Cloning and Characterization of a p53 and DNA Damage Down-regulated Gene PIQ that Codes for a Novel Calmodulin-Binding IQ Motif Protein and Is Up-regulated in Gastrointestinal Cancers

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Abstract

We have identified a p53 and DNA damage–regulated gene that encodes a novel IQ motif protein, which we have named p53 and DNA damage–regulated IQ motif protein (PIQ). PIQ has two isoforms, long (PIQ-L) and short (PIQ-S), and both bind to calmodulin in the presence and absence of calcium. PIQ expression is down-regulated by p53 and DNA damage–inducing agents, whereas PIQ itself represses the expression of p53 up-regulated modulator of apoptosis that is a key mediator of p53-induced apoptosis. Thus, PIQ is a novel protein that may function to bridge a crosstalk between p53 and calmodulin-regulated cellular processes. We further show that PIQ expression is up-regulated in a number of primary colorectal and gastric tumors when compared with matching normal tissues, suggesting that PIQ may be involved in tumorigenesis and could serve as a valuable diagnostic/prognostic marker for gastrointestinal tumors. (Cancer Res 2005; 65(23): 10725-33)

Introduction

The p53 tumor suppressor is one of the most extensively studied genes (reviewed in refs. 1–5). It encodes a complex protein that functions as a transcription factor and modulates the expression of its downstream target genes. p53 has been implicated in a variety of processes but its role in growth arrest and apoptosis remains undisputed (1–5). To date, several key proapoptotic and antiapoptotic genes have been identified that are positively or negatively regulated by wild-type p53 (4). Recently, one of the p53 downstream target genes, named p53 up-regulated modulator of apoptosis (PUMA), has emerged as a key mediator of p53-dependent DNA damage–induced apoptosis (6–11). PUMA is a direct transcriptional target of p53 that encodes a BH3-only domain proapoptotic protein of Bcl-2 family. It is a key component of the intrinsic pathway that localizes to mitochondria and modulates apoptosis by interacting with antiapoptotic proteins, including Bcl-2 and Bcl-XL (6–8). PUMA is up-regulated in response to DNA damage in a p53-dependent manner (6–8) whereas other apoptotic insults, such as endoplasmic reticulum stress, serum withdrawal, and dexamethasone treatment, up-regulate its expression in a p53-independent manner (8, 12–14).

Others and we have recently reported that PUMA is also regulated by agents that affect cellular Ca2+ homeostasis, suggesting that PUMA may also participate in Ca2+ and calmodulin-dependent signaling pathways (12–14).

Calmodulin is a small (16700 daltons) highly conserved multifunctional Ca2+-binding protein that plays a central role in Ca2+ signaling (reviewed in refs. 15, 16). It interacts with several key proteins, some of which include enzymes, kinases, and phosphatases that affect diverse cellular functions (15, 16). Calmodulin binds to its target proteins in a Ca2+-dependent or Ca2+-independent manner. In general, proteins that bind to calmodulin in a Ca2+-dependent manner do so via a basic amphipathic helix of ~20 amino acids, whereas proteins that bind to calmodulin in a Ca2+-independent manner use an IQ motif that has a consensus sequence (FILV)QXXX(RK)GXXX(RK)XX(FIVWY) (15, 17). Some IQ motif–containing proteins have been reported to also bind to Ca2+-bound calmodulin (15).

In general, the function of calmodulin and IQ motif proteins is modulated via their mutual interactions. For example, calmodulin-binding, in the presence or absence of Ca2+, regulates the activity of several IQ motif proteins including L-type Ca2+ channel, conventional and unconventional myosins, dyneins, and Ras GRF 1 and 2. Conversely, some IQ motif proteins bind and modulate the availability and activity of calmodulin. For instance, PEP-19, a small neuronal IQ motif protein, binds to both Ca2+-bound and Ca2+-free forms of calmodulin and thereby sensitizes calmodulin to alterations in intracellular Ca2+ (18). RC3, also known as neurogranin, is another IQ motif protein that binds to calmodulin in both Ca2+-dependent and Ca2+-independent manner. The binding of RC3 does not affect the Ca2+ and calmodulin association rate but rather accelerates Ca2+ dissociation from calmodulin and consequently shortens the half-life of Ca2+-bound calmodulin (19). Thus, through modulation of calmodulin activity, the IQ motif proteins can also affect calmodulin-regulated cellular responses.

In this article, we report the cloning and characterization of a novel p53 and DNA damage down-regulated gene, which we have named p53 and DNA damage–regulated IQ motif protein (PIQ). We show that this novel IQ motif–containing protein binds to both the Ca2+-bound and Ca2+-free calmodulin, and its expression is down-regulated by p53 and genotoxic agents. Given that p53 downstream target gene PUMA is thought to be regulated by calmodulin-dependent signaling events (14), we further investigated PIQ effect on PUMA expression. Our results also indicate that PIQ transcriptionally down-regulates PUMA expression and thereby suggest that p53, by negatively regulating PIQ, may further enhance PUMA expression. Thus, PIQ is a novel protein that seems to bridge a crosstalk between p53 and calmodulin-regulated cellular processes.

Note: The nucleotide and amino acid sequence of both PIQ-L and PIQ-S have been submitted to the Genbank with accession numbers AY964668 and AY964667.

Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Materials and Methods

Cell lines and culture conditions. MCF-7 and H1299 are human breast and lung cancer cell lines. These cells were maintained in DMEM (Cellgro Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (FBS, Gemini Bio-Products, Woodland, CA). The human colon cancer cells HCT116 and their p53-null variants (20) were maintained in McCoy's 5A medium (Cellgro Mediatech) supplemented with 10% FBS. DLD-1, human colon cancer cells that carries tetracycline/doxycycline-inducible p53 (21), were maintained in DMEM supplemented with 10% FBS in the presence of 20 ng/mL doxycycline to suppress p53 expression.

Plasmids. To generate Myc-tagged PIQ-L and PIQ-S expression vectors, the following PCR primers were used for amplification of the respective open reading frames (ORF): sense: 5'-CGGCTCGAGAACTGGAAGAGTAGAA-AACAAACTCTATCCAGAAGGATCTGAAGAGCAAGGTACAAG-3'; antisense: 5'-CGCCGGAACCTCATCTGGTGGTTTGTTTCGACACAAA-3'. The amplified products were digested with XhoI and BamHI and then subcloned into the mammalian expression vector pCEP4. The resulting vectors were named pCEP-PIQ-L and pCEP-PIQ-S, and were sequenced for confirmation. PIQ promoter fragments, PIQ-pro1 and Qpro2, were generated by PCR amplification using human genomic DNA as a template. For pGL3-PIQpro1 reporter plasmid, a pair of primer, 5'-CGGCTGAGAAGATCTTATGAA-AAGCTTACATCAGGTACAGA-3' and 5'-CCGAACTTTACCTGGTTTACGACTAA-3', was used to generate a 2,038 bp fragment containing 5'-flanking region and part of exon 1 of PIQ gene. This 2,038 bp fragment was then digested with XhoI and HindIII and subcloned into pGL3-basicle luciferase reporter vector (Promega, Madison, WI) upstream of the promoter-less luciferase gene. For pGL3-PIQpro2 reporter construct, the primer pair 5'-CGGCTCGAGAAGATCTTATGAA-AAGCTTACATCAGGTACAGA-3' and 5'-CCGAACTTTACCTGGTTTACGACTAA-3' was used to amplify a 1,822 bp fragment containing the 5'-flanking region, exon 1, intron 1, and part of exon 2 of PIQ gene, and then subcloned into pGL3-basic vector at XhoI and HindIII sites. Both constructs were sequenced for confirmation.

PUMA promoter lucerase construct, pGL-Full, was kindly provided by Dr. Thomas Chittenden and contains a 2,104 bp 5'-flanking region of the human PUMA gene placed upstream of the promoter-less luciferase gene in pGL3-basic vector. The pGL-1010 PUMA promoter deletion construct was generated by digestion of the pGL-Full plasmid with SpeI and EcoRI restriction enzymes. The pGL-1010 deletion construct lacks 1,094 nucleotides corresponding to the 5' end of the 2 kb full-length PUMA promoter and contains one nuclear factor of activated T cells (NFAT) site and one cyclic AMP-responsive element-binding protein (CREB) site. The pGL-697 PUMA reporter construct was generated by the site-directed mutagenesis approach using QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). Briefly, 20 ng of pGL-Full and 125 ng of each of the following primers were mixed with the reaction buffer containing deoxynucleotide triphosphates and Phi29 DNA polymerase: 5'-TTCA-CAAAACACCACTACGCGGCTGCGTGTTTACGAGGCGCCGCT-3' and 5'-AGCCGCGGCCGTATGAACTGCAGCGGGCTGCTGACCCGAAATCC-3'. Reaction was done with the following cycling variables: one cycle of 95°C for 1 minute; 18 cycles of 95°C for 50 seconds, 60°C for 50 seconds, 68°C for 4 minutes and 40 seconds; and one cycle of 68°C for 7 minutes. About 10 units of Dnpi restriction enzyme was added into the amplification product and incubated at 37°C for 1 hour to digest the template. Approximately 2 µl of digested reaction mixture was used for bacterial transformation. Positive clones were selected in the presence of antibiotic ampicillin and confirmed by sequencing. The pGL-697 construct lacks the 1,407 nucleotides that correspond to the 5'-end of the 2 kb full-length promoter.

Detection of PIQ isoforms by reverse transcription-PCR. For first-strand DNA synthesis, ~1 µg of total RNA from HCT116 cells was reverse transcribed with iScript reverse transcriptase using iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) according to the instructions of the manufacturer. Approximately 2 µl of first-strand cDNA product was used as a template for PCR amplification to amplify both PIQ isoforms in the same reaction. A primer pair (sense 5'-GCAAGGTCGACAAGTTGCAAAA-3' and antisense 5'-CCCTCTTCTTCTCCTCTGCTA-3') corresponding to the sequence within exons 7 and 12 was used and PCR reaction was performed with Taq DNA Polymerase using the following cycling variables: one cycle of 94°C for 3 minutes; five cycles of 94°C for 30 seconds, 46.7°C for 30 seconds, 72°C for 30 seconds; five cycles of 94°C for 30 seconds, 44.6°C for 30 seconds, 72°C for 30 seconds; 20 cycles of 94°C for 30 seconds, 42.6°C for 30 seconds, 72°C for 30 seconds; and one cycle of 72°C for 6 minutes. Approximately 1 µg of total RNA that was first-strand cDNA synthesis was also used as a template for PCR reaction under the same conditions to ensure that there was no genomic DNA contamination. For positive controls, PIQ-L, and PIQ-S, cDNAs were used as PCR templates. Same primers and PCR conditions were also used for amplification of PIQ isoforms from human placenta cDNA library (Clontech, BD Biosciences, Palo Alto, CA). The PCR-amplified fragments were subcloned into pCR2.1 vector using TA cloning kit (Invitrogen, Carlsbad, CA) and confirmed by sequencing.

Northern blot analyses. RNA extraction and Northern blot analyses were done as we have previously described (14, 22–24). Briefly, total RNA was extracted using Trizol reagent (Invitrogen) and ~20 µg of total RNA for each sample were fractionated on 1.2% agarose gel and transferred to Nytran SuperCharge membranes (Schleicher & Schuell, Keene, NH). Human PUMA, PIQ, and p21 cDNA probes were labeled with 32P and prehybridizations and hybridizations were done in QuikHyb Solution (Stratagene) at 65°C. To detect PIQ expression in paired human normal and cancer samples, Cancer Profiling Array 1 and Cancer Profiling Array II were purchased from Clontech, BD Biosciences, and probed with PIQ cDNA probe as described above.

Calmodulin-binding assay. To detect PIQ interaction with calmodulin, calmodulin-agarose binding assay was done as described (25–27) with modification. Briefly, H1299 cells were transiently transfected with pCEP-PIQ-L, pCEP-PIQ-S, or pCEP4 vector using Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer. Cells were harvested and lysed in isyus buffer [50 mmol/L Tris-HCl (pH 7.5), 120 mmol/L NaCl, 1% Triton-X 100, 5 mmol/L MgCl2, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 µg/mL leupeptin, 1 mmol/L NaF, and 1.5 µg/mL aprotinin]. Approximately 300 µg of lysate was further diluted in binding buffer [50 mmol/L Tris-HCl (pH 7.5), 120 mmol/L NaCl, 2 mmol/L MgCl2, 100 mmol/L KCl, 1 mmol/L PMSF, 1 µg/mL leupeptin, 1 mmol/L NaF, and 1.5 µg/mL aprotinin], and then mixed with calmodulinagarose beads or control agarose beads (Sigma, St. Louis, MO) supplemented with 1 mM CaCl2 or 1 mmol/L EGTA. After 2-hour incubation at 4°C, the mixture was centrifuged at 500 × g for 2 minutes. Beads were further washed three times with binding buffer supplemented with 1 mmol/L CaCl2 or 1 mmol/L EGTA and then boiled in SDS sample buffer. Bound proteins were fractionated by SDS-PAGE and then transferred to nitrocellulose membranes. Myc-tagged PIQ-L and PIQ-S were detected by Western blot analyses using mouse monoclonal anti-myc tag antibody, 9E10 (Santa Cruz Biotechnology, Santa Cruz, CA) according to standard procedures as we have previously described (22, 23).

Transfections and luciferase assays. H1299 human lung cancer cells were transiently cotransfected with indicated vectors using transfection reagent TransIT-LT1 (Mirus, Madison, WI) as per the protocol of the manufacturer and cultured for indicated time before harvesting. Luciferase assays were done using standard procedures as we have previously described (24, 28). Briefly, harvested cells were washed once with 1× cold PBS, then resuspended in 50 to 70 µL of K2PO4 solution (100 mmol/mL, pH 7.8) and lysed by three freeze and thaw cycles. Supernatants were separated from pellets by centrifugation at 16,000 × g for 30 minutes and protein concentrations were measured by the Bradford method. Approximately 5 to 20 µg of total protein per sample were added into 100 µL Luciferase Assay Reagent (Promega) and luciferase activity was measured using a luminometer (LUMAT LB 9507, Berthold Technologies, Germany).

Results

Cloning of the human PIQ gene. We had previously reported the cloning and characterization of DNA damage–regulated genes (29, 30) whose partial-length cDNAs were first identified by

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subtraction hybridization approach (31). Recent advances in genomics and bioinformatics have significantly facilitated the identification of novel genes of important functions. In our continued efforts to identify novel stress-regulated genes, we did a computer-based alignment of redundant expressed sequence tags in the databases to search for sequences that contain novel ORF. Using this approach, we delineated several expressed sequence tags with putative ORFs and then identified those that were regulated by genotoxic and nongenotoxic stresses. This approach enabled us to identify several novel stress-regulated genes, including the recently reported PDRG (24). Here, we report a novel gene identified by this approach, which we have named PIQ. PIQ encodes an IQ motif protein and is down-regulated by p53 and genotoxic stress.

PIQ has two isoforms, a long isoform (PIQ-L) and a short isoform (PIQ-S; Fig. 1A). PIQ-L, corresponds to a protein of 69.0 kDa with isoelectric point (pI) of 9.3, whereas PIQ-S corresponds to 53.8 kDa protein of 8.84 pI. PIQ gene resides on chromosome 3q13 and, based on genomic organization, PIQ-L contains 15 exons whereas PIQ-S, lacking exons 8, 9, and 10, contains only 12 (Fig. 1B).

PIQ is down-regulated by genotoxic stress and p53. To investigate genotoxic stress regulation of PIQ, MCF-7 and HCT116 cells, harboring wild-type p53, were treated with DNA damage–inducing agents Adriamycin and etoposide and Northern
analyses were done. As shown in Fig. 2B, Adriamycin and etoposide down-regulate PIQ expression in both MCF-7 and HCT116 cells. PUMA and p21 are up-regulated by DNA damage–inducing agents in a p53-dependent manner. We further probed the same blot with PUMA and p21 cDNA probes and found (as expected) that the expression of both PUMA and p21 was induced by Adriamycin and etoposide (Fig. 2B). These results, therefore, indicate that genotoxic stress down-regulates PIQ expression.

Because p53 is a major modulator of cellular response to genotoxic stress, we, therefore, also investigated whether p53 regulates PIQ expression. To that end, we used DLD1 human colon cancer cells that harbor inducible p53. Cells were cultured in the presence or absence of doxycycline and harvested at the indicated times. Total RNA extraction and Northern blot analyses were done as described in Materials and Methods. The membrane was sequentially hybridized with 32P-labeled human PIQ, PUMA, and p21 cDNA probes. Ethidium bromide staining of the gel shows RNA integrity and comparable loading.

Figure 2. A, tissue distribution of human PIQ mRNA. A Northern blot containing poly(A)+ RNA from the indicated human tissues (Clontech) was hybridized with a 32P-labeled PIQ cDNA probe. B, expression of PIQ is down-regulated by DNA damage–inducing agents. Northern blot showing Adriamycin and etoposide down-regulate PIQ expression in MCF-7 and HCT116 cells. Cells were either left untreated (C) or treated with 1 μmol/L adriamycin (Adr) or 30 μmol/L etoposide (Etop) for ~24 hours. RNA extraction and Northern analyses were done by standard procedures as described in Materials and Methods. The blot was sequentially hybridized with 32P-labeled human PIQ, PUMA, and p21 cDNA probes. Ethidium bromide staining of the gel shows RNA integrity and comparable loading; Columns, quantification of the signals that were normalized with respect to untreated controls. C, a representative Northern blot showing p53 suppression of PIQ in DLD1 human colon cancer cells that harbor inducible p53. Cells were cultured in the presence or absence of doxycycline and harvested at the indicated times. Total RNA extraction and Northern blot analyses were done as described in Materials and Methods. The membrane was sequentially hybridized with human PIQ and PUMA cDNA probes. RNA integrity and comparable loading are indicated by ethidium bromide staining of the gel. Columns, quantification of the signals that were normalized with respect to uninduced controls. D, DNA damage–inducing agents down-regulate PIQ expression in a p53-dependent manner. Isogenic HCT116 p53 wild-type and p53-null cells were either left untreated or treated with the indicated concentrations of Adriamycin and etoposide for ~24 hours. Cells were then harvested and Northern blot analyses were done as described in Materials and Methods. The membrane was first probed with PIQ and then with PUMA cDNA probes. Ethidium bromide staining of the gel shows RNA integrity and comparable loading.
colon cancer cells that harbor “Bidirectional Tet Expression Vector” system controlling the inducible expression of exogenous wild-type p53 (21). In these cells, the expression of p53 is repressed when cells are grown in the presence of tetracycline or doxycycline but induced when the antibiotic is removed from the growth medium. As shown in Fig. 2C, cells grown in the presence of doxycycline (wild-type p53 is repressed) exhibited easily detectable levels of PIQ mRNA but cells grown in the absence of this antibiotic (wild-type p53 is induced) exhibited down-regulation of endogenous PIQ. These results indicated that, indeed, p53 induction suppressed PIQ expression. The same blot was also probed with PUMA cDNA probe and, as is shown (Fig. 2C), PUMA expression was increased following doxycycline removal when wild-type p53 was induced, thus confirming that the inducible p53 was functional. In separate experiments, we have also confirmed the induction of p53 in these cells by Northern and Western blotting (data not shown). Thus far, these results indicate that, indeed, p53 induction or genotoxic stress.

To further investigate the role of p53 in down-regulation of PIQ following genotoxic stress, we used a pair of isogenic p53 wild-type (p53+/+) and p53 null (p53−/−) HCT116 cell lines (20). Both p53+/+ and p53−/− cells were treated with adriamycin and etoposide and Northern blot analyses were done. As shown in Fig. 2D, both agents down-regulated PIQ expression only in p53+/+ cells but not in p53−/− cells, suggesting that genotoxic stress regulation of PUMA occurs in a p53-dependent manner. It is well known that genotoxic stress regulation of PUMA occurs in a p53-dependent manner. We, therefore, also investigated the effect of these agents on PUMA expression in these cells and, as expected, PUMA expression was induced by both agents only in p53+/+ cells but not in p53−/− cells (Fig. 2D). Taken together, these results confirm that down-regulation of PIQ following genotoxic stress predominantly occurs in a p53-dependent manner.

**p53 down-regulates PIQ promoter activity.** Our results thus far have established that genotoxic stress down-regulates PIQ expression in a p53-dependent manner and that inducible activation of wild-type p53 also represses PIQ expression (Fig. 2). Next, we sought to investigate whether p53 transcriptionally down-regulates PIQ expression. To that end, first, we cloned and sequenced two genomic fragments corresponding to the 5′-flanking region representing the PIQ gene promoter. The first fragment named PIQpro1 is 2,038 bp in length and contains the 5′-flanking region and part of the exon 1 (Fig. 3A). The second fragment, named PIQpro2, is 1,822 bp in length and contains the 5′-flanking region, exon 1, intron 1, and part of the exon 2 (Fig. 3A).

**Figure 3.** A, schematic illustration of PIQ gene structure and PIQ promoter reporter constructs. B, the 5′-flanking region of human PIQ gene harbors promoter activity. H1299 cells cultured in six-well plates were transiently transfected with 1.0 μg of pGL3-basic, pGL3-PIQpro1, or pGL3-PIQpro2 for ~36 hours and luciferase assays were done as described in Materials and Methods. C, wild-type p53 suppresses PIQ promoter activity. p53-null H1299 cells were transiently cotransfected with 1.0 μg pGL3-PIQpro1 and the indicated amount of p53 expression vector. The total DNA amount was normalized to 2.0 μg using pCMV control vector. Cells were harvested and luciferase assays were done ~36 hours after transfection. D, p53 activates reporter activity from PG13-luciferase construct harboring several copies of p53 response element but not from MG15-luciferase that contains only mutated binding sites. H1299 cells were transfected with 1.0 μg PG13-luciferase or MG15-luciferase construct alone or with 0.5 μg p53 expression vector and the total DNA was normalized to 2.0 μg using pCMV control vector. Transfected cells were harvested ~36 hours later and luciferase assays were done as described. Columns, mean of three independent determinations; bars, SE.
H1299 cells were transiently transfected with expression vectors (pCEP-PIQ-L or pCEP-PIQ-S) carrying myc-tagged PIQ cDNA or the same vector without PIQ cDNA insert. Western blot analysis was done by standard procedures using anti-myc-tag antibodies. PIQ-L and PIQ-S were subjected to SDS-PAGE. Agarose beads without calmodulin and cell lysates were similarly processed to show that PIQ does not bind to agarose beads.

The PIQ promoter function was specific and not due to generalized squelching or toxic effects and was in line with its negative effect on endogenous PIQ expression. As shown in Fig. 3B, p53 clearly suppressed PIQ promoter activity in a dose-dependent manner and similar results were obtained with pGL3-PIQpro1 and increasing amounts of p53 expression vector. As shown in Fig. 3D, p53 clearly up-regulated PG13-promoter activity but did not affect MG15-mediated reporter activity. For this purpose, H1299 cells that both isoforms of PIQ were capable of interacting with calmodulin. Furthermore, calmodulin interactions with both PIQ-L and PIQ-S took place in the presence (1 mmol/L CaCl2) and absence (1 mmol/L EGTA) of Ca2+, indicating that PIQ seemed to interact with calmodulin in both Ca2+-dependent and Ca2+-independent manner. As an additional control, we did the same assay using agarose beads without calmodulin and, as shown in Fig. 4B, both PIQ-L and PIQ-S isoforms coprecipitated with agarose-conjugated calmodulin only in the lysates of cells transfected with PIQ expression vector (Fig. 4B, lanes 4 and 5) but not in the vector-only transfected cells (Fig. 4B, lanes 3 and 5). These results confirmed that, indeed, both isoforms of PIQ interacted with calmodulin.

Figure 4. A, schematic illustration of PIQ-L and PIQ-S. IQ motifs are indicated as dark areas in the respective isoform. The sequence of IQ motifs in PIQ-L and PIQ-S matches perfectly with the consensus sequence of IQ motif. B, calmodulin (CaM) agarose pull-down assay show that both PIQ isoforms interact with calmodulin. H1299 cells were transiently transfected with expression vectors (pCEP-PIQ-L or pCEP-PIQ-S) carrying myc-tagged PIQ cDNA or the same vector without PIQ cDNA insert (V). Cell lysates were precipitated with agarose-conjugated calmodulin in the presence of 1 mmol/L CaCl2 or 1 mmol/L Ca2+ chelator EGTA and precipitates were subjected to SDS-PAGE. Agarose beads without calmodulin and cell lysates were similarly processed to show that PIQ does not bind to agarose beads.

To confirm that these fragments harbor promoter activity, we placed them upstream of promoterless luciferase cDNA into a pGL3-basic vector and the resulting plasmids, named pGL3-PIQpro1 and pGL3-PIQpro2, harboring PIQ promoter luciferase constructs, were transiently transfected into H1299 and MCF-7 cells. As shown in Fig. 3B, both PIQpro1 and PIQpro2 constitutively exhibited higher luciferase activity than the pGL3-basic, which contains promoter-less luciferase construct. These results confirmed that both PIQpro1 and PIQpro2 harbored promoter activity. Furthermore, pGL3-PIQpro2 consistently displayed a higher activity than pGL3-PIQpro1 (Fig. 3B), suggesting that either intron 1 contains additional transactivation site(s) or that there are inhibitory sites residing within the distal 5’-flanking region.

Having established that the 5’-flanking region of PIQ harbors promoter activity, we next investigated whether p53 would regulate PIQ promoter function. For this purpose, H1299 cells with p53-null status were transiently cotransfected with pGL3-PIQpro1 and increasing amounts of p53 expression vector. As shown in Fig. 3C, p53 clearly suppressed PIQpro1 activity in a dose-dependent manner and similar results were obtained with pGL3-PIQpro2 (data not shown). To confirm that p53-mediated suppression of PIQ promoter activity was not due to squelching or toxic effects, we also similarly investigated the effect of p53 on PG13-luc and MG15-luc reporter vectors (32) that contain multiple copies of wild-type and mutated p53-binding sites, respectively. As shown in Fig. 3D, p53 clearly up-regulated PG13-luciferase activity but did not affect MG15-luc reporter function. These results thus confirmed that p53 suppression of PIQ promoter function was specific and not due to generalized squelching or toxic effects and was in line with its negative effect on endogenous PIQ expression.

PIQ contains IQ motifs and binds to calmodulin. Amino acid sequence analysis reveals that PIQ-L contains two IQ motifs whereas PIQ-S carries one (Fig. 4A). The IQ motif corresponds to 11 to 14 residue sequence with consensus (FILV)QXXX(RK)-GXXX(RK)XX(FIVWY) that is used by various proteins to interact with calmodulin (15, 17). Next, we sought to investigate the calmodulin-binding potential of PIQ. To that end, we first generated the expression vectors carrying myc-tagged PIQ-L and PIQ-S and confirmed their expression by transient transfection followed by Western blotting using anti-myc tag antibodies (data not shown). Then, we did calmodulin-agarose binding assay to explore whether myc-tagged PIQ-L and/or PIQ-S would interact with agarose-conjugated calmodulin. H1299 cells were transiently transfected with expression vectors carrying PIQ-L or PIQ-S or the expression vector without PIQ inserts and the lysates of transfected cells were incubated with agarose-conjugate calmodulin to explore the PIQ and calmodulin interactions. As shown in Fig. 4B, both the PIQ-L and PIQ-S isoforms coprecipitated with agarose-conjugated calmodulin only in the lysates of cells transfected with PIQ expression vector (Fig. 4B, lanes 4 and 5) but not in the vector-only transfected cells (Fig. 4B, lanes 3 and 5). These results confirmed that, indeed, both isoforms of PIQ interacted with calmodulin. As an additional control, we did the same assay using agarose beads without calmodulin and, as shown in Fig. 4B, neither PIQ-L nor PIQ-S coprecipitated with agarose beads alone (Fig. 4B, lane 2). These results thus confirmed that both isoforms of PIQ were capable of interacting with calmodulin. Furthermore, calmodulin interactions with both PIQ-L and PIQ-S took place in the presence (1 mmol/L CaCl2) and absence (1 mmol/L EGTA) of Ca2+, indicating that PIQ seemed to interact with calmodulin in both Ca2+-dependent and Ca2+-independent manner (Fig. 4B).

PIQ down-regulates PUMA expression. We have shown that PIQ is down-regulated in response to DNA damage in a p53-dependent manner. Furthermore, p53 down-regulates endogenous PIQ as well as PIQ promoter function. It is, therefore, possible that PIQ may serve to modulate p53 function particularly in response to DNA damage. In this context, it is of note that among the p53-regulated proapoptotic genes, PUMA has emerged as a key mediator of p53-dependent DNA damage-induced apoptosis (9–11). We and others have recently reported that PUMA is also regulated by Ca2+-mobilizing agents (12–14). We have also reported that PUMA gene promoter harbors several NFAT and CREB-binding sites (14). NFAT and CREB are transcription factors that modulate the expression of target genes in response to signals that involve Ca2+ and calmodulin. It is, therefore, reasonable to suspect that PUMA may also participate in Ca2+-regulated signaling pathways. It is interesting that both PIQ isoforms interact with calmodulin in the presence or absence of Ca2+, suggesting that PIQ may also modulate calmodulin-dependent signaling events. These findings prompted
us to investigate whether PIQ modulates PUMA expression particularly via calmodulin-dependent NFAT/CREB signaling pathway. To this end, we used PUMA promoter luciferase constructs (Fig. 5A) to assess the effect of PIQ on PUMA promoter activity. First, p53-null H1299 cells were transiently cotransfected with a reporter construct carrying 2,104-bp-long PUMA promoter fused to a promoterless luciferase gene (pGL-Full; Fig. 5A) and increasing amounts of PIQ expression vector. Our results, shown in Fig. 5B, indicated that PIQ clearly repressed PUMA promoter activity.

Next, we investigated whether PIQ negatively regulated PUMA promoter function by interfering with calmodulin-dependent NFAT/CREB signaling pathway. For this purpose, we used two different PUMA promoter deletion constructs named pGL-1010 and pGL-697 that we have recently generated (14). The reporter construct pGL-1010 carries PUMA promoter in which four NFAT and one CREB response elements are deleted (Fig. 5A), whereas the pGL-697 construct is devoid of all NFAT and CREB sites (Fig. 5A). We reasoned that if PIQ negatively regulates PUMA promoter activity via NFAT/CREB signaling pathway(s), then the removal of these response elements should affect PIQ modulation of PUMA promoter activity. As is shown in Fig. 5C, PIQ down-modulated PUMA promoter-luciferase activity from pGL-Full construct and removal of the distal 1,094 nucleotides, deleting four NFAT and one CREB response elements (pGL-1010), further potentiated PIQ repression of PUMA promoter function. The extent of PIQ repression of PUMA promoter activity did not change following the deletion of last CREB and NFAT sites (pGL-697). These results thus suggest that PIQ seems to down-regulate PUMA promoter function via a novel mechanism that may not involve CREB/NFAT signaling pathway. Because endogenous PUMA is constitutively expressed at low levels (Fig. 2B and C) and the fact that PIQ negatively regulates PUMA expression, it was not possible to evaluate the effect of exogenous PIQ on endogenous PUMA levels.

**PIQ is overexpressed in primary colon cancers.** p53 is mutated, deleted, or otherwise deregulated in a wide variety of tumors. Because PIQ is down-regulated by p53 and exogenous PIQ represses the PUMA promoter function, it is, therefore, relevant to investigate whether PIQ has a role in tumorigenesis. As a first step to investigate this issue, we analyzed the expression of PIQ in cancer profiling arrays of tumor and matching normal tissue samples representing 13 different tissue types. Our results indicated that PIQ expression was up-regulated in tumors of the gastrointestinal tract. Overall, PIQ up-regulation was noted in ~54% (21 of 39) of colon, 39% (17 of 37) of rectal, and 46% (11 of 28) of gastric tumors (Fig. 6). The clinicopathologic features of the tumors exhibiting PIQ up-regulation are presented as Supplementary Tables S1, S2, and S3.

**Discussion**

PIQ is a novel gene that codes for an IQ motif-containing protein. There are two PIQ isoforms, PIQ-L and PIQ-S, each with a molecular mass of 69.0 and 53.8 kDa, respectively. The PIQ-L transcripts are highly expressed whereas the PIQ-S transcripts exist in low abundance. Although the molecular mechanisms responsible for the differential expression of these isoforms remain to be elucidated, both isoforms harbor IQ motifs and, as shown here, both also interact with calmodulin. Thus, PIQ-L and PIQ-S,
expression of antiapoptotic genes, such as IAP2, Survivin, IAP1, and Bcl2, to enhance its apoptotic effects (36–38). p53 is believed to down-regulate its target genes via several potential mechanisms. For example, p53 has been shown to inhibit gene transcription by (a) direct sequence-specific DNA-binding to repressor elements, (b) directly interfering with the function of other transcription factors, and (c) sequestering certain cotranscription factors. However, the exact mechanisms by which p53 down-regulates the expression of its target genes remain to be fully elucidated. Our results indicate that PIQ is down-regulated in response to DNA damage in a p53-dependent manner and that p53 itself also down-regulates PIQ expression. Although our findings also indicate that p53 transcriptionally down-regulates PIQ gene expression, whether p53 mediates this effect directly or indirectly is an issue that remains to be elucidated. In this context, it is of note that the PIQ gene promoter does not harbor the repressive head-to-tail p53-binding element (39) or the p53 transcriptional repressor element (40) but contains five CCAAT sites and three putative Sp1-binding GGGGCGGGG sites (Fig. 3A). Recent evidence suggests that p53 binds to CCAAT-binding transcription factor NF-Y and thereby represses the activity of CCAAT-containing promoters (41, 42).

p53 has also been reported to suppress Sp1-mediated transcription of cyclin B1 gene via its interactions with Sp1 (43). It is, therefore, possible that p53 may suppress PIQ transcription via similar mechanisms and future studies are likely to provide a greater insight into this issue.

Our results also indicate that PIQ represses PUMA expression, which is a bona fide downstream target of p53. Based on our findings, it is reasonable to suggest that in response to genotoxic stress, p33 may enhance PUMA expression by (a) direct transcriptional regulation as well as (b) down-regulation of PIQ. Thus, PIQ may serve to function as a prosurvival molecule and its down-regulation may be one of the mechanisms that modulate p53-dependent genotoxic stress–induced apoptosis. We have also found that PIQ expression is up-regulated in human gastrointestinal malignancies, including colorectal and gastric cancers, and, thus, it may play a role in tumorigenesis. Previous studies have shown that p53 is frequently inactivated in gastrointestinal tumors (44). Although an experimental correlation between p53 status and PIQ expression remains to be established, it is, nevertheless, relevant to suggest that gastrointestinal tumors may exhibit PIQ overexpression partly due to the lack of functional p53; future studies will provide valuable insight into this issue.

We have recently reported that PUMA promoter contains CREB and NFAT-binding sites (14). NFAT and CREB are transcription factors that modulate the expression of target genes in response to signals that involve Ca²⁺ and calmodulin. Accordingly, we have also reported that PUMA transcription is regulated by agents that affect Ca²⁺-dependent signaling events (14). Our present results indicate that PIQ, a calmodulin-binding protein, down-regulates PUMA expression, deletion of CREB and NFAT-binding sites from the PUMA promoter does not relie from PIQ-mediated repression. These results, therefore, suggest that PIQ regulation of PUMA expression may not involve CREB/NFAT-dependent signaling events. We have recently reported that the PUMA promoter devoid of CREB/NFAT-binding sites (pGL-697) is still responsive to agents that affects intracellular Ca²⁺ concentrations (14). These findings, coupled with our present results, would suggest that PIQ seems to regulate PUMA expression via a novel mechanism that may still involve its interaction with calmodulin. Further studies are, therefore, needed to elucidate these possibilities.

PIQ-L contains two IQ motifs whereas PIQ-S contains one; accordingly, both interact with calmodulin in the presence or absence of Ca²⁺. IQ motif–containing proteins are known to interact with calmodulin in a Ca²⁺-dependent or Ca²⁺-independent manner and different IQ motif proteins mediate different functions in response to intracellular Ca²⁺ signals. A change in calmodulin conformation induced by Ca²⁺ binding is known to regulate the activity of IQ motif proteins, such as myosin and L-type Ca²⁺

via their interactions with calmodulin, are likely to be involved in calmodulin-regulated cellular processes.

We report here that PIQ expression is down-regulated by DNA damage in a p53-dependent manner. It is now well established that DNA damage–induced apoptosis involves p53-dependent up-regulation of certain proapoptotic genes, including PUMA, NOXA, BAX, and DR5 (6–8, 33–35). p53 transcriptionally up-regulates the expression of these genes via direct sequence-specific DNA binding to its canonical response elements. p53 also down-regulates the expression of antiapoptotic genes, such as Bcl2, Survivin, IAP1, and IAP2, to enhance its apoptotic effects (36–38). p53 is believed to down-regulate its target genes via several potential mechanisms. For example, p53 has been shown to inhibit gene transcription by (a) direct sequence-specific DNA-binding to repressor elements, (b) directly interfering with the function of other transcription factors, and (c) sequestering certain cotranscription factors. However, the exact mechanisms by which p53 down-regulates the expression of its target genes remain to be fully elucidated. Our results indicate that PIQ is down-regulated in response to DNA damage in a p53-dependent manner and that p53 itself also down-regulates PIQ expression. Although our findings also indicate that p53 transcriptionally down-regulates PIQ gene expression, whether p53 mediates this effect directly or indirectly is an issue that remains to be elucidated. In this context, it is of note that the PIQ gene promoter does not harbor the repressive head-to-tail p53-binding element (39) or the p53 transcriptional repressor element (40) but contains five CCAAT sites and three putative Sp1-binding GGGGCGGGG sites (Fig. 3A). Recent evidence suggests that p53 binds to CCAAT-binding transcription factor NF-Y and thereby represses the activity of CCAAT-containing promoters (41, 42).

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via their interactions with calmodulin, are likely to be involved in calmodulin-regulated cellular processes.
channels, that modulate the ATPase activity of myosin or opening of the L-type channels. PEP-19 and RC3 are IQ motif proteins that interact with both Ca\(^{2+}\)-bound and Ca\(^{2+}\)-free calmodulin and, thus, modulate the availability of calmodulin by affecting its Ca\(^{2+}\) association and dissociation. Although it remains to be elucidated whether PIQ modulates calmodulin activity or whether calmodulin affects PIQ function, our results nevertheless indicate that PIQ down-modulates the expression of p53 target gene PUMA whereas PIQ expression is down-regulated by p53. We have also found that PUMA expression is regulated by Ca\(^{2+}\)-mobilizing agents via mechanisms that seem to involve NFAT/CREB–dependent and NFAT/CREB–independent signaling events (14). In total, our results suggest that PIQ is a novel player in the Ca\(^{2+}\) calmodulin–mediated signaling pathways and that it may also serve to bridge p53 and Ca\(^{2+}\)/calmodulin–dependent signaling events.

**Note added in proof:** While this article was in preparation, Otto et al. (45) reported the identification of an IQ domain protein that they have named NPHP5, which interacts with calmodulin and retinitis pigmentosa GTPase regulator and is frequently mutated in Senior-Loken syndrome patients. We have found that the sequence of PIQ and NPHP5 is identical.

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**References**


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Cloning and Characterization of a p53 and DNA Damage Down-regulated Gene PIQ that Codes for a Novel Calmodulin-Binding IQ Motif Protein and Is Up-regulated in Gastrointestinal Cancers

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