing variants, it is essential for interactions with the C-terminal region of p73β. Considering that the SH3 domain binds to the PxxP motif, the PxxP motif in C-terminal region of p73β may be involved in interaction with amphiphysin IIb-1. However, in our experiments, a mutant form (P338A) of the PxxP motif of p73β was not responsible for binding with the SH3 domain of amphiphysin IIb-1, while C-terminal region (amino acids 321–376) within p73β were essential for the binding process. Interestingly, this region of p73β contains the NLS (amino acids 338–348) (Yuan *et al*., 1999; Ozaki *et al*., 1999). Since the NLS sequence of p73β is also present in a region adjacent to the PxxP motif, it is suggested that the binding motifs in these two regions collectively interact with SH3 domain of amphiphysin IIb-1. Immunofluroscence assays revealed that amphiphysin IIb-1 is localized in the cytoplasm. The p73β protein was also observed in the cytoplasm in the presence of the amphiphysin IIb-1. Therefore, we further propose that amphiphysin IIb-1 masks the NLS region of p73β, a process that may be biologically significant in functional localization.

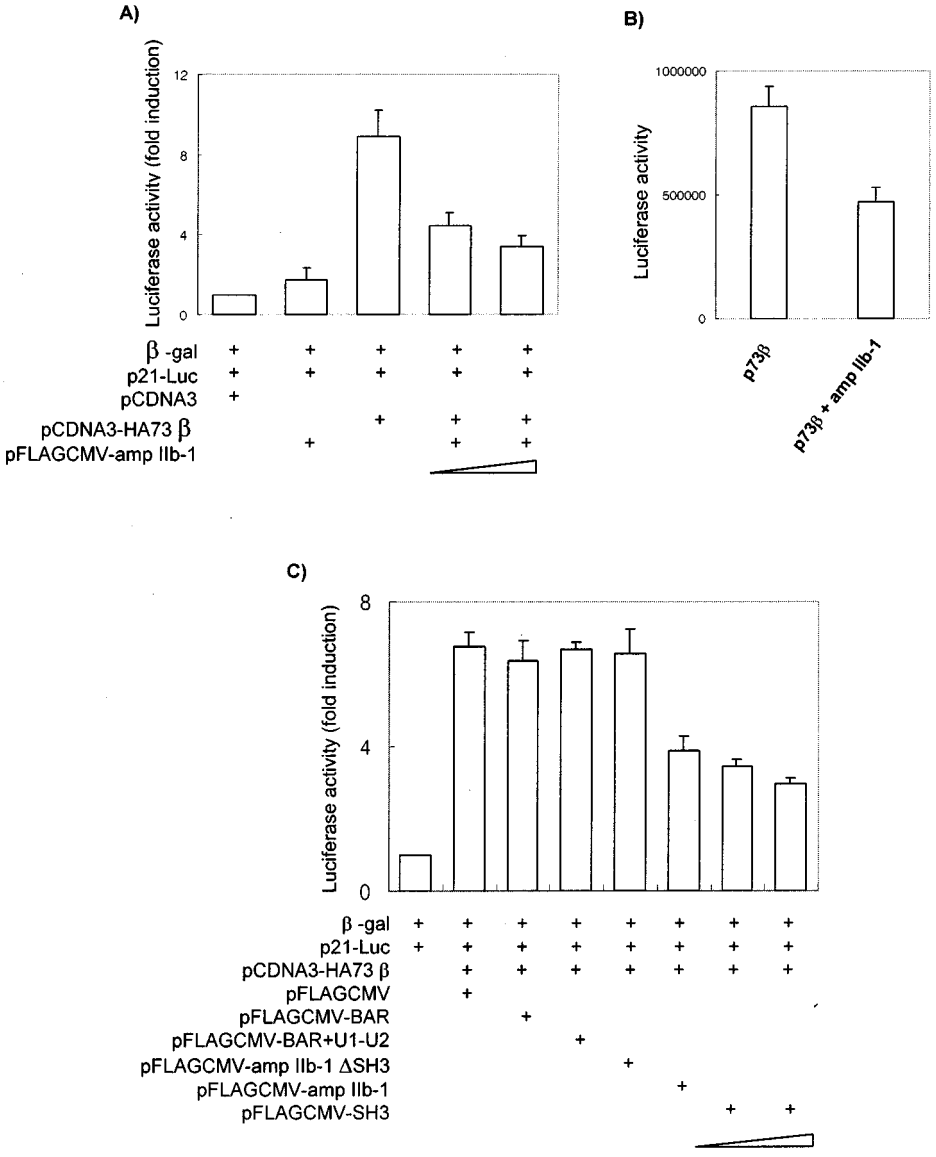

Figure 7 Inhibition of transactivation activity of p73 by amphiphysin IIb-1. (**a**) 293 cells were co-transfected with p21-Luc and pCMV-βgal, together with various sets of indicated plasmids. DNA concentrations were calibrated, using 1% agarose gel electrophoresis and a UV spectrophotometer. After 42 h transfection, luciferase activity was measured with a Lumat LB 9501 Berthold Luminometer (Sarstedt). Data were normalized, using β-galactosidase activity as an internal control. (**b**) H1299 cells were assayed with indicated plasmid combinations. (**c**) This experiment was also performed with deletion fragments of amphiphysin IIb-1

Amphiphysin IIb-1 is capable of inhibiting p73β-dependent transcriptional activation in a reporter containing the p21 promoter. The repressional effect of amphiphysin IIb-1 is probably due to interactions with p73β in the cytoplasmic region. Using a colony-forming assay, we further observed that amphiphysin IIb-1 inhibits the apoptotic function of p73β. Although the physiological significance of this assay is unclear, the distinction between the activity of p73β alone and in the presence of amphiphysin IIb-1 contributes significantly to characterization of the functional link between amphiphysin IIb-1 and p73β in the biological network.

Therefore, an interesting hypothesis is derived from our observations. Amphiphysin IIb-1 sequestrates nascent p73β protein into the cytoplasm by direct binding. Depending on the timing or signal for nuclear translocation, p73β protein is translocated into the nucleus, where it exerts transcriptional activity and cell death. Consistent with our proposal, several investigators suggest that one of the inactivation mechanisms of the tumor suppressor involves cytoplasmic sequestration (Moll *et al*., 1992; Darzynkiewicz *et al*., 1992; Li *et al*., 1998). In the case of p53, the tumor-suppressor protein is found in the cytoplasm in tumor tissue from breast cancer, and neuroblastoma (Ostermeyer *et al*., 1996; Moll *et al*., 1996). It is suggested that p53 might be sequestered by a truncated form of importin α, responsible for NLS-dependent nuclear protein import

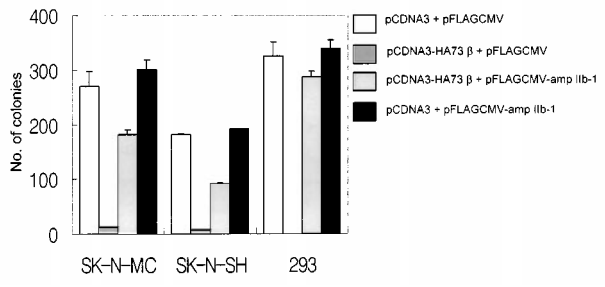

Figure 8 Effect of amphiphysin IIb-1 on p73β-mediated cell death. Cells were transfected with a combination of indicated plasmids, and subsequently incubated with medium containing G418 (500 μg/ml). pFLAGCMV vectors do not contain a selection marker for G418. After 4 weeks, colonies were stained with methylene blue and counted

(Kim *et al*., 2000). Similarly, amphiphysin IIb-1 may mask the NLS region of p73β and regulate functional localization of the protein. However, further studies are required to verify this postulation.

In conclusion, we have demonstrated the biological significance of interactions between p73β and amphiphysin IIb-1. The C-terminal region of p73β plays an important role in functional regulation. Although there are still many unanswered questions on the details of the molecular mechanism of amphiphysin IIb-1, the protein is thought to be involved in the cytoplasmic regulation of p73β. Further intensive studies are required for characterizing the physiological effects of amphiphysin IIb-1 on p73 isoforms.

Materials and methods

Cell culture and transfection

COS-7, 293 human embryonic kidney cell line, and SK-N-MC neuroblastoma cell line were obtained from ATCC, and maintained in DMEM supplemented with 10% FBS (GIBCO-BRL) and penicillin-streptomycin (50 U/ml). SK-N-SH and H1299 cell lines were maintained in RPMI1640 supplemented with 10% FBS. Transient transfection was performed by lipofectamine (GIBCO-BRL) with plasmid DNA combinations, according to manufacturer's instructions.

Bait construction

A pLexA vector for bait cloning was kindly provided by Dr JH Choi, Korea Advanced Institute Science & Technology (KAIST, Taejeon, Korea). For the cloning of full-length p73β, a *Eco*RI/*Not*I fragment from pCDNA3-HA73β (generously provided by Dr M Kaghad, Sanofi Recherche, France) was cloned into the pLexA yeast expression vector. The pLexA-73β plasmid was cut with *Eco*RI and relegated. The resulting plasmid, containing the C-terminal region of p73β, was denoted pLexA-73C, and used as bait in the library screening. To confirm bait expression in the yeast system, yeast proteins were prepared, using the Horvath and Riezman method (Horvath and Riezman, 1994). Yeast cells were cultured for 18 h in a 30°C incubator with shaking, and harvested. Lysis buffer (60 mM Tris [6.8], 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 40 mg bromophenol blue) was added and heated at 100°C for 5 min. SDS–PAGE was followed by Western blot analysis, using pLexA antibody (Clontech). Human brain library in pB42AD was purchased from Clontech.

Yeast two-hybrid screening

The yeast strain used in this study was EGY48 (MAT, his3, trp1, ura3, $LexA_{op(x6)}$-LEU2), a reporter host strain carrying wild-type LEU2 gene under control of LexA operators (Estojak *et al*., 1995). Yeast cells were grown in YPD (1% yeast extract, 2% bacto-peptone, 1% glucose) or Synthetic Dropout (SD) medium lacking the appropriate supplements to maintain selection. Yeast transformation was performed with the PEG/lithium acetate method, as previously described (Gyuris *et al*., 1993). For the transformation of bait plasmid, several colonies of EGY48 [p8op-LacZ] on the SD/Glu/-Ura plate were cultured in YPD medium. Competent cells were transformed with 1 μg pLexA-73C and 100 μg salmon sperm DNA, and the target plasmid library was sequentially transformed into EGY48 [p8op-LacZ + pLexA-73C]. Approximately 2×10^6 transformants were screened for positive colonies growing on SD/Gal/Raf/-Ura/-His/-Trp/-Leu containing X-gal (200 μg/ml). To further confirm the presence of interacting proteins, positive transformants were examined for expression of the LacZ reporter gene, using a filter assay or whole plate assay.

Plasmid rescue

Yeast plasmid DNA was extracted using glass beads, and pB42AD-positive plasmid was transformed into *E. coli* DH5α (Hoffmann and Winston, 1987). The plasmid was isolated, sequenced and re-tested for interactions with the p73β bait plasmid, using back transformation through the whole plate assay in selection plates.

Domain construction

To generate predictive binding domains of amphiphysin IIb-1, PCR amplification was performed on a pB42AD-amphiphysin IIb-1 template plasmid with the following primers: S1 (5′-TTATGCCTCTCCCGAATTCGCG-3′), AS1 (5′-TCTCGAGGTTGAGCTTGCTCAT-3′), AS2 (5′-TCTCGAGCTGCTTGACTTCCTT-3′), AS3 (5′-TCTCGAGCAGGTCCAAGCG-3′) S2 (5′-GGAATTCGCTGGAGCCCAG-3′), AS4 (5′-TCTCGAGTACCATCATAACCA-3′), a.a.2–229: S1 + AS1, a.a.2–324: S1 + AS2, a.a.2–442: S1 + AS3, a.a.397–490: S2 + AS4. PCR products were eluted, digested with *Eco*RI/*Xho*I and ligated into pB42AD yeast expression vector. Deletion constructs of p73C were generated by polymerase chain reaction, with wild-type p73C as template. While the same N-terminal primer was used for most constructs (5′-CATTGAAGGGCTGGCGGTTGGG-3′), C-terminal primers for PCR of the truncated forms included: 5′-NNCTCGAGACTCCATCAGCTCCAGG-3′ (amino acids 248–376), 5′-NNCTCGAGTTGGCGGAGCTCTCGTT-3′ (amino acids 248–321), and 5′-NNCTCGAGCCATTGTTGTTGAG-3′ (amino acids 248–456). The resulting DNA fragments were cloned into pLexA yeast expression vector.

In vitro *binding assay*

A pB42AD yeast-inducible vector fragment was cut and ligated into *Eco*RI/*Xho*I-digested pGEX4T1 prokaryotic

expression vector. The fusion protein was expressed in BL21, and purified on glutathione-sepharose 4B beads (Promega). Protein-protein interaction assays were performed, as previously described (Wadgaonkar and Collins, 1999). *In vitro* translated full-length p73β (TNT, Promega) was incubated with GST-fusion proteins, coupled to glutathione-sepharose bead. After 3 h incubation at 4°C, mixtures were washed in PBS containing 1 mM DTT (three times), followed by TNN containing 1 mM DTT (three times). Beads were boiled in 5 × Laemmli buffer, proteins resolved by 10% SDS–PAGE, and gels was exposed to X-ray film after drying.

In vivo *binding assay*

293 cells were transfected with a combination of indicated plasmids for 42 h and lysed by sonication. For immunoprecipitation assays, 500 μg cell lysates were incubated (with rotating) with 2 μl appropriate antibody in 500 μl PBS for 3 h at 4°C. Following the addition of 30 μl protein A/G agarose beads, the reaction was incubated overnight at 4°C, with rotating. Beads were washed three times in PBS containing 1 mM DTT, resuspended in 20 μl SDS sample buffer, and boiled for 5 min. Samples were analysed by Western blotting.

Luciferase assay

293 cells were cultured in 60 mm dishes and transfected with the firefly luciferase reporter gene (500 ng), and pCMV-βgal control (500 ng) (Promega), together with pCDNA-HA73β and pFLAGCMV-amphiphysin IIb-1. After 40 h transfection, cells were lysed in 200 μl 1 × CCLR buffer (Promega). Cell extracts (20 μl) were analysed with Luciferase reporter assay system, using a Lumat LB 9501 Berthold Luminometer (Sarstedt). Luciferase activities of the p21-luciferase vector were normalized, based on β-galactosidase activity of the cotransfected vector to correct transfection variation.

Immunofluorescence staining

COS-7 cells were grown on a sterile coverslip in 60 mm dishes and transfected with indicated expression vectors using lipofectamine (GIBCO-BRL). Two days after transfection, cells were fixed with 80% acetone and incubated with mouse anti-FLAG IgG antibody (1 : 500) (Santa Cruz), followed by Cy3-conjugated goat anti-mouse IgG (Amersham Pharmacia). Cells were simultaneously incubated with 0.5 g 4′,6-diamidino-2-phenylindole (DAPI; Sigma) per ml for 30 min. Plates were washed three times in PBS and drained. Fluorescence was photographed using a NIKON microscope (Nikon).

Cell death assay

Cells were transfected with various combinations of plasmids at 30% confluence. After 42 h transfection, cells were washed with 1 × PBS, transferred to growth medium containing G418 (500 μg/ml), and incubated for 4 weeks. Colonies were stained with methylene blue in 100% methanol and counted. Experiments were repeated three times.

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References

Agami R, Blandino G, Oren M and Shaul Y. (1999). *Nature,* **399,** 809–813.
Bauer F, Urdaci M, Aigle M and Crouzet M. (1993). *Mol. Cell Biol.,* **13,** 5070–5084.
Butler MH, David C, Ochoa G-C, Freyberg Z, Daniell L, Grabs D, Cremona O and De Camilli P. (1997). *J. Cell. Biol.,* **137,** 1355–1367.
Darzynkiewicz Z, Bruno S, Del Bino G, Gorczyca W, Hotz MA, Lassota P and Traganos F. (1992). *Cytometry,* **13,** 795–808.
David C, McPherson PS, Mundigl O and De Camilli P. (1996). *Proc. Natl. Acad. Sci. USA.,* **93,** 331–335.
Davison TS, Vagner C, Kaghad M, Ayed Caput D and Arrowsmith CH. (1999). *J. Biol. Chem.,* **274,** 18709–18714.
De Laurenzi V, Costanzo A, Barcaroli D, Terrinoni A, Falco M, Annicchiarico-Petruzzelli M, Levrero M and Melino G. (1998). *J. Exp. Med.,* **188,** 1763–1768.
Di Como CJ, Gaiddon C and Prives C. (1999). *Mol. Cell Biol.,* **19,** 1438–1449.
Dobbelstein M, Wienzek S, Konig C and Roth J. (1999). *Oncogene,* **18,** 2101–2106.
Elliott K, Sakamuro D, Basu A, Du W, Wunner W, Staller P, Gaubatz S, Zhang H, Prochownik E, Eilers M and Prendergast GC. (1999). *Oncogene,* **18,** 3564–3573.
Estojak J, Brent R and Golemis EA. (1995). *Mol. Cell Biol.,* **15,** 5820–5829.
Field S and Song OK. (1989). *Nature,* **340,** 245–246.
Ge K and Prendergast GC. (2000). *Genomics,* **67,** 210–220.
Gong J, Costanzo A, Yang H-Q, Melino G, Kaelin Jr WG, Levrero M and Wang JY. (1999). *Nature,* **399,** 806–809.
Gyuris J, Golemis E, Chertkov H and Brent R. (1993). *Cell,* **75,** 791–803.
Horvath A and Riezman H. (1994). *Yeast,* **10,** 1305–1310.
Haupt Y, Maya R, Kazaz A and Oren M. (1997). *Nature,* **387,** 296–299.
Hoffmann CS and Winston F. (1987) *Gene,* **57,** 267–272.
Ichimiya S, Nimura Y, Kageyama H, Takada N, Sunahara M, Shishikura T, Nakamura Y, Sakiyama S, Seki N, Ohira M, Kaneko Y, McKeon F, Caput D and Nakagawara A. (1999). *Oncogene,* **18,** 1061–1066.
Jost CA, Marin MC and Kaelin Jr WG. (1997). *Nature,* **389,** 191–194.
Kadlec L and Prendergast AM. (1997). *Proc. Natl. Acad. Sci. USA,* **94,** 12390–12395.
Kaghad M, bonnet H, Yang A, Yang A, Creancier L, Biscan J-C, Valent A, Minty A, Chalon P, Lelias J-M, Dumont X, Ferraa P and Caput D. (1997). *Cell,* **90,** 809–819.
Kim I-S, Kim D-H, Han S-M, Chin M-U, Nam H-J, Cho H-P, Chio S-Y, Song B-J, Kim E-U, Bae Y-S and Moon Y-H. (2000). *J. Biol. Chem.,* **275,** 23139–23145.
Kleihues P, Schauble B, zur Hausen A, Esteve J and Ohgaki H. (1997). *Am. J. Pathol.,* **150,** 1–13.
Le Douarin B, Prerrat B, vom Baur E, Chambon P and Losson R. (1995). *Nucleic Acids Res.,* **23,** 876–878.

Li S, Ku C-Y, Farmer AA, Cong Y-S, Chen C-F and Lee W-H. (1998). *J. Biol. Chem.,* **273,** 6183–6189.
Licitra EJ and Liu JO. (1996). *Proc. Natl. Acad. Sci. USA,* **93,** 12817–12821.
Martin MC, Jost CA, Irwin MS, DeCaprio JA, Caput D and Kaelin Jr WG. (1998). *Mol. Cell. Biol.,* **18,** 6316–6324.
Moll UM, Riou G and Levine AJ. (1992). *Pro. Natl. Acad. Sci. USA,* **89,** 7262–7266.
Moll UM, Ostermeyer AG, Haladay R, Winkfield B, Frazier M and Zamberri G. (1996). *Mol. Cell. Biol.,* **16,** 1126–1137.
Ostermeyer AG, Runko E, Winkfield B, Ahn B and Moll UM. (1996). *Proc. Natl. Acad. Sci. USA,* **93,** 15190–15194.
Ozaki T, Naka M, Takada N, Tada M, Sakiyama S and Nakagawara A. (1999). *Cancer Res.,* **59,** 5902–5907.
Ramjaun AR and McPherson PS. (1997). *J. Neurochem.,* **70,** 2369–2376.
Roth J, Konig C, Wienzek S, Weigel S, Ristea S and Dobbelstein M. (1998). *J. Virol.,* **72,** 8510–8516.
Sakamuro D, Elliott KJ, Wechsler-Reya R and Prendergast GC. (1996). *Nat. Genet.,* **14,** 69–77.
Steegenga WT, Shvarts A, Riteco N, Bos J and Jochemsem AG. (1999). *Mol. Cell. Biol.,* **19,** 3885–3894.
Strano S, Munarriz E, Rossi M, Cristofanelli B, Shaul Y, Castagnoli L, Levine AJ, Sacchi A, Cesareni G, Oren M and Blandino G. (2000). *J. Biol. Chem.,* **275,** 29503–29512.
Tirode F, Malaguti C, Romero F, Attar R, Camonis J and Egly JM. (1997). *J. Biol. Chem.,* **272,** 22995–22999.
Tsutsui K, Maeda Y, Tsutsui K, Seki S and Tokunaga A. (1997). *Biochem. Biophys. Res. Commun.,* **236,** 178–183.
Ueda Y, Hijikata M, Takagi S, Chiba T and Shimotohno K. (1999). *Oncogene,* **18,** 4993–4998.
Wadgaonkar R and Collins T. (1999). *J. Biol. Chem.,* **20,** 13760–13767.
Wienzek S, Roth J and Dobbelstein M. (2000). *J. Virol.,* **74,** 193–202.
Yuan Z-M, Shioya H, Ishiko T, Sun X, Gu J, Huang YY, Lu H, Kharbanda S, Weichselbaum R and Kufe D. (1999). *Nature,* **399,** 814–817.
Zeng Z, Chen L, Jost CA, Maya R, Keller D, Wang X, Kaelin WG, Oren M, Chen J and Lu H. (1999). *Mol. Cell. Biol.,* **19,** 3257–3266.