UV-induced binding of ING1 to PCNA regulates the induction of apoptosis

Michelle Scott[1], Paul Bonnefin[1], Diego Vieyra[1], Francois-Michel Boisvert[1], Dallan Young[1,2], David P. Bazett-Jones[1] and Karl Riabowol[1,2,*]

[1]Departments of Biochemistry and Molecular Biology, Faculty of Medicine, The University of Calgary, 3330 Hospital Drive, NW, Calgary, Alberta T2N 4N1, Canada
[2]Department of Oncology, Faculty of Medicine, The University of Calgary, 3330 Hospital Drive, NW, Calgary, Alberta T2N 4N1, Canada
*Author for correspondence (e-mail: karl@ucalgary.ca)

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SUMMARY

Previous studies have shown that UV-induced binding of $p21^{WAF1}$ to PCNA through the PCNA-interacting protein (PIP) domain in $p21^{WAF1}$ promotes a switch from DNA replication to DNA repair by altering the PCNA protein complex. Here we show that the $p33^{ING1b}$ isoform of the ING1 candidate tumour suppressor contains a PIP domain. UV rapidly induces $p33^{ING1b}$ to bind PCNA competitively through this domain, a motif also found in DNA ligase, the DNA repair-associated FEN1 and XPG exo/endonucleases, and DNA methyltransferase. Interaction of $p33^{ING1b}$ with PCNA occurs between a significant proportion of ING1 and PCNA, increases more than tenfold in response to UV and is specifically inhibited by overexpression of $p21^{WAF1}$, but not by $p16^{MTS1}$, which has no PIP sequence. In contrast to wild-type $p33^{ING1b}$, ING1 PIP mutants that do not bind PCNA do not induce apoptosis, but protect cells from UV-induced apoptosis, suggesting a role for this PCNA-$p33^{ING1b}$ interaction in eliminating UV-damaged cells through programmed cell death. These data indicate that ING1 competitively binds PCNA through a site used by growth regulatory and DNA damage proteins, and may contribute to regulating the switch from DNA replication to DNA repair by altering the composition of the PCNA protein complex.

Key words: Apoptosis, DNA damage, ING1, PCNA, PIP

INTRODUCTION

The proliferating cell nuclear antigen (PCNA) was first described as a protein whose synthesis correlated closely with the growth state of cells and was later shown to be an essential processivity factor for DNA polymerases δ and ε, functioning in both DNA replication and nucleotide excision repair (Kelman and Hurwitz, 1998; Araujo et al., 2000). It is highly conserved evolutionarily, essential for viability and forms a sliding homo-trimeric clamp encircling DNA as part of a large multiprotein complex. PCNA interacts with proteins through different faces of the homo-trimer (Warbrick, 1998), but has no known enzymatic activity. It is believed to orient other proteins such as the FEN-1 exo/endonuclease (Hosfield et al., 1998; Gomes and Burgers, 2000), the CAF-1 chromatin assembly/remodelling factor (Shibahara and Stillman, 1999), DNA ligase I (Montecucco et al., 1998), the BASC super complex of proteins (which includes the BRCA1 tumor suppressor; Wang et al., 2000), the related MyD118 and Gadd45 proteins (Vairapandi et al., 2000) and the $p21^{WAF1}$ cyclin-dependent kinase activator/inhibitor (Waga et al., 1994; Podust et al., 1995; Chen et al., 1996; Zheleva et al., 2000) in DNA replication and repair complexes (Gibbs et al., 1996). Several of these proteins contain a common octapeptide motif (QXX[ILM]XXF[FY]) called the PCNA-interacting-protein (PIP) domain, through which they bind competitively to the interdomain connector loop of PCNA (Warbrick, 1998; Tsurimoto, 1999). In the case of $p21^{WAF1}$, a peptide that competes effectively for PCNA binding has been recently reported that corresponds to residues 141-160 of the carboxyl region of the p21 protein (Zheleva et al., 2000).

The ING1 candidate tumor suppressor was isolated based upon the ability of antisense ING1 sequences to promote focus formation in vitro and tumor formation in vivo (Garkavtsev et al., 1996). ING1 overexpression inhibits cell cycle progression in some cell models and promotes p53-independent and c-Myc-inducible (Helbing et al., 1997), as well as p53-dependent (Shinoura et al., 1999) apoptosis. The *ING1* gene maps near the telomere on chromosome 13q34 (Garkavtsev et al., 1997) and is well conserved throughout evolution, particularly within a 3′ exon common to different isoforms (Loewith et al., 2000; Zeremski et al., 1999; Gunduz et al., 2000). Three homologs of *ING1* exist in the yeast *S. cerevisiae* and two are known in *S. pombe.* We recently found that an ING1-related yeast protein (Yng2) could interact with Tra1, the yeast ortholog of human TRRAP (Loewith et al., 2000). TRRAP is well conserved evolutionarily and is significantly homologous to the PI-3 kinase family in a 328 amino acid C-terminal domain (McMahon et al., 1998). This family also includes DNA-dependent protein kinase (DNAPK) and ataxia telangiectasia mutated (ATM) serine-threonine kinases that appear to function in diverse pathways including those impinging upon recombination, G_1/S cell cycle checkpoint, DNA repair and transcription (Jeggo et al., 1998). Like ING1, ATM has been implicated in apoptosis (Herzog et al., 1998). Both yeast Tra1 and human TRRAP are found in complexes containing histone

acetytransferase (HAT) activity that is due to association with yeast Esa1 (Loewith et al., 2000) and hGCN5 (McMahon et al., 2000), respectively, suggesting that both yeast and mammalian ING1 proteins may be involved in chromatin remodelling by virtue of regulating the acetylation of histones and/or other regulatory factors in the nucleus.

Human cells predominantly express two ING1 mRNA isoforms by alternative splicing, encoding $p47^{ING1a}$ and $p33^{ING1b}$, as well as a truncated protein, $p24^{ING1c}$ that is believed to be produced by initiation at an internal ATG (Ma et al., 1999; Gunduz et al., 2000) (Fig. 1A). All isoforms of ING1 contain a plant homeodomain (PHD) form of zinc finger that is commonly found in chromatin-associated proteins (Aasland et al., 1995) and a nuclear localization signal (NLS) containing two distinct nucleolar translocation sequences (NTS) that target ING1 proteins to the nucleolus in response to some forms of DNA damage (Scott et al., 2001). Consistent with a role in regulating cell growth, a structurally related gene (*ING1-L*) located on human chromosome 4q35.1 is expressed aberrantly in a subset of colon cancers (Shimada et al., 1998). Expression of *ING1* is regulated through the cell cycle (Garkavtsev and Riabowol, 1997); its expression is reduced from two to tenfold in primary breast tumours (Toyama et al., 1999) and lymphoid malignancies (Ohmori et al., 1999), and missense mutations are seen in squamous cell carcinomas (Gunduz et al., 2000), consistent with a role as a class 2 tumor suppressor (Sager, 1997). Since our recent results and those of other groups (Cheung et al., 2000) had indicated that *ING1* expression was induced in keratinocytes in response to DNA damage, we asked whether the ING1 proteins might physically and/or functionally associate with proteins known to be involved in DNA repair. We report here that a major splicing isoform of the *ING1* gene encoding $p33^{ING1b}$, but not the $p47^{ING1a}$ isoform, physically interacts with PCNA following UV exposure and that this interaction promotes the entry of cells into a programmed cell death pathway.

MATERIALS AND METHODS

Immunoprecipitation western blotting

Nondenaturing immunoprecipitations (IPs) were performed as described previously (Riabowol et al., 1989). In some assays cells were exposed to 25 J/m^2 of UV, a dose determined to induce p53 maximally without killing cells, in trial experiments. Proteins were quantitated using a Bradford assay (BioRad) and results were corroborated by visualization of samples using Coomassie Blue staining after SDS-polyacrylamide (PAGE) gel electrophoresis. Proteins separated by PAGE through 12.5% gels were analyzed by western blotting as described (Garkavtsev and Riabowol, 1997) using an undiluted mixture of four mouse monoclonal ING1 antibody hybridoma supernatants (Boland et al., 2000) or with a 1:1000 dilution of different rabbit ING1 polyclonal antibodies (Garkavtsev et al., 1996).

Indirect immunofluorescence and confocal microscopy

Cells were fixed with 1.0% paraformaldehyde in PBS (pH 7.5) at room temperature for 5 minutes and permeabilized in PBS containing 0.5% Triton X-100 for 5 minutes. ING1 was visualized using four mouse monoclonal antibodies (Cab1-4), p53 was labelled with a 1:500 dilution of rabbit polyclonal antibody (Santa Cruz, FL-393) and PCNA was visualized using a 1:200 dilution of human antiserum (a gift from M. Fritzler, University of Calgary). After washing, cells were incubated with secondary antibodies: goat anti-rabbit (Cy3, Chemicon), goat anti-mouse (Alexa 488, Cedarlane or Cy5, Chemicon) or goat anti-human (Cy3, Chemicon). After rinsing, samples were mounted in 1 mg/ml of paraphenylenediamine in PBS/90% glycerol that also contained the DNA-specific dye DAPI at 1 μg/ml. Digital confocal imaging was performed using a 14-bit cooled CCD camera (Princeton Instruments) mounted on a Leica DMRE immunofluorescence microscope as described (Bazett-Jones et al., 1999). VayTek Microtome digital deconvolution software was used to remove out of focus contributions and image stacks were projected into a one image plane using Scion Image software. For quantitative analysis of colocalization, raw images were analyzed before deconvolution. The signal representing 10% of the nucleus for each labelling was set at a threshold value of 1, the rest becoming 0. The two images were than subjected to a boolean AND using ERGOvista software v4.4. The resulting number of pixels with a value of 1 was divided by the number of pixels representing 10% of the nucleus. This value represents the percentage of colocalization between the two proteins. For signal density quantitation, the nuclear signal of acetylated histones was integrated for injected and non-injected cells, using ERGOvista v4.4.

Cells, antibodies and DNA constructs used

Cells used were obtained from the ATCC in the case of the primary diploid fibroblast strain Hs68 (ATCC CRL #1635) or were a kind gift from P. Forsyth, University of Calgary (SNB-19 Glioblastoma cells). All experiments were done with Hs68 cells, and SNB-19 cells were used to confirm coimmunoprecipitation results for experiments shown in Fig. 3. ING1 expression constructs containing full-length cDNA versions of the human ING1 genes (Ma et al., 1999) were all driven by CMV promoters. In experiments listing vector DNA as negative controls, the parental vector for the expression constructs, pCI^{neo} (Promega) was used. ING1 polyclonal rabbit antibodies and the mouse Cab-1-4 ING1 monoclonals have been described previously (Boland et al., 2000). We also used mouse monoclonal (Santa Cruz #sc-56) and rabbit polyclonal (Santa Cruz #sc-7907) α-PCNA, α-CBP/p300 (Santa Cruz #sc-583) and α-p53 (Chemicon #AB565, Santa Cruz #sc-6243). Both rabbit and mouse α-ING1 antibodies were raised against a GST-ING1 fusion protein and both recognize native and denatured forms of $p33^{ING1b}$ and $p47^{ING1a}$, as well as the truncated $p24^{ING1c}$ isoform. Like the ING1 monoclonals, the DO1 and PAb421 α-p53 monoclonal antibodies (gifts from D. Lane (University of Dundee) and P. Lee (University of Calgary)) were not frozen before use. Horseradish peroxidase-conjugated secondary antibodies for western blotting were from Amersham-Pharmacia Biotech and were all used at 1:1000 dilution.

Glycerol gradient centrifugation

Primary Hs68 fibroblasts were scraped in ice-cold PBS containing 0.05% NP-40 and 0.05% Tween-20 and triturated to release nuclei. Nuclei were pelleted by spinning for 5 seconds at 4000 rpm (~3500 ***g***) in a refrigerated Eppendorf centrifuge, resuspended in three pellet volumes of nuclear homogenization buffer (NHB: 20 mM Hepes pH 7.5, 10% v/v glycerol, 0.05% NP-40, 0.05% Tween-20, 100 mM NaCl, 40 mM KCl, 1 mM $MgCl_2$, 0.5 mM DTT, 1 μg/ml of aprotinin and leupeptin, 100 μM PMSF and 10 mM NaF) and sonicated on ice with a microprobe. Lysates (~2×10^6 cells/condition) were centrifuged for 15 seconds at 5000 rpm (~4400 ***g***) at 4°C and supernatants (400 μl) were applied over linear 4 ml gradients of 15-35% glycerol containing the same components as NHB. After centrifugation for 18 hours at 28,000 rpm (80,000 ***g***) in an SW60 rotor at 4°C, gradients were fractionated into 0.25 ml aliquots and stored at –80°C.

RESULTS

ING1 and PCNA reside in similar sized protein complexes

Since the expression of ING1 through the cell cycle shows

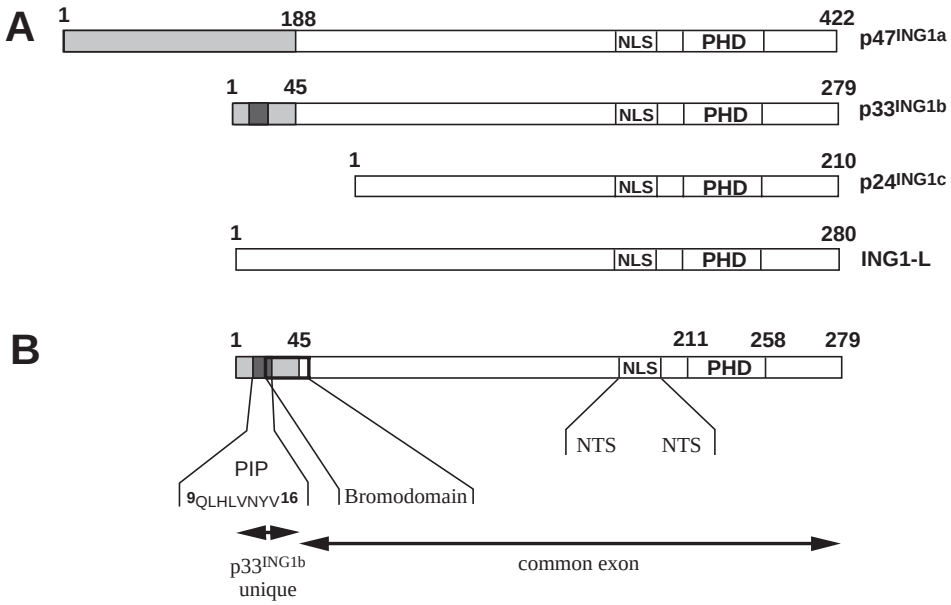

Fig. 1. ING1 protein structure. (A) Schematic diagram of three characterized isoforms of ING1 expressed in most tissue culture cells including Hs68 fibroblasts and SNB19 cells and a recently cloned ING1-like (ING1-L) gene. All proteins contain PHD and nuclear localization signal (NLS) domains; $p33^{ING1b}$ contains a PIP domain (P). Darkened regions represent isoform-unique regions and open bars represent a common exon. (B) functional regions of $p33^{ING1b}$ are indicated including the PIP sequence, a partial Bromodomain and the NLS, which consists of two independent nucleolar transport sequences (Scott et al., 2001).

similarities to that of PCNA and because we noted that one ING1 splicing isoform contained a PIP sequence ($p33^{ING1b}$; Fig. 1B), we asked whether these proteins might interact. To better understand the size of the protein complexes that ING1 isoforms and PCNA were contained in, we separated total nuclear proteins on glycerol velocity gradients under non-denaturing conditions. Fig. 2a shows that the two major ($p33^{ING1b}$, $p47^{ING1a}$) and several minor ($p24^{ING1c}$ and other) isoforms of ING1 were detected in broad, overlapping fractions corresponding to relative masses ranging from approximately 30 kDa up to greater than 430 kDa. In these gradients, denatured proteins were recovered in 1 or 2 fractions only (data not shown). Fig. 2b shows that the distribution of PCNA was nearly identical to that seen for $p33^{ING1b}$, suggesting that they were contained in protein complexes of similar mass.

UV induces a PCNA-$p33^{ING1b}$ interaction

To test whether the ING1 and PCNA proteins were contained in the same complexes, non-denaturing ING1 immunoprecipitates from UV-treated fibroblasts were probed with α-PCNA and showed a clear and inducible signal within 15 minutes of UV irradiation that progressively increased to more than tenfold by 24 hours after UV treatment as estimated by scanning densitometry (Fig. 3A). Similar results were obtained using rabbit α-PCNA followed by blotting with mouse α-ING1, although signals were not robust due to the relatively high levels of PCNA in cells compared with endogenous levels of ING1 (data not shown). This showed that PCNA inducibly bound to ING1, but it did not clearly address the question of which of the major ING1 isoforms expressed in cultured cells interacted, since the rabbit polyclonal antibody used in this experiment can recognize at least three ING1 isoforms (Fig. 1A). We therefore transfected cells with expression constructs encoding $p47^{ING1a}$ or $p33^{ING1b}$, immunoprecipitated lysates with α-ING1 and blotted to detect PCNA. Fig. 3B shows that cells transfected with $p33^{ING1b}$, but not with $p47^{ING1a}$ or with vector, bound abundant PCNA in ING1 complexes. Bands corresponding to PCNA were not seen in cells transfected with vector since these cells were not exposed to UV to induce an ING1b-PCNA interaction and blots were exposed for approximately 10% of the time as for untransfected cells shown in panel A to optimize signals seen. Control panels (Fig. 3C,D) confirm that PCNA was expressed at similar levels in

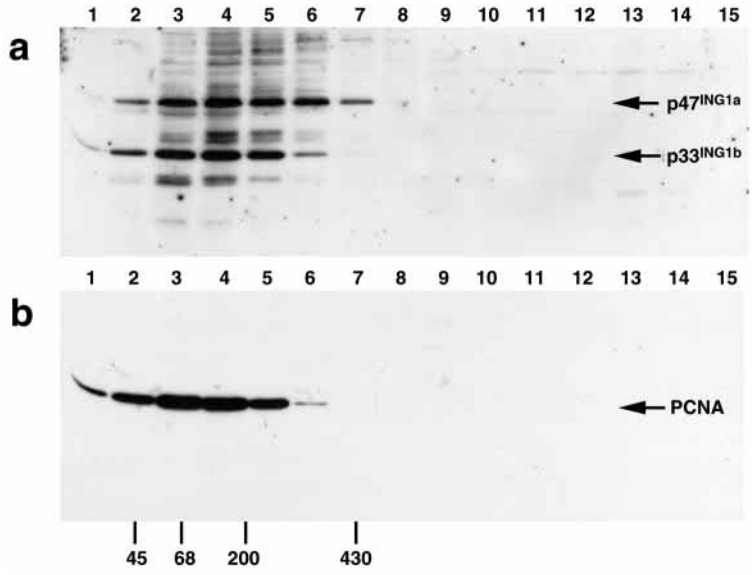

Fig. 2. Glycerol gradient fractionation of protein complexes containing ING1. Extracts prepared from Hs68 fibroblasts and sedimented, as outlined in Materials and Methods, were fractionated from the bottom of tubes. Fraction 15 represents the highest density fraction and fraction 1 represents the least dense fraction that was isolated last from the top of gradients. Samples corresponding to the 15 fractions isolated were electrophoresed through 12.5% gels, transferred to nitrocellulose and probed with (a) α-ING1 (Cab1-4) or (b) mouse α-PCNA antibodies and visualized by ECL. Numbers below panel b represent the relative migration of denatured protein standards run on control gradients.

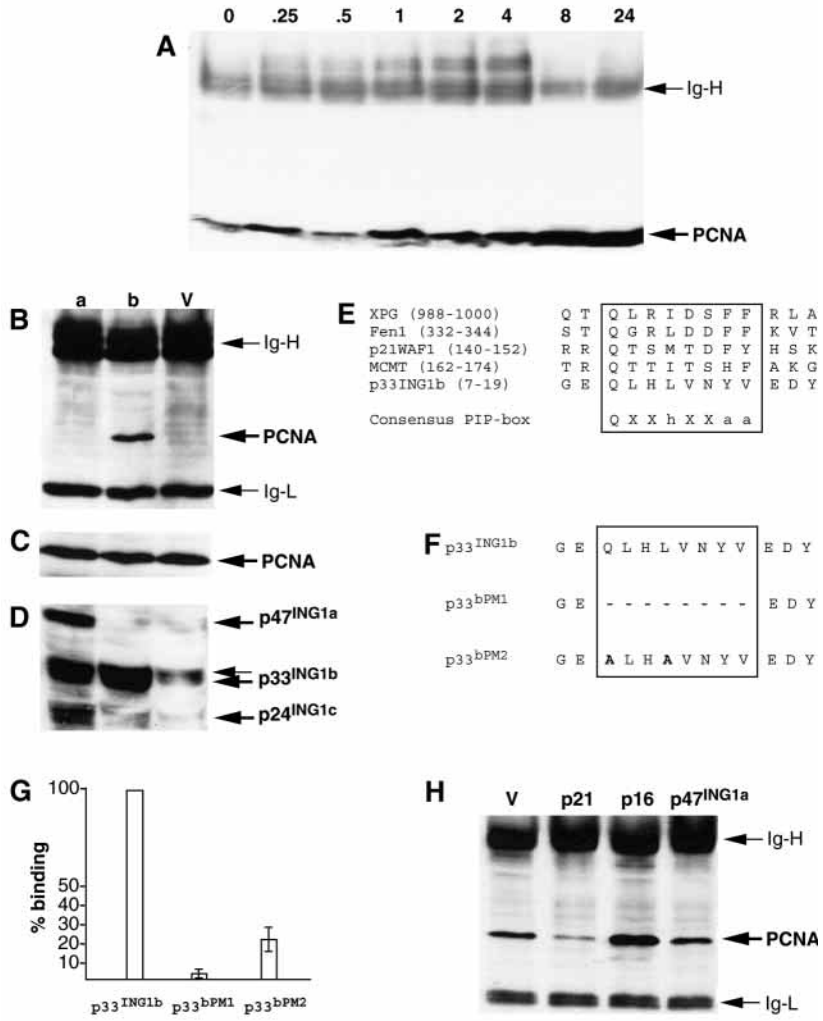

Fig. 3. Co-immunoprecipitation of $p33^{ING1b}$ with PCNA. (A) Nondenaturing immunoprecipitations of extracts from 5×10^6 cells with rabbit polyclonal α-ING1 were done in the absence (0) or at the number of hours indicated on the abscissa, after exposure of cells to 25 J/m^2 of UV. Immunoprecipitates were electrophoresed and blotted with mouse α-PCNA. IgG-H and IgG-L represent crossreactivity with heavy and light chains of immunoprecipitating antibodies, respectively. (B) Cells transfected with parental vector pCI^{neo} (V), or with expression constructs encoding $p47^{ING1a}$ (a) or $p33^{ING1b}$ (b) were harvested 24 hours after electroporation under nondenaturing conditions, immunoprecipitated with a mixture of four mouse α-ING1 monoclonals (Cab1-4) and electrophoresed and blotted with rabbit α-PCNA. (C) Lysates from the same experiment as shown in B blotted with rabbit α-PCNA. (D) Lysates from the same experiment as shown in B and C blotted for ING1 expression with Cab1-4. (E) The PIP motif in $p33^{ING1b}$ where 'X' represents any amino acid, 'h' represents a hydrophobic residue and 'a' represents aromatic residues. ING1b differs from the consensus sequence only by having a valine residue in place of an aromatic amino acid but both valine and phenylalanine have hydrophobic character. (F) Sequence of the PIP box mutations examined in G, showing the relative degree of interaction between PCNA and $p33^{ING1}$ variants. Values were generated by scanning densitometry, Results shown are the average of two experiments where data for each was generated by three scans at different exposures. (H) Effects of overexpressing the cyclin-dependent kinase inhibitors $p21^{WAF1}$ and $p16^{MTS1}$ on the ability of ING1 to bind PCNA. Cells transfected with vector, p21, p16 or $p47^{ING1a}$ were harvested 48 hours later and immunoprecipitated with α-ING1 monoclonals (Cab1-4) under non-denaturing conditions. Immunoprecipitates were electrophoresed and probed with rabbit α-PCNA. Experiments were done with both Hs68 and SNB19 cells, and gave similar results.

the samples (Fig. 3C) and that transfection resulted in overexpression of $p47^{ING1a}$ or $p33^{ING1b}$. Cells transfected with $p47^{ING1a}$ expression construct also produce truncated $p24^{ING1c}$ from internal initiation and a polypeptide of approximately 32 kDa that appears to be distinct from $p33^{ING1b}$, since it does not bind PCNA, and which may represent an additional ING1 isoform (Jager et al., 1999; Gunduz et al., 2000) or a degradation product of $p47^{ING1a}$.

Because ING1 appears to be inducible by UV in some cell types (Cheung et al., 2000), we asked whether the increased PCNA seen in ING1 immunoprecipitates after UV might be a function of increased amounts of ING1. Trial experiments to determine whether the ING1 gene or proteins increased in abundance in primary fibroblasts following exposure to UV showed no reproducible alterations in ING1 levels in response to 10-50 J/m^2 (data not shown), similar to previous observations for primary fibroblasts (Cheung et al., 2000).

$p33^{ING1b}$ binds PCNA through a PIP motif

Sequence analysis suggested that a candidate amino acid motif within $p33^{ING1b}$ that shares strong homology with regions in other proteins known to interact with PCNA through their PIP motifs (Warbrick, 1998; Tsurimoto, 1999) (Fig. 3E) may be required for binding. To test this idea, mutations (Fig. 3F) were introduced into this region of $p33^{ING1b}$ and constructs were transfected into fibroblasts. Wild-type $p33^{ING1b}$, but not PIP mutant 1 ($p33^{bPM1}$, a deletion of the PIP motif) clearly interacted with PCNA (Fig. 3G). Although binding was not abolished, as seen for the deletion mutant, perhaps due to binding through a secondary site, as seen for FEN1 (Gomes and Burgers, 2000) and $p21^{WAF1}$ (Zheleva et al., 2000), binding of $p33^{bPM2}$ (with two conserved residues within the PIP box mutated to alanine) was strongly inhibited. Decreased binding was not due to decreased expression of the mutant forms of $p33^{ING1b}$ as values in Fig. 3G show percent binding normalized to the amounts of $p33^{ING1b}$ and the mutants expressed. Furthermore, $p33^{ING1b}$ could compete for PCNA binding with another protein known to have a PIP box. Fig. 3H shows that transfection of the CDK inhibitor $p21^{WAF1}$ (which contains a PIP motif), but not the CDK inhibitor $p16^{MTS1}$ (which has no PIP motif), inhibited the interaction between $p33^{ING1b}$ and PCNA, similar to competition reported previously between $p21^{WAF1}$ and FEN1 for PCNA binding (Waga et al., 1994). Exposures in this panel are similar to those used in panel A

and therefore show an interaction between ING1b and PCNA when cells were transfected with vector only, similar to results in untransfected cells (panel 3A).

UV induces colocalization of ING1 and PCNA within the nucleus

To determine whether the UV-induced association of ING1 with PCNA coincided with alterations in nuclear distribution, fibroblasts were examined individually by confocal microscopy. Twenty-four hours after plating onto glass coverslips, one set of cells was treated with UV light, incubated for a further 15 minutes to 4 hours and fixed and stained for DNA, ING1 and PCNA, or for DNA, ING1 and p53. p53 was used as a control since it is UV-inducible, localizes to the nucleus and has been reported to physically associate with ING1 (Garkavtsev et al., 1998). Fig. 4 shows that UV induced a significant apparent increase in colocalization of ING1 with PCNA but not of ING1 with p53. Computer analyses of ten nuclei per condition showed that the UV-inducibility of the ING1-PCNA colocalization was similar in cells synchronized by serum deprivation and refeeding in G_0 and S phases of the cell cycle (data not shown). Owing to constraints of the experimental system, a baseline colocalization is detected for any two proteins examined. Examination of two proteins known to occupy different nuclear compartments, the CBP histone acetyl transferase and histone deacetylase-A (HDAC-A), showed that the degree of colocalization of these control proteins did not vary following UV exposure and resembled that of ING1 and p53 under all conditions examined. An additional possibility that we consider unlikely is that the apparent colocalization noted is due to the induction of a more homogenous distribution of pCNA, but not of control proteins in the nuclei of cells following UV that is separate from functional colocalization. This is especially unlikely since changes in the degree of diffuseness of staining is seen with p21 under different conditions despite a functional association between p21 and PCNA being seen (Li et al., 1996). Average values of apparent colocalization for 10 randomly selected nuclei of cells co-stained with α-ING1 and α-PCNA or with α-ING1 and α-p53 at different time points following UV exposure are shown in Table 1. These results corroborate data obtained previously using biochemical methods and both methods independently support the idea that there is a more than tenfold induction of $p33^{ING1b}$-PCNA binding/colocalization in response to UV irradiation.

Table 1. Relative association of proteins following UV treatment

	Hours after UV exposure							
Protocol	0	0.25	0.5	1	2	4	8	24
ING1-PCNA IP-Western	1	2.6	1.2	5.3	4.4	6.1	10.6	11.4
ING1-PCNA Confocal	1	2	5.5	10.5	14.5	12	ND	ND
ING1-p53 Confocal	0	1	1	0.5	0.5	0	ND	ND

Values for the IP-western experiment are all relative to the association seen between ING1 and PCNA in the absence of UV, which was set arbitrarily to a value of 1. Values were generated by using scanning densitometry and analysed with the NIH image program (Wong et al., 1994). Results for association as measured by confocal microscopy are given as a percentage and all have background values of 14% (for the control proteins CBP and HDAC-A) subtracted. Each value is the average of five separate nuclei measured during G_0 and five measured in S phase of the cell cycle and contains measuring errors ranging from ±2% to ±6%. ND, not determined.

Binding to PCNA correlates with the ability of $p33^{ING1b}$ to induce apoptosis

Since these data suggested that $p33^{ING1b}$ binding might effect a change in the function of PCNA, possibly from a role in DNA replication to a role in DNA repair, we next examined whether

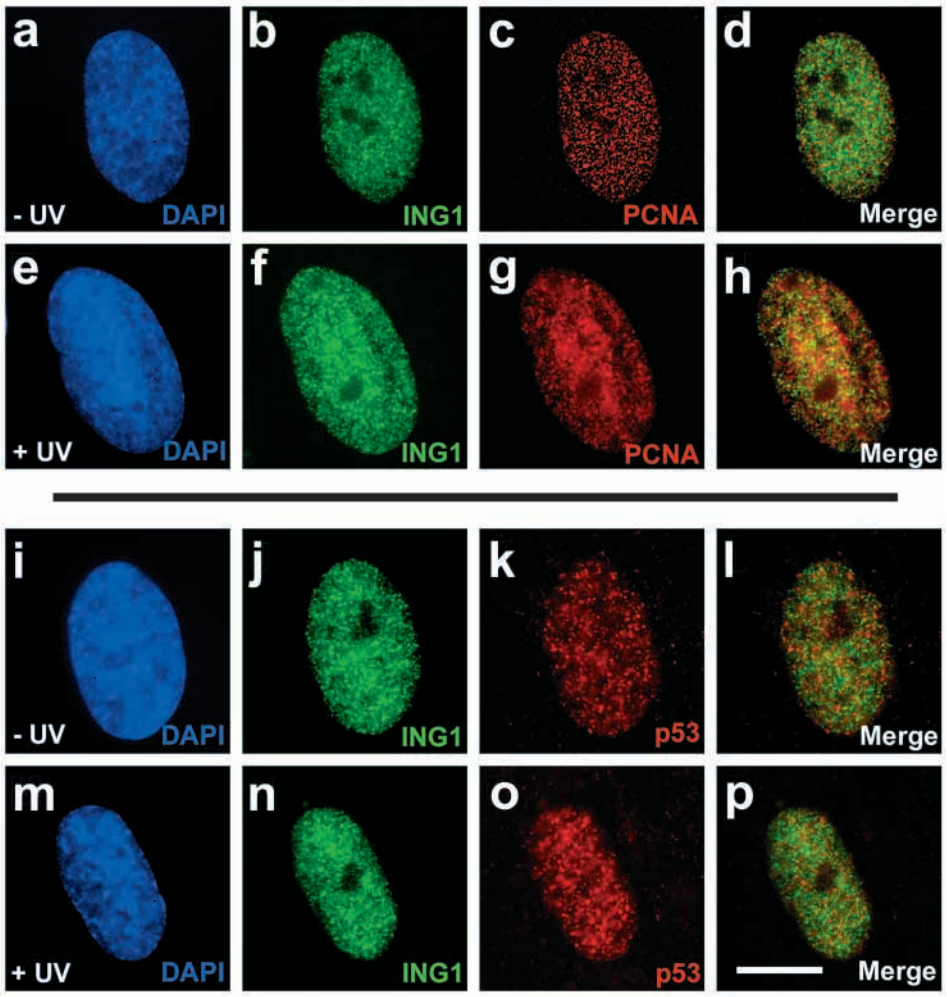

Fig. 4. UV-induced colocalization of ING1 with PCNA but not with p53. Half of a set of Hs68 fibroblasts plated for 24 hours were treated with 25 J/m^2 of UV, fixed 2 hours later and stained for DNA, ING1 and PCNA (a-h), or for DNA, ING1 and p53 (panels i-p). Confocal microscopy and calculation of the degree of colocalization was as described in Materials and Methods. Bar, 10 μm.

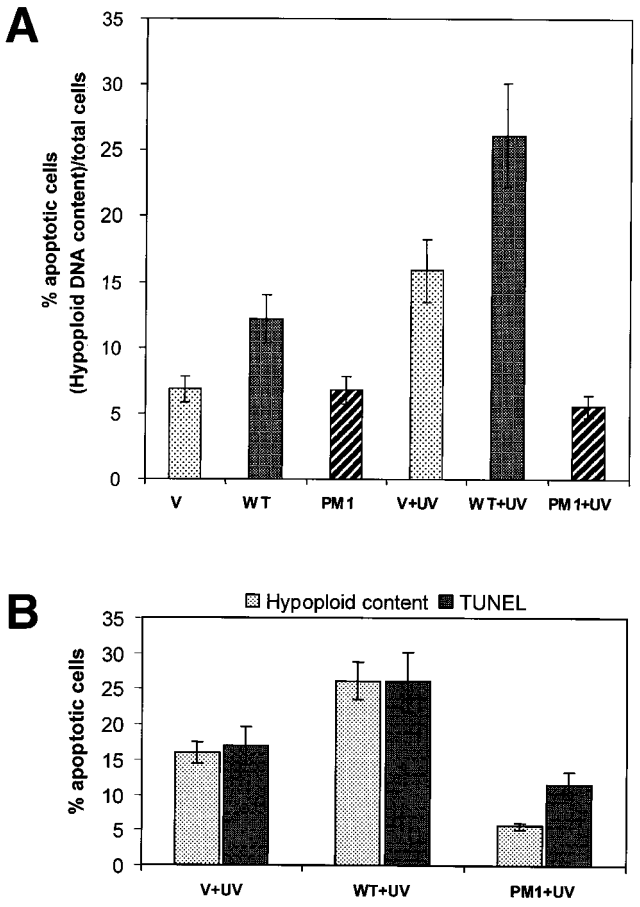

Fig. 5. p33^{ING1b} PIP mutants that do not bind PCNA do not induce apoptosis. Hs68 fibroblasts electroporated with constructs encoding GFP plus vector (V), wild-type (WT) or mutant (PM1) versions of p33^{ING1b} were allowed to recover for 24 hours and the subset indicated were treated with UV. 48 hours after electroporation, cells were harvested and analyzed by FACS (A), following propidium iodide staining to detect sub-G_1 DNA content as a measure of apoptosis. The graph shows the mean ± s.e. from five individual trials. (B) A comparison of results obtained by FACS (examining DNA content) and by TUNEL to confirm FACS results by examining DNA breakage.

overexpression of p33^{ING1b} or of different mutants of p33^{ING1b} might differentially affect the survival of cells exposed to UV irradiation. As shown in Fig. 5A, primary diploid cells co-transfected with empty control vector and a Green Fluorescent Protein (GFP) expression construct to identify cells taking up plasmid, showed similar levels of cells containing a sub-G_1 content of DNA as cells transfected with GFP plus mutant p33^{bPM1} which does not bind PCNA. By contrast, wild-type p33^{ING1b} increased the number of cells with sub-G_1 DNA staining by 78% in an average of three independent experiments. Although this degree of induction of apoptosis is less than that seen for other cell systems, this is expected since the induction of apoptosis in primary human fibroblasts differs from blood cells and established cell lines, generally showing lower levels and requiring prolonged time courses for apoptotic events that appear linked to transcriptional abnormalities (Di Leonardo et al., 1994; Andera and Wasylyk, 1997) and cell passage or state of senescence (Wang, 1995).

Other variants of p33^{ING1b} that were point mutated within the PIP motif, including p33^{bPM2}, gave intermediate levels of induction of apoptosis, consistent with their reduced efficiency of PCNA binding (Fig. 3G; data not shown). To determine if this effect was also seen in response to UV, cells were analyzed following transfection and UV exposure. As expected, following UV baseline levels of sub-G_1 cells increased in the vector control and expression of p33^{ING1b} further increased the number of cells showing sub-G_1 DNA staining. However, overexpression of p33^{bPM1}, rather than having an effect similar to vector, efficiently protected cells from UV in that cells had levels of sub-G_1 DNA content similar to unexposed controls. To test by a different method whether cells with a sub-G1 DNA content were undergoing apoptosis, the UV experiment was repeated with p33^{bPM1} but cells were analyzed by TUNEL. Very similar results were obtained (Fig. 5B) consistent with expression of the mutant, which does not bind PCNA, blocking cells from entering apoptosis.

DISCUSSION

This study indicates that the 33 kDa but not the 47 kDa isoform of ING1 strongly binds PCNA in a UV-inducible manner and that this binding is reflected by a major change in subnuclear localization. ING1 interacts with PCNA through a PIP sequence since mutation of the PIP sequence, but not other adjacent sequences of ING1, drastically affected binding. Therefore, ING1 should either interfere with, or block, the association of other proteins that bind this site such as p21^{WAF1}, the FEN1 and XPG nucleases, and DNA cytosine 5 methyltransferase (MCMT). Indeed, p21^{WAF1} overexpression, but not p16^{MTS1} overexpression, interferes with ING1b-PCNA association. However, these data do not rule out the possibility that the reduction of ING1-PCNA binding is due to the growth inhibitory effect of p21^{WAF1}. For example, ING1 and PCNA may bind in a cell cycle-regulated manner and so p21^{WAF1} might simply block cell cycle progression at a point where little interaction occurs. This possibility seems unlikely given the fact that both p21^{WAF1} and the p16^{MTS1} CDK inhibitors block cell growth within G_1 of the cell cycle when overexpressed.

The p21^{WAF1} protein can inhibit DNA replication by interacting with PCNA (Waga et al., 1994), and p33^{ING1b} is capable of inducing p21^{WAF1} expression (Zeremski et al., 1999) and of directly binding PCNA within minutes of exposure to UV (Fig. 3). Therefore, p33^{ING1b} may operate to block access to the PIP binding domain of PCNA in an acute manner through binding of p33^{ING1b}, and in a delayed manner through induction of p21^{WAF1} expression, which may be independent of, or synergistic with, UV-induced induction of p21^{WAF1} by p53. This competition could inhibit reassociation of the DNA polymerase core complex, which is essential for DNA replication (Reynolds, 2000) but not likely critical for DNA repair (Podust et al., 1995). This would favor DNA repair processes over DNA replication and possibly serve as a DNA damage checkpoint mechanism, similar to that proposed for p53. Alternatively, the binding of ING1b to PCNA may serve to inhibit the access of p21 to PCNA, promoting continued DNA synthesis of damaged DNA, which could subsequently trigger apoptosis.

How the rapid interaction of $p33^{ING1b}$ with PCNA is regulated is unknown but our preliminary data indicate that $p33^{ING1b}$ phosphorylation occurs rapidly following UV exposure, which may regulate its interaction with PCNA as reported recently for $p21^{WAF1}$ (Scott et al., 2000), perhaps through the action of PCNA-bound CDK2 (Koundrioukoff et al., 2000). The $p33^{ING1b}$-PCNA association would also alter the ratio of free $p33^{ING1b}$ to $p47^{ING1a}$, which could have the effect of differentially regulating gene expression since different isoforms of ING1 have been reported to have opposite effects upon the activation of p53 as a transcription factor (Zeremski et al., 1999).

The details of the mechanism(s) by which wild-type but not PIP mutant forms of $p33^{ING1b}$ promotes apoptosis upon interaction with PCNA is presently unknown, but a report indicating that the wild-type but not mutant forms of $p21^{WAF1}$, which do not interact with PCNA, promotes apoptosis (Asada et al., 1999) suggests functional similarities between the formation of ING1-PCNA and p21-PCNA complexes. Since overexpression of both $p21^{WAF1}$ and $p33^{ING1b}$ block cell cycle progression, both might contribute to regulating the induction of apoptosis by growth inhibition through binding PCNA. Whether the major role(s) of $p33^{ING1b}$ when bound to PCNA is a function of displacing other molecules from the complex, recruiting additional proteins to PCNA, binding different sites on PCNA, as seen for FEN1 (Gomes and Burgers, 2000) or altering the ratio of free ING1 isoforms, awaits clarification. Although these questions can be addressed through the use of regulatable expression constructs encoding $p33^{ING1b}$ and $p47^{ING1a}$, such experiments shall also have to be interpreted in light of reports of additional ING1 isoforms and related genes such as ING1L (Shimada et al., 1998) and ING2 (Jager et al., 1999).

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