EXPERIMENTAL STUDY

Molecular cloning of a functional promoter of the human plakoglobin gene

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Abstract

Objective: Plakoglobin (Pg) is the only cytoplasmic protein component common to both junctional complexes mediating cell–cell adhesion, adherens junctions and desmosomes. In these complexes Pg appears to act as a linker protein anchoring transmembrane proteins of the cadherin superfamily to the actin cytoskeleton and intermediate filament system respectively. Intercellular adhesion is frequently disturbed in skin diseases and in carcinomas, enabling tumour progression and metastasis. Whereas Pg expression is lost in some thyroid tumours and carcinoma cell lines, little information on Pg gene regulation is currently available owing to a lack of promoter studies.

Design and methods: We have cloned and sequenced genomic DNA from a human library that resulted in 979 bp upstream of the published Pg cDNA. The transcriptional start was mapped by rapid amplification of cDNA ends. Methylation-specific PCR of bisulfite-modified cell line DNA was applied to probe the methylation status of a promoter-associated CpG island. Reporter-gene constructs of various promoter fragments were transiently transfected in thyroid carcinoma cell lines and their activities were determined by luciferase measurements.

Results and conclusions: A 1 kb DNA fragment harbouring a functional promoter of the human Pg gene was cloned and characterized. The sequence lacks a canonical TATA box, but contains putative CCAAT boxes as well as various putative binding sites for transcription factors, among them SP1 and AP2, proximal to the transcriptional start. Considerable promoter activity was found in thyroid cell lines and deletion analysis indicated that a 300 bp region proximal to the 5'-untranslated region of the mRNA represents the minimal promoter of the human Pg gene. As cells lacking endogenous Pg expression were found to contain methylated CpG dinucleotides in a CpG island located around the transcriptional start site, it is suggested that epigenetic mechanisms such as DNA methylation contribute to dysregulated Pg expression.

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Introduction

Plakoglobin (Pg), also termed γ -catenin, forms with its closest relatives β -catenin and *Drosophila* segment polarity protein, armadillo, a subgroup of the large armadillo protein family that share a 42-amino-acid motif repeated several times (1). As a major cytoplasmic protein, Pg occurs in a soluble and a membrane-associated form, where it was first identified in both adhesive intercellular junctions, desmosomes and adherens junctions (2, 3). In adherens junctions, Pg and β -catenin form mutually exclusive complexes with the cytoplasmic tail of E-cadherin and provide a link to α -catenin that, in turn, establishes a connection to actin, which is a prerequisite for stable cadherin-mediated cell adhesion to occur (4). Pg, unlike the other catenins, is in addition a major component of the desmosomal plaque

(2) where it binds to the desmosomal cadherins, desmogleins and desmocollins, to anchor them through desmoplakins to the intermediate filament (5). The crucial roles of Pg for protein sorting, desmosomal plaque assembly and intermediate filament anchorage have clearly been demonstrated with Pg null-mutant mouse embryos that die early during development as a result of severe heart defects (6, 7). Recently, a homozygous mutation of the Pg gene leading to a truncation of the protein has been identified in patients suffering from Naxos disease, an autosomal recessive form of arrhythmogenic right ventricular cardiomyopathy (8). Even though Pg is an important common component of both cell–cell adhesion complexes, which are essential for tissue integrity and are frequently negatively affected in invasive and metastatic carcinomas (4), the gene structure has only very recently been

published (9), and factors regulating its promoter are still unknown. Previously, we and others observed a decreased expression of Pg in thyroid carcinomas and cell lines, which could partially be restored by inhibition of DNA methylation (10–14). To better understand regulation of the gene encoding Pg, we cloned the 5'-flanking region of the human Pg gene, characterized its activity by transient transfection analysis in thyroid carcinoma cell lines and investigated the DNA methylation status of an associated CpG island.

Materials and methods

Cloning and sequence analysis

To clone the promoter region of the human Pg gene, five human DNA libraries of the Genome Walker kit (Clontech, Heidelberg, Germany) were used. Each library consists of uncloned, restriction enzyme-digested, adaptor-ligated genomic DNA fragments. PCR was performed with each of the libraries with the adaptor-specific primer AP1 and the Pg-specific reverse primer 1 (5'-GGTGGGAGCCGGCGCGGACAGC-3'; start at position 69 in Pg cDNA, accession no. M23410). Following dilution, the products were used as templates for a second PCR with nested adaptor primer AP2 and Pg-specific reverse primer 2 (5'-GAACTGAGCTCGGCGCGGTCCGG-3'; start at position 47 in Pg cDNA). Amplification conditions were as described in the manufacturers protocol and Tth polymerase mixes as well as other reagent components were also obtained from Clontech. An amplified DNA fragment of 0.7 kbp obtained from the ScaI library was cloned in the T/A cloning vector pCRII (Invitrogen, Leek, The Netherlands) and designated pPG1.

DNA sequencing was performed on both strands using the Thermo sequenase radiolabeled terminator cycle sequencing kit (Amersham Life Sciences, Freiburg, Germany). To further extend the obtained sequence a second PCR walk was performed as before but using primer pairs AP1/Pg3 (5'-ACAGACGCCTT-CACCGGCCAGCCAAC-3') and AP2/Pg4 (5'-TCAGCGT-CAGGCATGATGCTTCTTTGGGCT-3') in the first and second PCR respectively, whereby the Pg-specific primers were derived from the pPG1-upstream sequence. As before, the product was cloned to give pPG2, subsequently sequenced, and the complete sequence assembled.

Nucleotide homology searches were performed using the Fasta3 program (15) (<http://www.ebi.ac.uk/fasta3>) and searches for potential transcription factor sites were carried out with the programs Mathinspector 2.2 (16) and FunsiteP (17) (both at <http://transfac.gbf.de/transfac/programs>) using the TRANSFAC database (18). The programs Grail 1.3 (<http://compbio.ornl.gov/grail-1.3>) and CpGplot/report (<http://www.ebi.ac.uk/emboss/cpgplot>) were used for CpG island prediction.

5'-Rapid amplification of cDNA ends

To characterize the transcriptional start an established 5'-rapid amplification of cDNA ends (5'-RACE) procedure (19) was applied using a commercial kit (Gibco Life Technologies, Eggenstein, Germany) according to the manufacturers instructions. Briefly, 3 µg total RNA isolated from 8505C cells was reverse transcribed by Superscript RT II (Gibco Life Technologies) in separate reactions using the Pg-specific antisense primers 361R (5'-CTGGCTGTGTGGA-CATCTG-3') or 203R (5'-CGAGTGGATACCCGAGTC-GTA-3'). The cDNAs were treated with RNase H, purified and dC-tailed in a terminal transferase reaction. Primary PCR was performed with abridged anchor primer and the Pg-specific nested primers 203R or 174R (5-TCTGCTGCCACTCAGTCACCT-3'). Following secondary PCR using universal abridged anchor primer and Pg-specific nested primers 174R or 141R (5-CCATCAGGTTTCATCACCTCCAT-3') the respective PCR products were subcloned in pCR2.1 (Invitrogen) and the corresponding nucleotide sequences of a total of 15 clones were determined by dideoxy sequencing using primer 141R and universal M13 primers.

Reporter-gene constructs

For *in vitro* transient expression assays a number of fragments were subcloned into the SmaI site of the promoter- and enhancerless luciferase (LUC) expression vector pGL3Basic (Promega, Mannheim, Germany).

In brief, the cloned fragment was cut from pPG1 with EcoRI, the ends filled in with Klenow DNA polymerase and the resulting fragment cloned in both orientations in the SmaI site in front of the LUC gene of pGL3Basic yielding constructs pGLPg1 and pGLPg2 (reverse orientation). Using unique SmaI sites in the fragment and the adaptor, pGLPg1 and pGLPg2 were digested with SmaI, the inserts removed and the vectors religated to give the deletion constructs pGLPg3 and pGLPg4 (reverse orientation). The dissected SmaI fragment of this step (~400 bp) distal to the coding region was subcloned in SmaI-digested pGL3Basic, yielding the constructs pGLPg5 and pGLPg6 (reverse orientation). The EcoRI fragment isolated from pPG1 was further cut with BspMI. Products of 0.24 kbp and 0.44 bp were isolated, their ends filled and cloned in SmaI-digested pGL3Basic, and named pGLPg7 and pGLPg9 respectively. The corresponding constructs with DNA fragments in reverse orientation were termed pGLPg8 and pGLPg10 respectively. Two DNA fragments ending proximal to the 5'-end of the published Pg cDNA were produced by PCR using pPG1 as template and a common reverse primer 5'-AAGGCAG-CAACTCAGTAACG-3' in combination with forward primers 5'-GTTAGGATGAGACCTCAAG-3' and 5'-ACGGGACAGTCAGGCGAGATA-3'. Both products were cloned in pCR2.1 (Invitrogen, Karlsruhe, Germany)

using a T/A cloning kit and as before their respective EcoRI fragments were filled in and subcloned into the SmaI site of pGL3Basic to give transfection constructs pGLPg11 and pGLPg12 respectively. The orientations of cloned fragments were checked by restriction enzyme digestions and all constructs were sequenced.

A complete fragment of approximately 1 kbp consisting of PG1 and PG2 was produced by PCR using human genomic DNA and cloned in pCR2.1 (Invitrogen). Thereof, an EcoRV/SmaI-fragment was isolated harbouring PG2 and a part of PG1 and subcloned in the SmaI site of pGLPg5 to yield pGLPg0. Three pGLPg0 clones were sequenced and used for transfections.

Cell culture, transient transfections and promoter activity measurements

Human thyroid carcinoma cell lines FTC-133 (20) (a generous gift from P. E. Goretzki, Düsseldorf, FRG), C643 (21) (generously supplied by N. E. Heldin, Uppsala, Sweden) and TPC-1 (22) (from S. Jhiang, Columbus, OH, USA) were cultured in DMEM/HamF12 (1:1) supplemented with 10% foetal bovine serum, penicillin (100 IU/ml) and streptomycin (100 µg/ml) at 37 °C in a humidified atmosphere with 5% CO₂. The human thyroid carcinoma cell line 8505C (23) (DSMZ, Braunschweig, Germany) was grown in RPMI 1640 with supplementations as above. Cells (2 × 10⁵) were plated in six-well plates 1 day before transfection. Transfections mediated by Eugene 6 (Roche, Mannheim, Germany) were carried out according to the manufacturers protocol using 1.5 µg of each promoter construct. To correct for transfection efficiency differences 1.5 µg β-galactosidase expression plasmid pSVβGal was cotransfected. The vector pGL3Ctrl (Promega, Mannheim, Germany) served as a positive control in parallel transfections conferring strong LUC expression driven by the SV40 promoter. Enzyme activities were determined 48 h post-transfection using a LUC assay kit and a β-galactosidase assay kit (both from Promega). All LUC activities were normalized to β-galactosidase activity and were given relative to the basal activity of empty pGL3Basic, which was set to unity. Measurements were performed in duplicate and repeated in 3–8 independent experiments. The data shown represent mean values (± s.e.m.).

Methylation analysis

To detect DNA hypermethylation of the Pg gene the methylation-specific PCR protocol was used (24). Briefly, genomic DNA of cell lines was isolated using a commercial kit (Qiagen, Hilden, FRG) and 1 µg was treated with sodium bisulfite as described (25). The modified DNA was subjected to PCR in separate reactions using the primer pairs PG-M (5'-TAGTCGTCGTTTCGATCGCGTC-3' and 5'-AACCGAATCGAAATCGAACCG-3') and PG-U

(5'-TTGGAGTAGTTGTTGTTTGTATTGTGTT-3' and 5'-CAAACCAAATCAAATCAAACCAA-3') designed to distinguish methylated from unmethylated DNA. The PCR mixtures contained 1 × PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 200 µM dNTPs, 1.5 mM MgCl₂, 10 pmol each primer per reaction, 0.5 U PlatinumTaq DNA polymerase (Gibco Life Technologies) and 50 ng template in a final volume of 25 µl. Amplification was carried out after an initial enzyme activation step (5 min at 95 °C) for 40 cycles (30 s at 95 °C, 45 sec annealing, 30 s at 72 °C), followed by a final extension (5 min at 72 °C). Annealing temperatures of 62 °C and 60 °C were used for PG-M and PG-U reactions respectively. The PCR products were resolved on a 6% polyacrylamide gel, stained by ethidium bromide and visualized under ultraviolet illumination. Expected PCR product sizes are 76 bp (position +30 to +105; cf. Fig. 1) in the case of presence of methylated CpGs in the Pg gene region covered by the PG-M primers or 84 bp (position +24 to +107; cf. Fig. 1) in the case of presence of unmethylated CpG dinucleotides using the PG-U primers.

Protein extraction and Western blotting

Cell lines were treated in parallel with and without 5-Aza-2'-deoxycytidine (AzaC; Sigma Chemicals, Taufkirchen, Germany) at a final concentration of 1 µM in growth medium for 4–5 days. For total protein extract preparations cells were briefly washed with phosphate-buffered saline (PBS; 150 mM NaCl, 10 mM sodium phosphate pH 7.4) and lysed by addition of hot Laemmli sample buffer (26). The lysates were briefly sonicated, heated (5 min, 95 °C) and centrifuged (10 min 14 000 × g) to remove insoluble material. The resulting supernatants were used for Western blotting. Protein contents were estimated according to Lowry (27) following trichloroacetic acid precipitation. Equal amounts of protein (10 µg/lane) were separated by 7.5% SDS-PAGE (26) and transferred electrophoretically to polyvinylidene difluoride membranes (28). After blocking with 5% non-fat dry milk in 0.1% Tween/PBS the membranes were incubated overnight at 4 °C with mouse monoclonal antibodies against β-catenin or Pg (Transduction Lab, Lexington, KY, USA). Following incubation with secondary anti-mouse IgG conjugated to either horseradish peroxidase or alkaline phosphatase bands were detected by chemoluminescence using an ECL kit (Amersham) or CSPD plus Emerald-Enhancer (Oncogene Science, San Diego, CA, USA) respectively, according to the manufacturers' protocols.

Results

A PCR-based approach was used to clone the 5'-flanking region of the human Pg gene. The assembled sequence consists of 1026 nucleotides (Fig. 1) and includes at the 3'-end a stretch of 46 nucleotides,

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                                PEA3
-965 CTGGTTTGGT GGTCTTCAA ATGAGAGGAA GGAGGTTCTA GCTTGGGGTG TGCCACTTTG
-905 TAGTTTGTG ACTTCTCTGA ACTTCGGTTT CTTCATCTTT ACAATGAGGG TCGTTTTCTC
-845 ATCCTAACTA TTTTTCAGAG CTGTCACAAT AATTAAAAAA AAACATACCG TTAATTTTAA
-785 TTTCTAACCT TTAGCAGTTT CTGAGTGCTT TCACAAACCT GACCTCATTT AATCCCCACA
-725 TACTCCAGTG AGGTAGATAT GGCTAATGTA TCCAGTTTAC AGAAGAGGAG AAACAGGGTG

                                NKX2.5
-665 AAGTAACTTG CCTAAAATCA TACCAAGTGG GAGAAGACCA AACCTGGCCT TCTAATTCTA

                                AP2
-605 AATCCCAGGA GTACTCCATC TTTTCGGGAAA AAAATCCCAC CCCAGCACAA AGCCTATGAA
-545 ATGCTATACA AATATGTGAT TACTACTTTG GGGCAAGGAT GAATAAGCCC AAAGAAGCAT
-485 CATGCCTGAC GCTGAGTTGG CTGGCCGGTG AAGGCGTCTG TCCCTTCCCT TGGTATCCCT

                                MyoD
-425 ATGACTTACC TGTTGGACAG GTAGGGGGAA GGGGAGAGTA ATGAGTCTCA CCTGCTCAGA

                                CCAAT
-365 GCAAGGGTGG GGCAAGACAC ACCCCATCCC TTCCATTGGT TTTTTCCTTA GTCTTACTGA

                                CCAAT
-305 CAGAGCCTTG TCCAATCAGG AGGAAGTAAC TTTCTATCTG CCAATAGATG CAATGTTAGG

                                AP2 CCAAT
-245 ATGAGACCTC AAGTTAGAGT CCATCCCTAG AGCCGACTGG CAGTCCCCGG GGCCAATGGC

                                SP1 SP1
-185 AAGCGGATAA ACAGAGGCGG CCGTGGAAGA GGACTGGAGG CGAGGCTCCG CCCCTCCACG

                                AP2 SP1
-125 GGACAGTCAG GCGAGATAGC CAGTGAGCTC GCACCAGAGG GTGGGCGTCT CCCCAGGGG
-65 CGGAGCTTCG AGGTGGCGAG GGGCGTGGCT TGGCTGTCAG GTCTCTTCGC CTTTGTTCG

* *
-5 GTTACTGAGT TGCTGCCTTG GCCAGAGTCC GGAGCAGCCG CCGCCCGACC GCGCCGAGCT
+56 CAGTTC

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Figure 1 Nucleotide sequence of the 5'-flanking region of the human plakoglobin gene. Asterisks mark both major transcriptional positions mapped by 5'-RACE and numbering refers to the most distal transcriptional start point (+1). The sequence in italics represents the overlap with the published human plakoglobin cDNA (3), which starts at -119 relative to the translational start codon. Putative CCAAT boxes are shown underlined and putative regulatory elements are indicated in bold letters above the underlined sequence.

which are identical to the start region of the corresponding human Pg cDNA (positions 2–47; accession no. M23410), published previously (3). This overlap confirms that the amplified DNA region represents the 5'-flanking region of the Pg gene. Thus we extended the 5'-flanking region of this gene by 979 bp. The sequence of the 5'-flanking region of the human Pg gene (Fig. 1) has been deposited with the EMBL Nucleotide Sequence Databases (accession no. AJ276892). Whereas the overall GC-content of the sequence is 50%, the GC-content of 300 bp located at the 3'-end is considerably higher (66%) and this short segment notably harbours 24 out of a total 31 CpG dinucleotides. According to established criteria (29) computer analysis by the CpGplot program predicts one CpG island of at least 330 bp that starts at position -219 (Fig. 1) and extends into exon 1 of the Pg gene. The promoter sequence lacks a canonical TATA box, but contains four putative CCAAT boxes, of which the most downstream CCAAT box located 200 bp proximal to the start of the corresponding Pg cDNA shows the least differences in the flanking nucleotides to published

CCAAT consensus sequences (30, 31). Computer searches for putative regulatory regions resulted in a number of probable transcription factor binding sites, most of them localize to the second half of the sequence. Putative regulatory factors include stimulator protein-1 (SP1), activator protein-2 (AP2), PEA3, MyoD and Nkx2.5 (Fig. 1).

The transcriptional site was mapped by the 5'-RACE procedure (19) using RNA isolated from 8505C, a human anaplastic thyroid carcinoma cell line (23), and different combinations of Pg mRNA-specific primers. Four of 15 sequenced clones extended the Pg cDNA sequence by 14 nucleotides (designated position +1 in Fig. 1), four clones started at position +2, and six started in pos. +15, the published 5'-end of Pg cDNA (3), with the remaining clones being scattered between these regions or were shorter probably due to degradation. On the basis of these results, we assigned two major transcriptional start points at positions +1/+2 and +15 (cf. Fig. 1).

To study the promoter activity the complete 1026 bp fragment as well as seven deletion fragments were

Figure 2 Promoter activity measurements. (A) Schematic of reporter-gene constructs used in transient transfection assays. Various fragments of the 5'-flanking region of the human Pg gene were fused in front of the LUC gene in the pGL3Basic vector. Numbering of nucleotides is given as in Fig. 1. (B) Functional analysis of Pg promoter-LUC constructs in 8505C cells. Cells were transiently transfected with 1.5 μ g pGL3Basic containing fragments of the 5'-flanking region of the human Pg gene in front of the LUC gene, or 1.5 μ g empty pGL3Basic, and 1.5 μ g pSV β Gal vector respectively. Expression vectors carrying the respective fragment in antisense orientation were available for pGLPg-constructs 1, 3, 5, 7 and 9. All LUC activities were measured 48 h post-transfection, and were normalized to the β -galactosidase activity. The LUC activity of empty pGL3Basic was set to unity and the activity of each promoter construct was expressed as a fold increase over that value. (C) Cell lines FTC-133, 8505C and TPC-1 were transiently transfected with empty pGL3Basic vector or Pg-LUC constructs pGLPg0, 1, 5 or 11 in combination with pSV β Gal and activity measurements were performed as in Fig. 2B.

cloned into the promoterless LUC expression vector pGL3Basic. The reporter-gene vectors (cf. Fig. 2A) were used for transient transfections of 8505C cells, and the transcriptional activation was determined by LUC activity measurements. As shown in Fig. 2B, the complete sequence (pGLPg0) was able to efficiently drive LUC expression, about 96-fold compared with the empty pGL3Basic vector. Comparison of the data for the full-length construct (pGLPg0) and the 5'-deletion

constructs (pGLPg1, 5 and 9) indicates the presence of a silencing element in the first 360 nucleotides (positions -965 to -594). Constructs lacking this 5'-region, but cloned in reverse orientation displayed either no or reduced stimulation of LUC expression (Fig. 2B). These results thus confirmed that the DNA region -593 to +61 functions as a promoter element. The central third of the complete sequence (nucleotides -593 to about -220; pGLPg3, pGLPg7) did not

Figure 3 Protein expression and DNA-methylation status. (A) Cells were treated with (+) and without (–) 1 μ M AzaC for 4–5 days. Total protein extracts containing equal amounts of proteins of treated/untreated cell lines were analyzed by Western blotting using antibodies directed against β -catenin (β -cat) and Pg. Note that the applied protein amounts for 8505Cs used for Pg blotting had to be reduced to allow comparison. (B) Methylation-specific PCR. Genomic DNA of cell lines was extracted, modified by bisulfite treatment and subjected to PCR with primer pairs PG-U (U) and PG-M (M) specific for unmethylated and methylated alleles of a Pg promoter-associated CpG island respectively. PCR products were analysed by nondenaturing 6% PAGE. Expected product sizes were 76 bp and 84 bp for PG-M and PG-U primers respectively. The numbers on the left indicate the size of markers (bp).

stimulate LUC transcription efficiently when used without the last part indicating essential functions of the latter region. The high LUC activity (about 140-fold when compared with basal activity of pGL3Basic) measured with fragments harbouring the 3'-end regions (pGLPg9, pGLPg5) is consistent with this interpretation. Thus, most of the promoter activity can be attributed exclusively to the last third of the sequence (nucleotides about –220 to +61). Further 5'-deletions led to a progressive decrease of LUC activity (pGLPg11, pGLPg12). Notably, only in these two constructs have the 46 nucleotides corresponding to the 5'-UTR of the Pg mRNA also been deleted. Obviously this GC-rich region positively regulates transcription, as indicated by the threefold lower stimulation of LUC expression by construct pGLPg11 when compared with the otherwise similar construct, pGLPg5 (Fig. 2B). Further deletion of regions harbouring the most downstream CCAAT box and GC-rich regions, probably representing binding sites for SP1 and AP2, considerably decreased LUC expression (pGLPg12).

In order to better understand the variable down-regulation of Pg expression in different thyroid tumours

and cell lines reported previously (10–14), we used four constructs (pGLPg0, 1, 5 and 11) for transient transfections of three human thyroid carcinoma cell lines (FTC-133, TPC-1 and 8505C) which endogenously express very low, medium and high levels of Pg (Fig. 3A) respectively and were derived from follicular, papillary and anaplastic thyroid tumours respectively (20, 22, 23). The overall activity patterns of transfected constructs were comparable in the three cell lines (Fig. 2C). Again a lower LUC expression of the full-length construct (pGLPg0) compared with the 5'-deletion constructs lacking the first 360 nucleotides (pGLPg1 and 5) may be attributed to the presence of a silencing element in that region. As well, the strongly decreased LUC activity of construct pGLPg11 might again be indicative of a regulatory role of the 5'-UTR of Pg. The results with all constructs also indicate, that a loss of Pg expression in FTC-133 and TPC-1 is not caused by the lack of essential transcription factors, as transfectants displayed considerable LUC activity (TPC-1) compared with transfected 8505C, or even approximately twofold higher LUC activity in the case of FTC133, that has almost completely lost Pg expression.

To identify an underlying mechanism for loss of Pg, cell lines including C643, an anaplastic thyroid carcinoma cell (21), were treated with the well-known cytosine analog AzaC, which after being incorporated into DNA inhibits DNA (cytosine-5)-methyltransferase at the target site for DNA methylation, CpG dinucleotides, and thereby indirectly leads to demethylation of methylated CpG islands (32). A partial restoration of Pg expression was detectable in FTC133 and C643 by Western blotting (Fig. 3A) as well as by Northern blotting (data not shown). The AzaC-mediated increase of Pg gene expression appears to be specific as the amounts of β -catenin (Fig. 3A), α -catenin and p120^{cas} (data not shown) remain unaffected. Therefore, Pg down-regulation might result directly from aberrant DNA hypermethylation of its gene. To investigate this possibility, genomic DNA of cell lines was modified by bisulfite treatment, which converts unmethylated cytosine residues to uracil without affecting 5-Methylcytosine residues, and subsequently subjected to PCR with primer pairs PG-M and PG-U intended to discriminate the methylation status of CpG dinucleotides located to the CpG island extending into exon 1. As shown in Fig. 3B, 8505C and TPC-1, which both were AzaC-unresponsive and endogenously express Pg, contain only unmethylated alleles. By contrast, the Pg-negative and AzaC-responsive FTC133 and C643 clearly displayed CpG methylation in addition to unmethylated alleles.

Discussion

The present study reports the cloning and characterization of a 1 kb DNA region representing the human Pg

promoter region, which to our knowledge has not yet been published. In previous investigations the human Pg cDNA was cloned (3), and the human Pg gene was mapped to chromosome 17q12-q22 in proximity to the breast cancer susceptibility gene 1, BRCA1 (33). Very recently, when this work was finished, the complete genomic organization of the human Pg gene has been reported showing that the Pg gene probably consists of 13 exons covering a 17 kb-long DNA region (9). The start region of the sequence obtained (accession no. AF233882) is identical with our nucleotide sequence, but did not cover the first 152 nucleotides, as reported here. In addition, a working draft sequence generated from a chromosome 17-derived clone in the course of the human genome project became accessible in the EMBL HTG nucleotide database (accession no. AC012192) which at present (version 14) consists of 183 kb DNA in 15 unordered contigs; a part of contig 45 was found to be identical (99.8%) to the DNA sequence reported here and extends our sequence in both directions. In agreement with our data, both sequences show the same nucleotide difference at the start position of the published Pg cDNA sequence (pos. +15 in Fig. 1; G instead of a C).

These data clearly confirm that we achieved the cloning of a part of a functional human Pg promoter. In contrast to the gene encoding the related β -catenin (34) no obvious TATA box is present, but the cloned region includes in the 3'-half CCAAT boxes, GC-rich regions, the presence of putative transcription factor binding sites, and a high frequency of CpG dinucleotides located to a CpG island, which in combination are characteristic features commonly found in many promoters of housekeeping and tissue-specific genes (29–31).

Two closely neighboured major transcriptional start sites of the Pg gene were mapped by 5'-RACE their positions are compatible with the results of the transfection experiments (cf. Fig. 2) and the location of the most proximal CCAAT box (position –193, cf. Fig. 1), although the latter is more frequently found at ~position –70 in promoters lacking TATA boxes (30). Transfection of thyroid cell lines with Pg reporter-gene constructs results in a strong transcriptional activation that depends both on orientation and size of the respective fragments. Although a direct participation of trans-acting factors remains to be shown, the major CCAAT box-binding protein, NF-Y (30), as well as the general transcriptional activators, AP2 and SP1, binding to GC-rich regions following the proximal CCAAT box might contribute to the transcriptional activation observed.

Because of its comparable roles in adherens junctions and desmosomes Pg is commonly found in epithelia of various tissues but its expression is not restricted to them (5). Essential functions of Pg for heart development which can not be compensated for by its

homologue, β -catenin have been revealed in studies with knockout mice (6, 7). Interestingly, a sequence element (position –645; cf. Fig. 1) could represent a binding site for a heart-specific transcription factor, termed Nkx2.5 (also called Csx), a homeobox-containing gene expressed in cardiocytes and their progenitors and found to be essential for cardiac development (35, 36). As two additional Nkx2.5 binding sites are present in a region located further upstream, covered by the sequence AC012192 (see above), it is tempting to speculate that the Pg gene represents a downstream target for Nkx2.5 in the heart.

Loss of cadherin/catenin-dependent intercellular adhesion is found frequently in many carcinomas and represents one key step in tumour progression and metastasis (4). Pg as an essential cytoplasmic anchoring protein for classic cadherins (e.g. E-cadherin) in adherens junctions and desmosomal cadherins (desmogleins and desmocollins) in desmosomes participates in these processes. Reduced Pg expression correlates with malignant transformation in certain tumour cell lines (10, 12, 37, 38) and Pg overexpression can decrease the tumorigenicity and proliferation of transformed cells (39, 40). This might also relate to the participation of Pg and β -catenin in the wnt signal transduction pathway that is frequently dysregulated in certain carcinomas (41). Whereas the Pg-related β -catenin represents a potential oncogene, Pg appears to play a tumour suppressor role (33, 39, 40). Even though the contribution of dysregulated Pg expression to thyrocyte transformation and thyroid carcinoma progression is unknown at present, studies in differentiated and anaplastic thyroid carcinoma tissues (11, 14, 42) and in cell culture models (10, 12, 13) demonstrated frequent loss of Pg, in part along with de-differentiation. Mechanisms are mostly unknown so far and could include gene mutations/deletions, altered RNA or protein stability, hormonal dysregulation, loss/overexpression of transcriptional regulators and epigenetic silencing. This study addressed the latter two mechanisms. First, comparison of reporter-gene activity in different cell lines (cf. Fig. 2C) shows no obvious correlation to the endogenous Pg levels, making it unlikely that lack of essential transcription factors plays a role. The data indicate that a silencing element may reside in the first 360 nucleotides of the promoter. In addition, a putative binding site for MyoD (position –375) might be shared by the transcription factor Snail, that has recently been shown to down-regulate E-cadherin expression in tumour cells (43, 44) and may in another cellular context be involved in Pg gene regulation. Second, in accordance with previous investigations (10, 12) AzaC-mediated DNA demethylation leads to partial restoration of Pg expression in Pg-negative thyroid carcinoma cell lines. The cloning of the Pg promoter region enabled us to identify an associated CpG island, and to demonstrate by methylation-specific PCR that Pg gene methylation around the

start point of transcription is involved in Pg gene silencing in thyroid carcinoma cell lines. This is the first report concerning epigenetic regulation of the Pg gene. Whether these mechanisms are involved in the down-regulation of the Pg gene expression during tumour formation in the thyroid, as was shown for the E-cadherin gene (45), has to be investigated in future studies analysing thyroid tumour biopsies.

In summary, we have cloned the 5'-flanking region of the human Pg gene, characterized its promoter activity in thyroid cell lines and have identified promoter hypermethylation as a mechanism that participates to Pg expression. Further work is required to define the interaction of suggested factors contributing to the regulation of this important gene as well as a detailed analysis of the methylation pattern in carcinoma tissues, which will assist in tumour profiling.

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