Subcellular Targeting of p33^{ING1b} by Phosphorylation-Dependent 14-3-3 Binding Regulates p21^{WAF1} Expression

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ING1 is a type II tumor suppressor that affects cell growth, stress signaling, apoptosis, and DNA repair by altering chromatin structure and regulating transcription. Decreased ING1 expression is seen in several human cancers, and mislocalization has been noted in diverse types of cancer cells. Aberrant targeting may, therefore, functionally inactivate ING1. Bioinformatics analysis identified a sequence between the nuclear localization sequence and plant homeodomain domains of ING1 that closely matched the binding motif of 14-3-3 proteins that target cargo proteins to specific subcellular locales. We find that the widely expressed p33^{ING1b} splicing isoform of ING1 interacts with members of the 14-3-3 family of proteins and that this interaction is regulated by the phosphorylation status of ING1. 14-3-3 binding resulted in significant amounts of p33^{ING1b} protein being tethered in the cytoplasm. As shown previously, ectopic expression of p33^{ING1b} increased levels of the p21^{Waf1} cyclin-dependent kinase inhibitor upon UV-induced DNA damage. Overexpression of 14-3-3 inhibited the up-regulation of p21^{Waf1} by p33^{ING1b}, consistent with the idea that mislocalization blocks at least one of ING1's biological activities. These data support the idea that the 14-3-3 proteins play a crucial role in regulating the activity of p33^{ING1b} by directing its subcellular localization.

ING1 is a tumor suppressor that affects regulation of the cell cycle (14), induction of apoptosis (21), and repair of DNA damage (8, 42). The mechanisms by which the five ING gene family members (20) exert their biological effects are not fully understood, although splicing isoforms of the ING1 gene differentially regulate levels of histone acetylation through interactions with histone acetyltransferase and histone deacetylase complexes (3, 10, 29, 45, 50). In addition to such epigenetic effects, different ING proteins have been reported to bind a number of proteins, including PCNA (42), p53 (15, 28), Sir2 (27), and NF- κ B (16), which directly impinge upon pathways targeted during tumorigenesis. The ING1 class II tumor suppressor (39) gene produces two major alternatively spliced mRNAs encoding proteins that localize to the nucleus and that are growth inhibitory. ING1 maps to chromosome 13q33-34 (17), a site frequently associated with loss of heterozygosity in several types of cancers (7, 33, 48). In human cells, p47^{ING1a} and p33^{ING1b} are the major ING1 splicing isoforms expressed, and recent studies suggest that they display different or even opposite properties in their activities in apoptosis and senescence (4, 51), perhaps through opposite roles in regulating histone acetylation levels (10).

Suppression of p33^{ING1b} expression promotes focus formation and anchorage-independent growth in vitro and tumor formation in vivo, while ectopic expression of this protein was shown to block cell cycle progression by arresting transfected cells in G₁ of the cell cycle (14, 31). Clinical data have shown that reduced levels of p33^{ING1b} are seen in primary breast tumors, lymphoid malignancies, testis tumors, and squamous cell cancers, consistent with ING1 acting as a class II tumor

suppressor (7, 24, 26, 37, 46, 47). Additionally, p33^{ING1b} also displays properties of a regulator of apoptosis in different experimental systems. Both the apoptotic and cell cycle regulatory properties of p33^{ING1b} may involve the tumor suppressor p53, with which p33^{ING1b} and the closely related p33^{ING2} were found to be capable of physically and/or functionally interacting (15, 18, 21, 27, 44), affecting the levels of p53 target genes such as p21^{WAF1} (15, 27) and Bax (9). A role in determining the fate of cells that have sustained DNA damage was also supported by the observation that p33^{ING1b} binds to the proliferating cell nuclear antigen (PCNA) in a DNA damage-inducible manner that was directly linked to the ability of p33^{ING1b} to induce apoptosis (42). Moreover, decreased nuclear levels and aberrant cytoplasmic localization of p33^{ING1b} were observed in invasive breast cancer and brain tumor cells (36, 52). Only two studies to date have addressed how human ING proteins may be localized within the cell. The first analysis of ING1 localization identified two functional nucleolar targeting signals (see Fig. 3) within a nuclear localization sequence (NLS), which were required for the nucleolar localization of ING1 upon overexpression or in response to UV-induced DNA damage (43). A second study found that rare phosphoinositide signaling molecules bind to the plant homeodomain (PHD) region of cytoplasmic ING2, promoting its translocation to the nucleus and its activation of p53 as a transcription factor in response to DNA damage (18).

The 14-3-3 proteins are a family of conserved, ubiquitously expressed proteins with monomeric molecular masses of approximately 30 kDa. To date, seven highly conserved 14-3-3 family members (β , ϵ , γ , η , δ , τ , and ζ) encoded by seven distinct genes have been identified in mammalian systems. 14-3-3 proteins mainly reside in the cytoplasmic compartment of the cell, forming homodimers or heterodimers with other 14-3-3 family members (1, 12). Most reports indicate that many

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of the 14-3-3 members are interchangeable with respect to protein binding, and emerging evidence suggests that 14-3-3 proteins are central regulators of the cell cycle, especially at cell cycle checkpoints, where they might function as regulators of DNA damage checkpoints (25, 30, 35). In fission yeast, the 14-3-3 proteins Rad24 and Rad25 are required for checkpoint responses and are essential for cell survival (11). In addition, one of the 14-3-3 isotypes, 14-3-3 δ , is strongly up-regulated following genotoxic stress and is a downstream target of the tumor suppressor p53 (22).

Given the links of both the ING proteins (15, 18, 21, 27, 44) and the 14-3-3 proteins (22, 53) to p53 activity, the phosphorylation-dependent binding of 14-3-3 proteins (reviewed in reference 32), we asked whether the major p33^{ING1b} isoform of ING1 interacted with different members of the 14-3-3 family. We find that p33^{ING1b} binds 14-3-3 under basal conditions, and the interaction is dependent upon the phosphorylation state of p33^{ING1b}. These findings define a major new ING1 regulatory mechanism that is mediated by the phosphorylation-dependent binding of 14-3-3 proteins.

MATERIALS AND METHODS

DNA constructs and mutagenesis. Human 14-3-3 cDNA clones were a kind gift of Johanna Zilliacus (Karolinska Institute) and Mark Bedford (MD Anderson Cancer Center). The human ING1 cDNAs (31) were subcloned into the pCI vector (Promega). To obtain a His-tagged human p33^{ING1b}, primers 5'-TGAA TTGATGCATCACCACCATC and 5'-TATTTCTAGACTACCTGTTGT AAGCCCTCTC were used to add 6 histidines to the N terminus of p33^{ING1b} by the PCR method. Mutagenesis of ING1b serine 199 to alanine was carried out by the PCR overlap extension method (23), and primers 5'-TATTGAATTC ATGTTGAGTCCTGCCAAGC, 5'-TATTTCTAGACTACCTGTTGTAAGC CCTCTC, 5'-AGAGGCGGCTCCTGCCGAC, and 5'-GTCGGCAGGAGCCG CCTCT were used. The mutation was confirmed by DNA sequencing. To obtain mammalian expression constructs of 14-3-3, we cloned the glutathione *S*-transferase (GST)-tagged 14-3-3 cDNAs into a pcDNA 3.1 vector and a Flag-pcDNA 3.1 vector (Invitrogen). All constructs were checked by DNA sequencing in the Southern Alberta DNA Services Laboratory to confirm cloning steps.

Cell culture and transient expression. HEK293 and HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 100 units/ml penicillin, 100 mg/ml streptomycin, and 10% fetal calf serum in a 5% CO₂ incubator at 37°C. Cells were seeded onto 6-cm or 10-cm dishes 18 to 24 h prior to transfection and then transiently transfected at 60 to 70% confluence using a standard CaPO₄ protocol (40). At 24 h after transfection, cells were harvested for further assays.

Cell fractionation. Cell monolayers were rinsed with ice-cold phosphate-buffered saline (PBS) (150 mM NaCl, 20 mM Na₂PO₄, pH 7.4) and then fractionated or harvested in 300 μ l of cell lysis buffer (50 mM Tris-Cl, 2 mM EGTA, 1% Triton X-100, 0.4 mM Na₂VO₄, 50 mM NaCl, plus 1:1,000 protease inhibitor mixture [Sigma]) on ice. Cell lysates were sonicated on ice and centrifuged at 14,000 $\times$ g at 4°C for 15 min, and the supernatants were used immediately in the indicated assays. Fractionation of HeLa cells was as previously described (2). Cell monolayers, washed as above, were scraped in ice-cold PBS and split into two portions. One third of the cells were pelleted and lysed in Laemmli sample buffer (whole-cell lysates). The other two thirds were pelleted for 10 s in an Eppendorf centrifuge and aspirated, and cell pellets (light yellow) were resuspended in 10 volumes of PBS containing 0.1% NP-40 on ice. After immediately triturating cells by passage through a 20-gauge needle and a 1-ml syringe five times on ice, nuclei (white) were pelleted by centrifugation for 10 s in an Eppendorf centrifuge. Supernatants composed of cell cytoplasm were removed and boiled with 1/5 volume of 5 $\times$ Laemmli sample buffer (cytoplasmic fractions). Nuclei were triturated a second time on ice in PBS containing 0.1% NP-40 to ensure that all cells had ruptured cytoplasmic membranes. Nuclei were pelleted as before, aspirated, and boiled in the same final volume as the cytoplasmic preparations, using 1 $\times$ Laemmli sample buffer. Cell samples were routinely examined by polyacrylamide gel electrophoresis (PAGE) and staining with Coomassie blue to verify protein concentration and integrity.

GST pull-down assays and coimmunoprecipitation. For the pull-down assays, cell lysates containing 1 mg of total protein or 3 μ g of recombinant His-tagged

ING1b protein were incubated overnight with 10 μ g of GST or GST-14-3-3 bound to glutathione-Sepharose. For coimmunoprecipitations, cell lysates from each sample were precleared by incubation with 10 μ l of protein A/G-Sepharose for 30 min at 4°C and then incubated with 5 μ g of specific antibody and 25 μ l of protein G-Sepharose or an equivalent amount of mouse anti-ING1 preincubated to 25 μ l of protein G-Sepharose at 4°C for 4 h on a roller system. The immunocomplexes recovered on beads were washed two times for 10 min with 1 ml of cell lysis buffer before the addition of Laemmli sample buffer.

Western analysis. Proteins were resolved by sodium dodecyl sulfate-PAGE and transferred to nitrocellulose membranes (Hybond; Amersham). Immunoblotting was performed with a cocktail of four mouse anti-ING1 monoclonal antibodies (5), mouse anti-p21, rabbit anti-14-3-3 K19, antiactin, anti-phosphoserine/threonine, anti-Flag, antinucleoporin, and anti-pyruvate kinase antibodies (Santa Cruz Biotechnology), and mouse or polyclonal rabbit antilaminin antibody (Sigma). Immunoreactive bands were visualized using enhanced chemiluminescence with ECL reagent (Amersham Biosciences).

Dephosphorylation with calf intestine alkaline phosphatase. HEK293 Cells were transfected with His-ING1b and harvested, and His-tagged ING1b protein was purified from the cell lysate using a nickel agarose column as described by the manufacturer (QIAGEN). The resulting 1-mg/ml His-p33^{ING1b} stock was used for subsequent assays. Equal amounts (3 μ g) of His-p33^{ING1b} were treated with or without a mixture of phosphatase inhibitors (NaF, orthovanadate, and glycerol phosphate). The untreated His-p33^{ING1b} was then incubated with increasing amounts of calf intestinal alkaline phosphatase (Promega) at 37°C for 1 h. Individual proteins were incubated with the GST fusion construct or with GST alone. Bound fractions of recombinant proteins were separated by denaturing sodium dodecyl sulfate-PAGE.

Indirect immunofluorescence. Cells were fixed by treatment with 4% formaldehyde in PBS, permeabilized with 0.5% Triton X-100 in PBS, and blocked with 3% bovine serum albumin in PBS. Immunofluorescence was performed by incubation with mouse anti-ING1 Cab-3 antibody (Santa Cruz Biotechnology) and polyclonal anti-14-3-3 antibody (Santa Cruz Biotechnology) antibody at a 1:50 dilution for 45 min at 37°C in PBS containing 1% bovine serum albumin. After washing in PBS, cells were incubated for 45 min at 37°C with secondary antibody (1:100 goat anti-mouse antibody conjugated with indocarbocyanine [Jackson ImmunoResearch] or 1:80 goat anti-rabbit antibody conjugated with fluorescein isothiocyanate [Santa Cruz Biotechnology]) and washed with PBS. 4',6'-Diamidino-2-phenylindole (DAPI) staining was performed with 0.5 μ g of DAPI per ml in PBS containing 1% bovine serum albumin for 10 min at 37°C. After mounting the samples, immunofluorescence images were captured with a Leica DM R microscope and a Photometrics 16-bit cooled digital camera.

RESULTS

14-3-3 avidly binds to p33^{ING1b}. An interaction between 14-3-3 and ING1 was first identified in vitro using a GST pull-down assay and overexpressed proteins. Because there are seven isoforms of 14-3-3 in human cells, we tried to determine if particular 14-3-3 family members were capable of binding to ING1. After coincubating lysates of HEK293 cells overexpressing p33^{ING1b} with the different 14-3-3s we found that, although they differ markedly in their affinity for p33^{ING1b}, all seven of the 14-3-3s were capable of pulling down p33^{ING1b} when precipitated with glutathione-Sepharose beads (Fig. 1A). The 14-3-3 η isoform bound most strongly to p33^{ING1b} in the GST pull-down assay, with only very weak binding seen with the 14-3-3 β , γ , and ζ family members. Binding of p33^{ING1b} to 14-3-3 was specific, since it did not bind nonspecifically to beads or to GST expressed at similar levels. Figure 1B confirms that the seven isoforms of 14-3-3 were efficiently expressed and that differential binding to p33^{ING1b} was not due to differential levels of the 14-3-3 proteins. Western blots of cell lysates confirmed that ING1 isoforms were expressed at similar levels in all lysates used for pull downs (data not shown). Since binding of p33^{ING1b} to the 14-3-3 η family member was robust, we concentrated further efforts upon analysis of the 14-3-3 η -p33^{ING1b} interactions.

FIG. 1. GST pull down assays of p33^{ING1b} using 14-3-3 isoforms. (A) Lysates of HEK293 cells overexpressing p33^{ING1b} were incubated with GST fusion proteins of all seven known members of the 14-3-3 family, which were preincubated with glutathione-Sepharose beads. Eluates were electrophoresed and blotted with ING1 antibodies. The input lane represents 5% of the lysate used in pull-down assays. Beads and GST control lanes were incubated with the same amount of lysate as each 14-3-3 lane. (B) Aliquots of each GST-14-3-3 protein used in the GST pull-down assay were electrophoresed and stained with Coomassie brilliant blue. M, molecular mass marker (from the top, 175, 83, 62, 47, 33, and 25 kDa).

To ask whether interactions could be observed between 14-3-3 and endogenous levels of p33^{ING1b}, HeLa cell lysates were applied to glutathione-Sepharose columns previously loaded with GST or with GST-14-3-3 η , the 14-3-3 isoform that bound most avidly to p33^{ING1b} in initial experiments. As shown in Fig. 2A, endogenous p33^{ING1b} was pulled down by GST-14-3-3 but not by GST alone. The interaction between p33^{ING1b} and 14-3-3 was then examined using only endogenous proteins to further confirm its specificity and potential relevance in vivo. HeLa whole-cell extracts were immunoprecipitated with anti-

FIG. 2. Association of endogenous ING1 and 14-3-3 η . (A) GST pull-down assays of endogenous p33^{ING1b} with GST-14-3-3 η . Equal amounts of HeLa cell nuclear extracts were incubated with GST or GST-14-3-3 η bound to glutathione beads. After extensive washing, samples were eluted, electrophoresed, and probed with ING1 antibodies. (B) Immunoprecipitation (IP)-Western blot assay of HeLa cell nuclear extracts. Upper panels show 14-3-3 Western blots of immunoprecipitations with nonspecific mouse immunoglobulin G (IgG) and mouse anti (α)-ING1 antibody or the reciprocal IP-Western assay. Lower panels show control Western blots of lysates used for immunoprecipitations.

FIG. 3. Diagram of the p33^{ING1b} protein. Amino acids 1 to 45 are unique to the p33^{ING1b} splicing isoform of ING1, while residues 45 to 279 are shared with p47^{ING1a}. The PCNA-interacting protein (PIP) motif, a partial bromodomain, the NLS with two functional nucleolar translocation signals (NTS), and the PHD are illustrated. The REAS PADLP motif, which has homology to 14-3-3 consensus binding sites is located between the NLS and PHD regions. Serine 199 represents a candidate phosphorylation site for regulating 14-3-3 binding.

ING1, anti-14-3-3, or nonspecific antibodies of the same species and concentration, and immunoprecipitates were blotted with the corresponding anti-14-3-3 or anti-ING1 antibodies of the opposite species (5). Figure 2B demonstrates a clear and specific association between 14-3-3 and p33^{ING1b} in the reciprocal immunoprecipitation (IP)-Western blots. Lower panels are control Western blots of lysates used in immunoprecipitations and confirm that 14-3-3 and p33^{ING1b} were at similar or greater levels in immunoprecipitates using nonspecific antibodies, further demonstrating the specificity of the interaction. These results corroborate those found using overexpressed proteins and using endogenous p33^{ING1b} in GST pull-down experiments. Furthermore, they support the idea that 14-3-3 family members bind p33^{ING1b} at levels found under physiologically relevant conditions.

Sequence analysis identifies a potential 14-3-3 binding site in ING1. The binding of 14-3-3 proteins to target proteins requires the specific motifs RSXS/T(P)XP or RXXXpSXP (34). Study of the 14-3-3-Raf interaction led to the identification of two potent consensus 14-3-3 binding sequences for that protein: RSXpS/TXP and RXXXpS/TXP, with arginine (R) at position -3 from the phosphorylatable serine or threonine as a minimal requirement (34, 54). An exhaustive sequence analysis identified one possible sequence, REAS/PADLP in ING1, which closely matched a canonical, phosphorylation-dependent 14-3-3 binding site. This site, with a target serine residue at amino acid 199 of p33^{ING1b}, is located between the NLS and the PHD regions (Fig. 3). Both the NLS and PHD motifs have been identified as sites important for subcellular localization of the ING1 proteins in response to DNA damage (18, 43).

Interaction between 14-3-3 and p33^{ING1b} requires serine 199. To test whether the potential 14-3-3 binding site identified above was functional, we used site-directed mutagenesis to convert the serine residue at position 199 into alanine (Ser199-A). Overexpression of wild-type and Ser199-A forms of p33^{ING1b} with either GST or GST-14-3-3 η and recovery of GST-bound proteins on glutathione-Sepharose indicated that p33^{ING1b} but not Ser199-A could bind 14-3-3 η , although both wild-type and mutant forms of p33^{ING1b} were expressed efficiently (Fig. 4A). To examine this in a reciprocal manner using an independent method, constructs encoding p33^{ING1b} and Ser199-A were co-expressed in HEK293 cells with 14-3-3 η , and lysates from transfected cells were immunoprecipitated with anti-ING1 or control antibodies. As shown in Fig. 4B, the Ser199-A mutant was unable to bind 14-3-3 despite being efficiently expressed in

FIG. 4. Mutation of serine 199 to alanine disrupts the 14-3-3/p33^{ING1b} interaction. (A) Lysates of HEK293 cells overexpressing p33^{ING1b} or a mutant in which serine 199 was mutated to alanine (Ser199-A) were incubated with GST or a GST-14-3-3 η fusion protein. After recovery on glutathione-Sepharose, precipitates were electrophoresed and probed with anti-ING1 antibody. The lower panel is a Western blot confirming similar expression levels of the ING1 constructs. (B) Immunoprecipitation-Western assays of HEK293 cells coexpressing 14-3-3 η with wild-type and Ser199-A mutant p33^{ING1b}. Immunoprecipitates indicated on the upper axis were electrophoresed and blotted with 14-3-3 antibody in an experiment done in the reciprocal manner to panel A. The lower two control panels show that the levels of p33^{ING1b} and 14-3-3 proteins expressed are similar. +, overexpressed; -, not overexpressed.

transfected cells. No 14-3-3 is detected in ING1 immunoprecipitates in the absence of ING1 overexpression, as was shown in Fig. 2, since exposures used to visualize overexpressed proteins are much shorter than needed for endogenous proteins. These data support the idea that serine 199 within the motif REASPLP mediates the interaction between 14-3-3 and p33^{ING1b}.

Binding of 14-3-3 to p33^{ING1b} depends on the phosphorylation status of p33^{ING1b}. It is well documented that phosphorylation on serine and threonine is important for proteins to interact with 14-3-3 (32). To further verify the specificity of the interaction between 14-3-3 and p33^{ING1b} and to ask whether the interaction might depend upon the phosphorylation status of p33^{ING1b}, we directly asked if dephosphorylation of purified p33^{ING1b} would alter its binding affinity for purified 14-3-3 η in vitro, in the absence of possible bridging proteins. Full-length human p33^{ING1b} with an N-terminal histidine tag was expressed in HEK293 cells and purified to homogeneity over a nickel agarose column. The η member of the 14-3-3 family was expressed as a fusion with GST and purified using glutathione-Sepharose. The purified 14-3-3 η and His-p33^{ING1b} proteins associated directly, but the phosphatase treatment of His-p33^{ING1b} led to a drastic and dose-dependent decrease in its ability to bind 14-3-3 η (Fig. 5). Blotting of the phosphatase-treated samples with anti-phospho-serine/threonine antibody suggested the existence of at least two distinct S/T phosphorylation sites on ING1 and that phosphatase treatment resulted in the expected dephosphorylation of ING1 (Fig. 5C) in parallel with loss of binding to 14-3-3 (Fig. 5A). This observation reflects the need for a phosphoserine within the sequences that are recognized by 14-3-3 proteins and further supports the idea that these proteins interact directly through the identified site, without the required participation of additional proteins.

14-3-3 η directs p33^{ING1b} to the cytoplasm. Binding of 14-3-3 to a number of its partners had previously been reported to induce their redistribution from the nucleus to the cytoplasm (13, 49). In HeLa cells transfected with either vector DNA or with the 14-3-3 η expression construct, the majority of ING1 staining was nuclear. However, a distinct subpopulation of ING1 was seen in the cytoplasm of cells overexpressing 14-3-

3 η , as highlighted Fig. 6h. This is consistent with previous reports of a small biologically active population of ING proteins residing in the cytoplasm and inducibly translocating to the nucleus in response to stress (18). To ask this question using an independent method, we prepared cytoplasmic extracts from HeLa cells transfected with a vector control or with a 14-3-3 η expression construct. Figure 7A shows that a significant amount of signal for p33^{ING1b} is seen in the cytoplasm of cells transfected with 14-3-3 η , whereas very little is seen in cells transfected with the control construct. Reprobing of the membrane with antibody against the cytoplasmic control laminin verified that loading and fractionation of the cell populations was similar. To ask what proportion of the total p33^{ING1b} protein might be translocated and whether 14-3-3 expression affected p33^{ING1b} levels, cells transfected with control vector or 14-3-3 η were fractionated into nuclear, cytoplasmic, or whole-cell fractions, as described in Materials and Methods, and

FIG. 5. Phosphorylation-dependent interaction between 14-3-3 η and p33^{ING1b}. (A) A His-tagged version of p33^{ING1b} was purified from HEK293 cells and treated with the indicated amounts of alkaline phosphatase before being subjected to in vitro GST pull-down assays with purified GST-14-3-3 η . Proteins binding to glutathione-Sepharose beads were eluted, electrophoresed, and blotted with anti-ING1 antibody. (B) Control Western blot of 5% phosphatase-treated samples showing equal amounts of His-p33^{ING1b} was used in all assays. Heat-inactivated alkaline phosphatase (Alk. PPTase) did not affect recovery of p33^{ING1b} in parallel assays (data not shown). (C) Control Western blot of 5% phosphatase-treated samples showing dephosphorylation of serine/threonine residues in p33^{ING1b}. +, present; -, absent.

FIG. 6. 14-3-3 η localizes a subpopulation of p33^{ING1b} to the cytoplasm. HeLa cells were transfected with a 14-3-3 η expression construct and, 24 h later, were fixed and stained with rabbit anti-14-3-3 (followed by goat anti-rabbit fluorescein isothiocyanate) (a and e), mouse anti-ING1 (followed by goat anti-mouse indocarbocyanine) (b and f), and DAPI (for DNA) (c and g). Panels d and h show the merging of the ING1 and DNA signals. The top row of panels show untransfected cells, and the bottom row shows cells transfected with 14-3-3 expression construct. The bar in panel h equals 50 μ m, and the arrow highlights the presence of extranuclear p33^{ING1b} staining.

blotted with antibodies against 14-3-3 to confirm expression and localization, antibodies against p33^{ING1b}, and against the nuclear and cytoplasmic control proteins nucleoporin and pyruvate kinase. As shown in Fig. 7B, a significant fraction of p33^{ING1b} was directed to the cytoplasmic fraction, as seen previously. This was not due to fractionation artifacts, since nucleoporin was totally nuclear and pyruvate kinase was completely cytoplasmic. Overexpression of 14-3-3 did not have any detectable effect upon the levels of p33^{ING1b}, and the signals for p33^{ING1b} and nucleoporin were approximately twice as strong as those seen for whole-cell lysate, as expected for proteins that localize primarily or exclusively in the nucleus. Signals for pyruvate kinase were greater in cytoplasmic extracts than in whole-cell extracts, as expected. In Fig. 7A and 7B, transfection efficiency was high and we were unable to detect ING1b in the cytoplasm at the exposures examined. Figure 7C shows an independent experiment in which the 14-3-3 transfection efficiency was reduced. The top panel shows a higher exposure for ING1b detection and results corroborate panels A and B, showing increased cytoplasmic ING1b in response to 14-3-3 that is expressed at two- to threefold-higher levels in the 14-3-3-transfected population of cells. Since panels A to C show results from populations of cells, we next asked what proportion of cells overexpressing 14-3-3 would also show cytoplasmic localization of p33^{ING1b}. Cells transfected with the p33^{ING1b} expression construct or with p33^{ING1b} and 14-3-3 expression constructs were examined to see what proportion of p33^{ING1b}-overexpressing cells showed cytoplasmic staining. Figure 7D shows that coexpression of 14-3-3 increased the number of cells showing detectable cytoplasmic staining by approximately 3.5-fold. Cytoplasmic staining of endogenous

p33^{ING1b} was not detectable using this method due to the low levels of p33^{ING1b} in cells.

Up-regulation of p21^{WAF1} by p33^{ING1b} is inhibited by 14-3-3 η . Overexpression of p33^{ING1b} is known to up-regulate p21^{WAF1} (also known as p21^{WAF1/Cip1/Sdi1}) (15, 27), particularly in the presence of DNA damage (X. Feng, S. Pastryeva, D. Muruve, D. Larocque, S. Richard, G. Tallen, M. Truss, A. von Deimling, and K. Riabowel, submitted for publication). To ask whether altering the localization of a subpopulation of ING1 proteins might affect levels of p21^{WAF1}, we coexpressed wild-type and ser199-A mutant forms of ING1 in the absence and presence of 14-3-3 η , followed by exposure of cells to 30 J/m² of UV irradiation. As shown in Fig. 8A, ectopic expression of p33^{ING1b} was capable of up-regulating p21^{WAF1}. However, when 14-3-3 η was transiently cotransfected with p33^{ING1b}, the p33^{ING1b}-dependent up-regulation of p21^{WAF1} was nearly completely eliminated. The Ser199-A mutant of p33^{ING1b} was unable to increase p21^{WAF1} levels in response to UV in the presence or absence of overexpressed 14-3-3 η . These data are consistent with 14-3-3 η inhibiting p33^{ING1b}-induced up-regulation of p21^{WAF1} upon UV-induced DNA damage. They also suggest that the integrity of serine 199 is needed for p33^{ING1b} to increase p21^{WAF1} levels. To ask if 14-3-3 would also affect the ability of p33^{ING1b} to regulate apoptosis (9, 10, 21, 42–44, 51), combinations of control vector, 14-3-3, the wild type, and the Ser199-A mutant of p33^{ING1b} were transfected into cells which were subsequently analyzed for apoptosis 24 h later. Figure 8B shows that expression of p33^{ING1b} increased the amount of apoptotic cells beyond the amount seen in response to Lipofectamine treatment (compare untransfected to Vector and Vector plus ING1b). Cotransfection of 14-3-3 with vector had

FIG. 7. Levels of p33^{ING1b} in subcellular fractions. (A) Cytoplasmic fractions of HeLa cells transfected with pcDNA vector or with pcDNA-14-3-3 η were electrophoresed and blotted with antibodies against p33^{ING1b} and laminin. The laminin cytoplasmic basement membrane glycoprotein served as a cytoplasmic marker and loading control. (B) Cells transfected with vector or the 14-3-3 η expression construct were fractionated as described in Materials and Methods, and protein from equal numbers of cells was electrophoresed for cytoplasmic, nuclear, and whole-cell lysates. Blotting for nucleoporin and pyruvate kinase serve as markers for the nucleus and cytoplasm, respectively, and the bottom panel confirms that 14-3-3 is overexpressed and localizes primarily to the cytoplasm. This blot used 25% of the total protein used in panel A to allow comparison of the ING1 signals in whole-cell and nuclear fractions in the absence and presence of 14-3-3 expression. (C) Cells transfected under conditions of lower efficiency and fractionated as in panel B were blotted for ING1b expression, and blots were developed much longer to visualize ING1b in vector-treated cells. This gives a better idea of the magnitude of difference, since only ~2-fold more 14-3-3 was seen in the transfected cell population (second panel) and a >2-fold difference was seen in cytoplasmic ING1b in response to 14-3-3 overexpression. Nucleoporin and pyruvate kinase blots confirm clean fractionation of cell preparations. (D) HeLa cells were transfected with an expression construct encoding p33^{ING1b} or with p33^{ING1b} and 14-3-3 η and were fixed and stained 24 h later for ING1. Cells in five random fields were scored for nuclear versus nuclear plus cytoplasmic staining. The bars show the percentage of transfected cells with detectable cytoplasmic staining (as seen in Fig. 6f), and error bars represent the standard deviations between counts.

no clear effect on apoptosis, but cotransfection of 14-3-3 with p33^{ING1b} protected cells from apoptosis. In contrast, cotransfection of the Ser199-A mutant of p33^{ING1b} with or without 14-3-3, showed that 14-3-3 did not protect against apoptosis if it was unable to bind p33^{ING1b} and that, as expected, the Ser199-A mutant was more efficient in inducing apoptosis, since it cannot be translocated by 14-3-3 to the cytoplasm.

DISCUSSION

In this study, we have shown that p33^{ING1b} specifically binds members of the 14-3-3 family in a manner that depends upon the phosphorylation status of serine residue 199 that is located within the amino acid motif REASPADLP. Dephosphorylation of p33^{ING1b} or mutation of serine 199 strongly inhibits the ability of p33^{ING1b} to interact with 14-3-3 proteins, and mutation of Ser199 to alanine results in a failure of p33^{ING1b} to induce levels of the p21^{Waf1} cyclin-dependent kinase inhibitor upon DNA damage-induced stress. 14-3-3 also inhibits the ability of p33^{ING1b} to induce apoptosis, although in this assay, the Ser199-A mutant is constitutively active in promoting apoptosis, differentiating the functions of p33^{ING1b} in p21 induction versus apoptosis. Finally, increasing 14-3-3 levels by transient transfection also results in cytoplasmic accumulation of p33^{ING1b}, as assessed by two independent methods. This correlates with inactivation of p33^{ING1b} as an inducer of p21^{Waf1}

and lends support to the idea that p33^{ING1b} is regulated by stress-induced phosphorylation that activates it to translocate to the nucleus and by phosphorylation-dependent sequestration in the cytoplasm by 14-3-3 binding. These studies provide the first evidence of regulation of the ING family of class II tumor suppressors by the 14-3-3 family which has been shown to affect cell cycle checkpoints (35).

Several independent groups have shown that the interaction of 14-3-3 proteins with numerous target molecules is regulated by phosphorylation. For example, the interaction of 14-3-3 and PDK1 requires phosphorylation of PDK1 (41). Recent studies with phosphopeptide libraries revealed the existence of additional preferred motifs for 14-3-3 binding, such as RXRXX pSXP or RXXpS (54). The p33^{ING1b} protein contains a 14-3-3 binding motif in its amino acid sequence 196-REASPADLP-204. Site-directed mutagenesis of the target serine revealed that serine 199 within the REASPADLP motif is required for p33^{ING1b}/14-3-3 binding using both in vitro GST pull-down assays and in vivo IP-Western blots in which the p33^{ING1b} mutant, Ser199-A, failed to be pulled down by either GST-14-3-3 or an anti-14-3-3 antibody. Many studies have also shown that 14-3-3 binding can have a direct effect on target protein activity (38, 41) and can mediate the relocalization of nuclear ligands (6). We discovered that the interaction of p33^{ING1b} with 14-3-3 leads to the translocation of p33^{ING1b} from the nucleus to the cytosol and/or anchors a population of p33^{ING1b}

FIG. 8. 14-3-3 inhibits p33^{ING1b}-induced up-regulation of p21^{Waf1/Cip1/Sdi1} and apoptosis. (A) HEK293 cells were transfected with constructs encoding p33^{ING1b}, 14-3-3 η , or the Ser199-A mutant. Twenty-four hours after transfection, cells were irradiated with 30 J/m² of UV. Cells were harvested 12 h later, and lysates were electrophoresed and blotted with the indicated antibodies. (B) HCT116 cells transfected with the indicated constructs plus a green fluorescent protein expression construct using Lipofectamine were cultured for 24 h, and total (floating and adherent) cells were harvested and stained with propidium iodide. Samples gated for green fluorescent protein expression were scored by flow cytometry for sub-G₁ content as a measure of apoptosis. Lipofectamine reproducibly induced a background level of apoptosis, as noted for the vector control. All transfections were done in parallel, and error bars are generated from two independent sets of transfections and analyses. +, overexpressed; -, not overexpressed.

in the cytoplasm. Also, p33^{ING1b} up-regulated p21^{Waf1} upon UV-induced DNA damage, in agreement with previous reports, and we find that 14-3-3 inhibits the up-regulation of p21^{Waf1} by p33^{ING1b}. It is well established that the p53-mediated increase in p21^{Waf1} levels is a major mediator of cell cycle arrest after DNA damage (3, 19). After UV irradiation, p33^{ING1b} shifts from the nucleoplasm to the nucleolus (43) and enhances p53-dependent repair (28) and apoptosis (9). The mechanism(s) by which ING1b stimulates the activity of p53 appears to be by increasing the stability of p53, either through disruption of the p53-MDM2 interaction (28) or through direct abrogation of ubiquitination-mediated degradation of p53 (Feng et al., submitted). Both of these proposed mechanisms of p53 stabilization require interaction between the proteins, and interaction requires a common intracellular locale. Studies from different groups have reported the localization of significant amounts of p33^{ING1b} to the cytosol which correspond with aggressiveness of tumors and poor prognosis (reviewed in

reference 10), and the existence of a small, biologically relevant pool of ING2 in the cytoplasm has been linked to the activation of p53 by rare phosphoinositide signaling molecules (18). 14-3-3 binding also inhibits the ability of p33^{ING1b} to efficiently induce apoptosis, although in contrast to its activities in transcriptional induction, the Ser199-A mutant of p33^{ING1b} is constitutively active for inducing apoptosis, showing that the function of p33^{ING1b} in transcription and apoptosis pathways is transduced by different effectors.

Our data show that 14-3-3 specifically interacts with p33^{ING1b} in a phosphorylation-dependent manner. These data are consistent with the previous proposal that 14-3-3 proteins may act as gatekeepers, through differential binding avidity, to subsets of multiply phosphorylated target proteins (55). Based upon our observations, it is possible that 14-3-3 keeps a population of p33^{ING1b} tethered in the cytoplasm, where it is ready to be further phosphorylated and bound by phosphoinositides in response to DNA damage. Activation would result in dissociation of 14-3-3 and translocation of ING protein to the nucleus, where it would increase p53 activity and, subsequently, induce p53-dependent gene expression. Either phosphorylation at a second site, or dephosphorylation of Ser199 on p33^{ING1b} could promote translocation of p33^{ING1b} to the nucleus. This possible mechanism needs to be further tested to determine if newly translocated ING1b proteins constitute the most active population of ING1b in the cell nucleus since our and previous data do not address this point directly. Based upon previous reports of both the 14-3-3 and ING proteins interacting with and altering p53 activity, the p33^{ING1b}/14-3-3 interaction represents an important aspect of the mechanism by which p33^{ING1b} may exert tumor suppressor function.

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