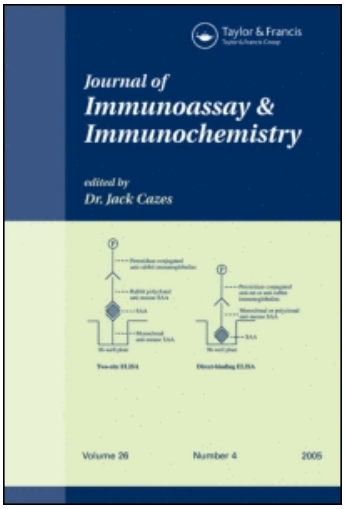

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Generation of a Polyclonal Antibody Specifically Against the p33^{ING1b} Tumor Suppressor

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ABSTRACT

The p33^{ING1b} tumor suppressor protein plays a prominent role in cellular stress responses including cell cycle arrest, DNA repair, apoptosis, and chromatin remodeling. As the main product of the inhibitor of growth 1 (*ING1*) gene, p33^{ING1b} is the most intensively studied protein of the ING family. So far, most ING1 antibodies have been raised against full-length proteins. Since all ING1 isoforms share an identical carboxyl-terminus, and commercially available ING1 antibodies often lack specificity, we sought to develop a polyclonal antibody capable of specifically recognizing the p33^{ING1b} protein. Here, we describe the development and characterization of the p33^{ING1b}-specific antibody.

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Key Words: Polyclonal antibody; $p33^{ING1b}$; Tumor suppressor; *ING1* gene.

INTRODUCTION

The inhibitor of growth 1 (*ING1*) gene was initially cloned through subtractive hybridization between breast cancer and non-transformed mammary epithelial cells.[1] Two distinct promoter regions within the *ING1* gene, as well as mRNA splicing events, give rise to at least three detectable isoforms of 24, 33, and 47 kDa, all of which share an identical C-terminus containing a PHD zinc finger motif.[2] The $p33^{ING1b}$ isoform is believed to be the most abundant,[3] and is ubiquitously expressed in various human tissues.[4] The $p33^{ING1b}$ plays a crucial role in inducing cell cycle arrest and promoting apoptosis. Flow cytometry analysis demonstrated a G_0/G_1 arrest of normal fibroblasts transfected with $p33^{ING1b}$, while antisense constructs were found to be capable of promoting cellular transformation.[1] In fact, $p33^{ING1b}$ can physically associate with p53 and dramatically enhance transactivation of the CDK inhibitor, $p21^{waf1}$, in a p53-dependent manner.[5] Moreover, using microarray technologies, it was recently found that the $p33^{ING1b}$ down regulates the expression of cyclin B1, which is required for mitotic initiation.[6] In another study, Shinoura et al.[7] demonstrated that adenoviral delivery of both $p33^{ING1b}$ and p53 induce apoptosis in two glioma cell lines that would not undergo apoptosis by overexpressing either gene alone. Using the host-cell reactivation assay and radioimmunoassay, we have recently demonstrated that $p33^{ING1b}$ greatly enhances the repair of UV-damaged DNA and that $p33^{ING1b}$ can physically associate with GADD45, which is known to be involved in DNA repair.[8]

All ING1 isoforms share a common carboxyl-terminus and, thus, polyclonal antibodies raised against a full-length $p33^{ING1b}$ protein can potentially recognize all ING1 isoforms. In order to further investigate the biological functions of the tumor suppressor $p33^{ING1b}$, we describe here the development and characterization of a polyclonal antibody raised specifically against the $p33^{ING1b}$ protein.

EXPERIMENTAL

Materials

The synthetic peptide N-LSPANEQLHLVNC-C corresponding to the N-terminus of the $p33^{ING1b}$ protein was synthesized by the University of British

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Columbia Nucleic Acid and Protein Service Unit. All other reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

Methods

Antibody Production

The synthetic peptide was initially conjugated to a carrier protein keyhole limpet hemocyanin (KLH). The KLH (20 mg) was denatured by boiling, in the presence of 5% (v/v) sodium dodecyl sulfate (SDS) and 10 mM sodium carbonate. The solution was then activated with N-hydroxysuccinimide-iodoacetate ester (NHS-Iac) at room temperature for 10 min. The activated species of the KLH was then purified by passing though a G15 Sephadex-bead column. Five milligram of the peptide were dissolved in 400 μL of 10 mM sodium phosphate [pH 8.5], added to the iodoacetate-activated KLH, and left on a rotator at room temperature overnight. The concentration of the conjugated protein solution was adjusted to 1 mg/mL in phosphate buffered saline (PBS). Two New Zealand rabbits were initially immunized with 1 mg of conjugated peptide in Freund's complete adjuvant, and every 2 weeks subsequently with Freund's incomplete adjuvant. After 6 weeks of immunization, rabbit serum was tested for immune response with enzyme-linked immunosorbent assay (ELISA), and the immunized rabbit was bled and immune serum was prepared for antibody purification.

Purification of ING1b-Specific Antibody

Affinity column construction: 10 mL of sepharose CL beads were activated by washing with 10 mM sodium carbonate, followed by 20 min incubation with 100 mg of sodium *m*-periodate. Beads were then washed with 0.1 M citrate buffer and conjugated with 10 mg of the synthetic peptide at pH 9.5, by rotating at room temperature for 30 min. Then, 10 mg sodium cyanoborohydride was added to the beads at room temperature overnight. Beads were washed with PBS prior to use.

Affinity purification of ING1b-specific antibodies: Approximately 80 mL of the anti-serum was passed through the affinity column. The column was then washed with 100 mL of PBS followed by 50 mL of 1 M NaCl. The specific anti-$p33^{ING1b}$ antibodies retained in the column were eluted with 3% acetic acid. The antibodies were then precipitated with 30% of ammonium sulphate and reconstituted with PBS.

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Enzyme-Linked Immunosorbent Assay

BamHI and EcoRI restriction sites were introduced upstream and downstream of *ING1b* by PCR (forward primer: 5′-CTCGAGGATCCCTG-CAGC-3′; reverse primer: 5′-CGAATTCCTACCTGTTGTAAGCCC-3′) from pCI-ING1b (a kind gift from Dr. K. Riabowol, University of Calgary). A glutathione-S-transferase (GST)-ING1b fusion vector was then obtained by subcloning *ING1b* into pGEX-2T (Amersham Bioscience). The vector was introduced into BL21 *E. coli*, grown to an optical density of 0.6, and induced with 1 mM IPTG for 2 h at 26°C with vigorous shaking. Cells were resuspended in a resuspension buffer (25 mM Tris [pH 7.5], 150 mM NaCl, 5 mM β-mercaptanol) and lysed on ice for 30 min in the presence of 50 ng/mL lysozyme and subsequent freeze/thaw cycles. $MgCl_2$ (5 mM) was added and DNA degraded with 50 ng/mL DNase I for 30 min on ice. Nonidet-40 (1% v/v) was added to the lysate, which was then rotated for 30 min at 4°C, centrifuged at 12,000*g* for 30 min at 4°C and fusion protein recovered from supernatant with glutathione beads. An ELISA plate was coated with GST or GST-$p33^{ING1b}$ in 20 mM Tris-Cl (pH 8.5) for 1 h at room temperature, washed with 0.05% (v/v) Tween 20/PBS (PBS-T), and blocked with 5% milk in PBS-T. The plate was then incubated with 0.2 μg/mL of anti-p33 antibodies, washed, and incubated with a HRP-conjugated goat anti-rabbit antibody (Santa-Cruz biotech). All antibody incubations were done at room temperature in 5% milk in PBS-T for 1 h. *o*-Phenylenediamine dihydrochloride was used as a substrate.

Cell Culture and Transfection

MMRU melanoma cells (a kind gift from Dr. R. Byers, Boston University School) were maintained in Dulbecco's modified Eagles medium, supplemented with 10% fetal bovine serum (Canadian Life Technologies, Burlington, ON), 100 units/mL penicillin, and 100 μg/mL streptomycin in a 5% CO_2 atmosphere at 37°C. Cells were transfected at 50–60% confluency with Effectene reagent (Qiagen, Mississauga, ON) at a ratio of 1 μg DNA to 25 μL Effectene. Plasmids used for transfection include pCI-ING1b and pCMV-ING1b-FLAG, constructed by inserting a BglII and KpnI restriction site up and downstream the *ING1b* construct by PCR (forward primer: 5′-GAAGATCTACCATGCTGAGTCCTGCCAAC-3′; reverse primer: 5′-CGGGGTACCCCTGTTGTAAGCCCTCTC-3′) and re-ligating in the pCMV-FLAG vector.

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Western Blot Analysis

Cells were harvested by scraping and solubilized with a triple-detergent lysis buffer (50 mM Tris-Cl [pH 8.0], 150 mM NaCl, 0.1% SDS, 1% Nonidet-40, 100 μg/L phenylmethylsulphonyl fluoride, 0.02% sodium azide, 1 μg/mL aprotinin, 1 μg/mL leupeptins, 1 μg/mL pepstatin A). The concentration of proteins was determined by using DC Protein Assay (Bio-Rad, Mississauga, ON). Fifty microgram per lane of proteins were separated on 12% polyacrylamide-SDS gels and electroblotted onto polyvinylidene difluoride (PVDF) filters. Filters were incubated with primary antiserum (1 : 750 dilution of a 0.3 μg/mL stock in PBS-T containing 5% BSA) for 1 h, followed with three washes in PBS for 5 min each, and then incubated with HRP-conjugated secondary antiserum for 1 h at room temperature. Signals were detected with SuperSignal ECL (Pierce, Rockford, IL).

Immunofluorescence

MMRU cells were grown on 22 mm × 22 mm cover slips at a density of 2 mm × 10^4 cells/well in a 6-well plate, and transfected with pCMV-ING1b-FLAG. After 24 h, cells were simultaneously fixed and extracted in 3.7% paraformaldehyde (v/v), 0.25% glutaraldehyde (v/v), and 0.5% Triton X-100 (v/v) in PEM buffer (40 mM PIPES, 2.5 mM EGTA, 0.5 mM $MgCl_2$ [pH 6.9]) for 10 min. Indirect immunolabeling was performed by inverting cover slips on antibody solutions (dissolved in PBS) at room temperature for 45 min, and by washing with PBS three times, 5 min each, between incubations. Cells were counterstained with HOECHST 33258 (2 mg/mL stock diluted 1 : 3000 in PBS) to visualize DNA, mounted in Permount mounting media (Fischer Scientific, Ottawa, ON), and visualized under a Zeiss Axioplan 2 microscope.

RESULTS AND DISCUSSION

A number of biological functions, such as senescence, cell cycle arrest, apoptosis, DNA repair, and chromatin remodeling, have been attributed to the *ING1* tumor suppressor gene.[9,10] Of the three protein products encoded by this tumor suppressor gene, the $p33^{ING1b}$ is the most abundant, and ubiquitously expressed in various human tissues[3,4] We here, describe the generation of rabbit antiserum specifically against the $p33^{ING1b}$ protein. The carboxyl-terminal of all three ING1 proteins is encoded by exon 2 of *ING1*. Peptides differ at their amino-terminus, which is encoded by exon 1b for $p47^{ING1a}$ and exon 1a for $p33^{ING1b}$. The $p24^{ING1c}$ variant is solely encoded by exon 2[2,11] A 12-amino acid peptide, corresponding to the amino-terminus of

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A

p47^{ING1a}:

MSFVECPYHSPAERLVAEADEGGPSAITGMGLCFRCLLFSFSGRSGVEGGRVDLNVFGSLGLQPWIGSSRCWGGPCS
SALRCGWFSSWPPPSKSAIPIGGGSRGAGRVSRWPPPHWLEAWRVSPRPLSPLSPXXFGRGFIAVAVIPGLWARGRG
CSSDRLPRPAGPARRQFQAASLLTRGWGRAWPWKQILKELDECYERFSRETDGAQKRRMLHCVQRALIRSQELGDEK
IQIVSQMVELVENRTRQVDSHVELFEAQQELGDTVGNSGKVGADRPNGDAVAQSDKPNSKRSRRQRNNENRENASSN
HDHDDGASGTPKEKKAKTSKKKKRSKAKAEREASPADLPIDPNEPTYCLCNQVSYGEMIGCDNDECPIEWFHFSCVGL
NHKPKGKWYCPKCRGENEKTMDKALEKSKKERAYNR

p33^{ING1b}:

MLSPANGEQLHLVNYVEDYLDSIESLPFDLQRNVSLMREIDAKYQEILKELDECYERFSRETDGAQKRRMLHCVQRALIR
SQELGDEKIQIVSQMVELVENRTRQVDSHVELFEAQQELGDTVGNSGKVGADRPNGDAVAQSDKPNSKRSRRQRNNE
NRENASSNHDHDDGASGTPKEKKAKTSKKKKRSKAKAEREASPADLPIDPNEPTYCLCNQVSYGEMIGCDNDECPIEW
FHFSCVGLNHKPKGKWYCPKCRGENEKTMDKALEKSKKERAYNR

p24^{ING1c}:

MLHCVQRALIRSQELGDEKIQIVSQMVELVENRTRQVDSHVELFEAQQELGDTVGNSGKVGADRPNGDAVAQSDKPN
SKRSRRQRNNENRENASSNHDHDDGASGTPKEKKAKTSKKKKRSKAKAEREASPADLPIDPNEPTYCLCNQVSYGEMI
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B

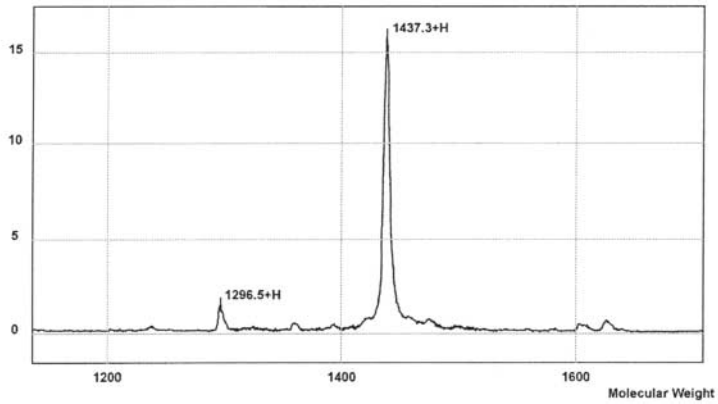

Figure 1. Peptide used for generation of p33^{ING1b}-specific polyclonal antibody. (A) Sequence of p47^{ING1a}, p33^{ING1b}, and p24^{ING1c} (accession number BAA82887, BAA82886, and BAA83496, respectively) proteins. Underlined amino acids indicate sequence used to raise anti-ING1b antibody. The protein sequence encoded by a common exon is shaded. (B) Mass spectrometry analysis of the peptide used to raise polyclonal antibodies recognizing the N-terminus of p33^{ING1b}. The predicted molecular weight of the peptide is 1437.56.

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$p33^{ING1b}$ was used to raise rabbit anti-sera specific for the $p33^{ING1b}$ isoform [Fig. 1(A)]. Note, that a glycine residue was not included in the peptide to avoid the formation of a cyclic structure upon synthesis. A cysteine residue was also added at the carboxyl terminus for KLH conjugation. SELDI mass spectrometry was performed to assess the purity of the synthesized peptide [Fig. 1(B)]. The observed molecular weight of the peptide corresponds to the predicted molecular weight of 1437.56. Minimal impurity was observed.

After immunization of the rabbits and purification of the antibody (see Methods), we characterized the specificity of this antibody. The affinity-purified polyclonal antibody was found to be highly specific for the $p33^{ING1b}$ protein. The antibody was capable of detecting a bacterially produced GST-$p33^{ING1b}$ fusion protein, but not GST alone, in an ELISA assay (Fig. 2). The high specificity of this antibody was further assessed through western analysis. Protein extracts were obtained from MMRU cells expressing a $p33^{ING1b}$-FLAG fusion protein or untagged $p33^{ING1b}$, resolved by SDS-PAGE, and transferred to membranes [Fig. 3(A)]. When membranes were probed with the antibody, very little background is observed. As expected, the FLAG fusion appears slightly higher than the untagged $p33^{ING1b}$ counterpart and can be detected by anti-FLAG antibody when the membrane is stripped and

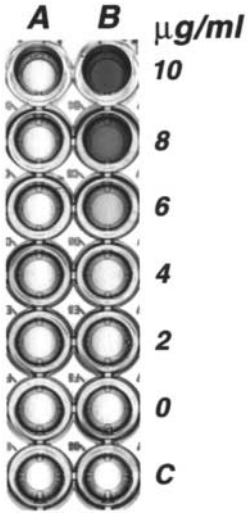

Figure 2. Enzyme-linked immunosorbent assay showing recognition of a bacterially produced GST-ING1b fusion protein by the anti-ING1b polyclonal antibody. Plate was pre-coated with serial dilutions of GST alone (A) or GST-ING1b (B), and incubated with 0.2 μg/mL of anti-$p33^{ING1b}$ antibody. (C) Buffer alone control.

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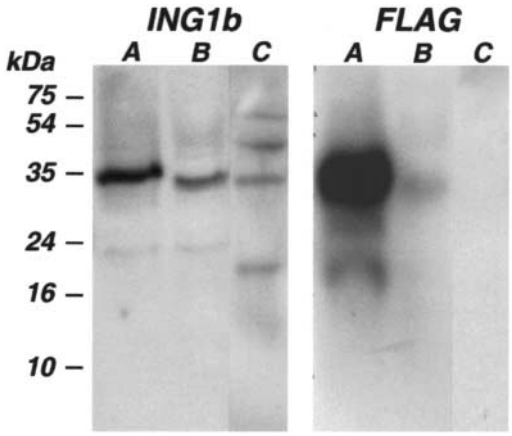

Figure 3. Western analysis of $p33^{ING1b}$ using the ING1b-specific polyclonal antibody. Lanes were loaded with protein extracts from MMRU cells transfected with pCMV-ING1b-FLAG (A) or ING1b (B). Extracts from untransfected cells are also shown (C). Membranes were probed with anti-ING1b antibody, striped and re-probed with anti-FLAG antibody.

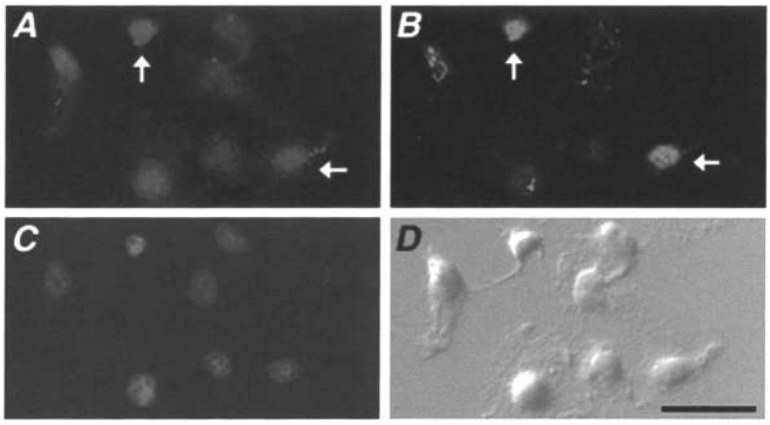

Figure 4. Immunofluorescent microscopic images of MMRU cells transfected with pCMV-ING1b-FLAG. As expected, $p33^{ING1}$ is found to be mainly a nuclear protein. Both endogenous and exogenous $p33^{ING1b}$ was detected with the $p33^{ING1b}$-specific rabbit polyclonal antibody (A). Exogenous $p33^{ING1b}$ was also detected using mouse monoclonal anti-FLAG antibody (B). FITC-conjugated anti-rabbit (A) and rhodamine-conjugated anti-mouse (B) antibodies were used respectively (Jackson Immuno-Research Labs). DNA was stained with HOECHST (C), and a Nomarski differential interference (DIC) picture taken (D). Arrows indicate transfected cells. Scale bar = 50 μm.

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re-probed. Endogenous $p33^{ING1b}$ is also detected by this antibody in untransfected cells. As expected from previous experiments,[12] indirect immunofluorescence using the designed polyclonal antibody, recognized $p33^{ING1b}$ by and large as a nuclear antigen (Fig. 4). Overexpressed exogenous $p33^{ING1b}$-FLAG detected by anti-FLAG antibody was found to largely overlap the signal obtained from the $p33^{ING1b}$-specific antibody. Endogenous levels of $p33^{ING1b}$ were also found to increase 24 h after ultraviolet irradiation of the cells (not shown).

Antibody generation is an important procedure for dissecting the biochemical functions of a protein. Since all ING1 proteins share an identical carboxy-terminus, the generation of a highly specific polyclonal antibody against the $p33^{ING1b}$ protein will enable one to specifically study this protein and reduce background due to non-specific antibody binding.

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