The Proline-Rich Acidic Protein Is Epigenetically Regulated and Inhibits Growth of Cancer Cell Lines

Jinqiu Zhang, Hangyee Wong, Srimat Ramaman, Denis Cheong, Adrian Leong, and Shing Chuan Hooi

ABSTRACT

The proline-rich acidic protein (PRAP) gene was found previously to be expressed in the epithelial cells of the mouse and rat gastrointestinal tracts, and pregnant mouse uterus. This article describes the isolation, distribution, and functional characterization of the human homologue. PRAP was abundantly expressed in the epithelial cells of the human liver, kidney, gastrointestinal tract, and cervix. PRAP expression was significantly down-regulated in hepatocellular carcinoma and right colon adenocarcinoma compared with the respective adjacent normal tissues. Treatment of the cells with butyrate, trichostatin A, and 5′-aza-2′ deoxycytidine caused increases in PRAP gene expression up to 30-fold, suggesting that the gene is suppressed through epigenetic mechanisms involving histone deacetylation and methylation. To determine the significance of PRAP expression in cancer cells, we cloned PRAP and its two major splice variants from human colon and liver, and overexpressed it in HeLa, HT29, and HepG2 cells. PRAP caused cell growth inhibition in the cancer cell lines in transient transfection assays, colony formation assays, and in the growth rates of stable clones. The data suggest that PRAP and its variants may play an important role in maintaining normal growth homeostasis in epithelial cells. The epigenetic suppression of PRAP expression in cancer may cause growth dysregulation, a hallmark of the carcinogenic process.

INTRODUCTION

Epigenetic mechanisms are integral to the process of tumor development (1). Epigenetic modifications do not change the DNA sequence itself but alter the transcriptional activity of genes, changing the repertoire of genes expressed by the cell. The two major epigenetic mechanisms operating in the cell are DNA methylation at CpG islands in the promoter and histone acetylation. Methylation at CpG islands silences gene transcription in most instances (2), but, rarely, it results in activation (2, 3). Similarly, deacetylation of histones is thought to result in transcriptional silencing because of the condensation of chromatin (reviewed in Refs. 4, 5; Refs. 6–8). Recent evidence suggests that the two processes are related. Methyalted DNA appears to preferentially associate with histone deacetylase protein complexes and histone methylases (9–11). Histone methylation has been shown to result in DNA methylation (12, 13).

The importance of epigenetic mechanisms in cancer development is evidenced by the reversal of the cancer phenotype when the cells are treated with drugs that modify epigenetic mechanisms (14–16). These drugs induce growth arrest and differentiation by altering the expression of a limited number of genes (17). This suggests that regardless of the initial genetic abnormality causing the cancer, there are differentiation switches in the cell that are amenable to reactivation. This has important implications to our understanding of the fundamental process of cell homeostasis in epithelial cells. The epigenetic suppression of PRAP expression in cancer may cause growth dysregulation, a hallmark of the carcinogenic process.

MATERIALS AND METHODS

5′- and 3′-RACE to Identify cDNA Ends.

We had described previously the cloning and characterization of the rat prap gene (20). Using the rat sequence, we performed a BLAST search of the National Center for Biotechnology Information GenBank database and identified several human expressed sequence tag sequences that were >50% homologous to a region of the rat prap gene. Two pairs of primers (prapF1, R1 and prapF2, R2) were designed based on the ~180 bp of conserved sequence (shaded in Fig. 1) to amplify the 5′- and 3′- ends of the PRAP cDNA using the SMART RACE cDNA Amplification kit (Clontech, Palo Alto, CA). In brief, 1 μg of total RNA obtained from human normal colon mucosa was reverse transcribed using primers provided in the kit. The 5′- end of human PRAP was amplified by two rounds of nested PCR using gene-specific primer prapR1, prapR2, and UPM and NUP, respectively (Fig. 1). UPM and NUP were provided in the kit. Similarly, the 3′- end of PRAP was amplified by two rounds of nested PCR using the 3′-specific primer prapF1, prapF2, and UPM and NUP, respectively. The products of 5′ and 3′ RACE were purified and cloned into pTOPO vector (Invitrogen, Carlsbad, CA). The DNA inserts were sequenced using a Rhodamine Terminator Cycle sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, CA) and an ABI Prism 377 Autosequencer.

End-to-End Amplification of Full-Length PRAP cDNA.

To confirm the presence of full-length PRAP, 3 μg of total RNA prepared from normal colon mucosa was used for RT-PCR. Forward Primer F0 and reverse primer R0 (Fig. 1) were used for the PCR reaction after reverse-transcription with oligo-deoxy-thymidylic acid. The product was gel purified and sequenced three times. It was used as a probe for Northern blot analysis.

Received 5/29/03; revised 7/2/03; accepted 7/24/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by grants from Academic Research Fund (R-185-000-039-112) and National Medical Research Council (R-185-000-039-112) of Singapore.

2 To whom requests for reprints should be addressed, at Department of Physiology, Blk MD9, 2 Medical Drive, Singapore 117597. Phone: 65-6874-3222; Fax: 65-6778-8161; E-mail: phlshc@nuhs.edu.sg.

3 The abbreviations used are: PRAP, proline-rich acidic protein; RT-PCR, reverse transcription-PCR; RACE, rapid amplification of cDNA ends; UPM, universal primer mix; NUP, nested universal primer; DAC, 5′-aza-2′ deoxycytidine; TSA, trichostatin A; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione S-transferase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SP, without signal peptide.
**Tissue Samples.** Anonymized human tissue specimens were obtained from the archives of the tumor bank of National University Hospital of Singapore. Nontumor tissues were obtained adjacent to the margin of resection (≥5 cm away from tumor). Necrotic parts of the tumors were removed. Colon mucosa was dissected away from muscle and connective tissue. The samples were snap frozen in liquid nitrogen and stored at −80°C. The remaining tissues were sent for histopathological analysis.

**Cell Lines and Treatments.** The HT29, HCT116, HepG2, and HeLa cell lines were purchased from American Type Culture Collection (Manassas, VA). HT29 and HCT116 cells were cultured in McCoy’s 5A (Sigma Chemical Co., St. Louis, MO), HepG2 in MEM, and HeLa in RPMI 1640, and all were supplemented with 10% fetal bovine serum (Life Technologies, Inc., Gaithersburg, MD), at 37°C in a 5% CO2 humidified atmosphere. Cells were treated with 5 mM sodium butyrate (final concentration in medium; Life Technologies, Inc.) or vehicle. Total RNA was prepared from the cells at the respective time points from three separate flasks. In a separate experiment, HT29 and HCT116 cells were treated with vehicle, Dac (1 μM; Sigma), TSA (300 nM; Sigma), or a combination of both drugs for 72 h. For the combined treatment, cells were treated with 1 μM Dac for 24 h followed by the addition of 300 nM TSA for another 48 h. Total RNA was extracted using the RNase kit (Qiagen). The experiment was performed in duplicate and the average result calculated.

**Northern Blot Analysis.** A multisite blot comprising polyadenylic acid RNA derived from various human tissues was used (MTN blot; Clontech) to determine the expression of PRAP in human tissues. In a separate experiment, total RNA from tissues and cell lines was prepared using the guanidinium thiocyanate method as described previously (21). Fifteen μg of RNA was separated by formaldehyde/agarose gel electrophoresis, transferred to nylon membrane (Qiagen, Hilden, Germany), cross-linked (Stratalinker; Stratagene, La Jolla, CA), and hybridized to cDNA probes, as described previously (22). The probes were obtained by labeling PCR-amplified PRAP cDNAs with 32P using the random priming method. Relative mRNA levels were quantified by Typhoon phosphoimager (Amersham Bioscience, Piscataway, NJ) and normalized against 18S levels. Normal/tumor ratios were obtained by dividing the normalized relative densitometric units of the normal mucosa by tumor.

**Real-Time Quantitative RT-PCR.** Primers for real-time PCR were designed using the LightCycler Probe Design Software, version 1.0 (Roche, Meylan, France). F3 and R3 were used for the amplification of PRAP (Fig. 1). Real-time PCR was performed on the LightCycler (Roche) using the LightCycler-RNA amplification kit SYBR Green I (Roche). The specificity of the amplification was assessed by electrophoretic separation of the amplified products and melting curve analysis. Standard curves of PRAP and GAPDH were constructed using serial dilutions of total RNA from 0.1 ng to 500 ng. The efficiencies of both amplifications were calculated according to the slope of the standard curves. Relative PRAP expression was quantified after normalization with GAPDH.

**Generation of Polyclonal Antibody.** Polyclonal antibodies against recombinant GST-PRAP (amino acid 21–150) were raised in New Zealand White female rabbits and affinity-purified against GST-PRAP immobilized on nitrocellulose membrane. The specific antibodies were eluted using IgG elution buffer (Pierce Biotechnology, Rockford, IL) and neutralized with 0.1 volume of 1 M Tris base.

**Immunohistochemistry.** Anonymized, archived samples of human kidney, liver, colon, and uterine cervix were obtained from patients with surgical resection. Tissues were formalin-fixed, paraffin-embedded, sectioned, and mounted on slides. Deparaffinized slides were treated with 3% hydrogen peroxide in PBS and pretreated at 95°C for 30 min in 10 μl/liter citrate buffer (pH 6.0). A rabbit polyclonal antibody specific to PRAP (1:1000 dilution) was the primary antibody. Staining was carried out by an avidin-biotinylated horseradish peroxidase complex method (DAKO, Glostrup, Denmark) using a goat-antirabbit secondary antibody. The PRAP antibody (1 μg/ml) preincubated with 10 μg/ml of GST-PRAP was used as a control.

**Western Blot Analysis.** Cells or tissues were extracted with radioimmunoprecipitation assay buffer (1% PBS, 1% NP40, 0.5% sodium deoxycholate, and 0.1% SDS; for protease inhibitors, phenylmethylsulfonyl fluoride, aprotonin, pepstatin A, and leupeptin were freshly added to the radioimmunoprecipitation assay buffer at the point of usage). Five μg of total protein was separated on 12% SDS polyacrylamide gel and blotted onto the Hybond-C extra nitrocellulose membrane (Amersham Life Science). The primary antibody used was a rabbit polyclonal antibody specific for PRAP (1:5,000) or mouse monoclonal antibody specific for GAPDH (Chemicon International, Temecula, CA). The secondary antibody was either horseradish peroxidase-conjugated antirabbit IgG (Bio-Rad, Hercules, CA; 1:10,000) or horseradish peroxidase-conjugated antimouse antibody (Santa Cruz Biotechnology, Santa Cruz Biotechnology, CA; 1:20,000). The proteins were visualized using the SuperSignal West Dura

**Fig. 1.** Nucleotide and predicted amino acid sequence of PRAP. The cDNA sequence and predicted open reading frame (single letter codes) are shown. Gene-specific primers used in the 5′- and 3′-RACE are indicated by arrows: forward primers propF1 and propF2, and reverse primers propR1 and propR2. The respective translation initiation and termination sites are indicated in bold. The primers used for amplification of the probe for Northern hybridizations (F0 and R0) and real-time RT-PCR (F3 and R3) are indicated by arrows. The conserved GenBank expressed sequence tag sequence is shaded. Potential casein kinase II phosphorylation sites are boxed.
Extended (Pierce) chemiluminescent substrate and exposed to an autoradiograph film.

ELISA. For the detection of PRAP in the culture supernatant, pcDNA3.1-PRAP and pcDNA3.1 empty vector were transfected into HepG2 cells in a T25 flask. After 24 h, the medium was replaced by serum-free medium containing 1% Insulin-Transferrin-Selenium (Life Technologies, Inc.) in DMEM. The cultures were incubated for another 48 h, and the supernatant was collected and passed through a 0.22-μm filter. The cleared supernatant was then concentrated 5-fold in a spin vacuum and used for coating the ELISA plates. Different volumes of supernatant from the two transfections were used for coating the plate. PRAP polyclonal antibody at a dilution of 1:1000 was used as the primary antibody.

Isolation and Identification of PRAP Variants in Normal Tissues. Three μg of RNA was extracted from normal colon mucosa and liver. RT-PCR was performed using oligodeoxythymidylic acid and specific PRAP primers. The primers used were F0 and R0 (Fig. 1). The amplified products were ligated into T-vector plasmid (Promega, Madison, WI). Ten positive clones from each sample were randomly selected and sequenced.

Cloning and Overexpression of PRAP Variants in Different Cell Lines. PRAP and its two major variants (PRAPV1 and PRAPV2) were cloned into the BamHI and EcoRI sites of the pcDNA 3.1 vector (Invitrogen, Carlsbad, CA). The PRAP-SP plasmid was generated by amplifying the PRAP coding sequence beginning from amino acid 21 to 151. The cDNA fragment was cloned into pcDNA 3.1. All of the plasmids were sequenced to confirm identity and exclude mutations. The plasmids were transfected into HepG2 and HeLa cells using Lipofectamine (Invitrogen), according to the manufacturer’s recommendations. Each experiment was performed in quadruplicate and repeated three times. After 72 h, the cells were harvested and counted by a hemocytometer after trypan blue staining. Whole cell extracts were prepared for Western blot analysis as described.

Colony Formation Assay and Generation of Stable Clones. HeLa cells were transfected with either 1 μg of pcDNA3.1/PRAP or pcDNA 3.1 using Lipofectamine plus reagent (Invitrogen). Cells were subjected to selection in 1200 μg/mL G418 and the number of colonies scored manually at the end of 3 weeks. Stable clones of HT29 cells expressing PRAP were selected in a similar way. We managed to expand 2 clones of the 22 selected. The expression of PRAP was confirmed by Western blot analysis.

Cell Proliferation Study by MTT Method. Stable clones of HT29 cells expressing PRAP were seeded in 24-well plates in McCoys 5A medium supplemented with 10% fetal bovine serum. The colorimetric MTT (Sigma) assay was used as a surrogate measure of cell number at various time points. The experiment was performed in replicates of six and repeated twice.

RESULTS

Cloning and Sequence Analysis of Human PRAP. We identified and sequenced 633 bp of the human PRAP cDNA (Fig. 1). The sequence has been deposited in GenBank (accession no. AF421885). The cDNA encoded a putative protein of 151 amino acids that was ~50% homologous to that of rat and mouse pregnant-specific uterine protein (GenBank accession no. U28486). There are two regions of the protein that showed a higher degree of conservation. The NH₂-terminus of the protein, predicted to be the signal peptide by the SignalP program (23), was ~70% conserved between human and rodent. The COOH-terminal 135–149 amino acid was 80% conserved between human and rodent. The predicted cleavage site of the protein was between amino acid 20 and 21. Two putative casein kinase II phosphorylation sites were identified by the PROSITE program (Fig. 1).

Tissue Distribution of Human PRAP. Northern Blot analysis showed hybridization to a specific band ~700 bp in length. PRAP was abundantly expressed in liver and kidney, and less in small intestine and colon. PRAP mRNA was not detected in the rest of the tissues (Fig. 2). In a separate study, we found high levels of PRAP in the mucosal layer of the stomach, caecum, and ascending colon. A summary of the expression in the stomach and colon is shown in Fig. 3. The difference between expression in the caecum and distal colon was ~3.5-fold. Levels in the stomach and proximal large intestine were comparable with that detected in liver and kidney (data not shown). The expression of PRAP was studied by immunohistochemistry (Fig. 4). Results showed that PRAP was expressed in the epithelial lining of the colon and cervix. PRAP was strongly expressed in the hepatocytes of the liver. Both the proximal and distal tubules were strongly stained for PRAP in the kidney. There was no expression of PRAP in the kidney glomerular cells.

PRAP Expression Is Down-Regulated in Two Types of Cancers. We studied the regulation of PRAP gene expression in colorectal and liver cancers. Our earlier results indicated that PRAP was highly expressed in the right colon (caecum and ascending) and liver. Seventeen paired samples of right colon carcinoma and adjacent normal mucosa were randomly selected from a tissue bank. Fig. 5 shows the expression of PRAP in the 17 pairs of samples. Although the basal level of PRAP expression showed substantial variation among the samples, PRAP expression was down-regulated in 14 of the 17 sets of samples. In several cases, PRAP expression in the cancer samples was barely detectable by Northern blot analysis. Overall, PRAP expression was decreased 3.5-fold (P < 0.01) in tumor tissues compared with matched normal mucosa. Real-time RT-PCR was used to quantify the expression of PRAP in liver carcinomas. The efficiencies of PRAP and GAPDH amplifications were comparable at 2.0 and 2.2, respectively. The expression of PRAP was determined for 9 sets of hepatocarcinomas and matched “normal” (nontumor-containing) liver tissue. Results are shown in Table 1. Overall, there was a 3.8-fold reduction of PRAP expression in hepatocarcinoma compared with matched “normal” livers (P < 0.01).
Regulation of PRAP Expression by Butyrate and Epigenetic Modifiers. We studied the regulation of PRAP gene expression in HT29 cells by butyrate. Treatment of HT29 cells with 5 mM butyrate has been shown to induce cell differentiation within 24 h after treatment (24–27). PRAP gene expression was significantly increased 20-fold within 24 h after butyrate treatment (Fig. 6). The increase in expression was sustained for up to 6 days after treatment, at which time the cells were beginning to show morphological differentiation. The expression of the PRAP gene at this time was 29-fold higher than in control nontreated cells (Fig. 6A). Using Western blot analysis, we showed that butyrate treatment resulted also in an increase in PRAP expression (Fig. 6B). Maximal PRAP expression was observed between 2 mM and 5 mM of butyrate treatment.

To determine whether PRAP gene expression was regulated by epigenetic mechanisms, HT29 and HCT116 cells were treated with 1 µM DAC, which blocks DNA methylation, or 300 nM TSA to inhibit histone deacetylase or both. PRAP expression was determined by a sensitive and specific quantitative RT-PCR, and the results are shown in Table 2. Treatment of cells with 300 ng of TSA resulted in an 8.4-fold increase in PRAP expression in HT29 cells. Similarly, treatment of DAC resulted in an 8.5-fold increase in PRAP expression in the cells. Combined treatment with both TSA and DAC did not result in an additional increase in PRAP expression. Interestingly, DAC treatment alone did not result in an increase in PRAP gene expression in HCT116 cells. However, HCT116 cells treated with TSA showed a 5.2-fold increase in PRAP gene expression. The combined treatment with both DAC and TSA resulted in a synergistic 19.3-fold increase in PRAP gene expression.

PRAP Is Secreted. PRAP, PRAP/-SP, and PRAP/-29 were transfected into HeLa cells. Western blot analysis showed that PRAP and PRAP/-SP transfected cells expressed a protein of similar size (Fig. 7A). This suggests that the signal peptide present in the full-length...
HUMAN PROLINE-RICH ACIDIC PROTEIN IN CANCER CELL GROWTH

Table 2. PRAP expression in HT29 and HCT116 treated with TSA and DAC
Real-time RT-PCR results of PRAP expression in HT29 and HCT 116 treated with TSA, DAC, or in combination for 72 h.

<table>
<thead>
<tr>
<th></th>
<th>TSA (300 ng)</th>
<th>DAC (1 µM)</th>
<th>TSA and DAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fold increase</td>
<td>8.4</td>
<td>8.5</td>
<td>8.3</td>
</tr>
<tr>
<td>HCT116 (untreated)</td>
<td>13.97</td>
<td>11.6</td>
<td>13.8</td>
</tr>
<tr>
<td>Fold increase</td>
<td>5.17</td>
<td>1.11</td>
<td>19.29</td>
</tr>
</tbody>
</table>

*PRAP*<sup>5</sup>-GAPDH<sup>5</sup> (*a*), crossing point of *PRAP* normalized by GAPDH (after normalization with efficiencies of *PRAP* and GAPDH, respectively).  
*Fold increase of *PRAP* expression relative to untreated HT29 or HCT116.

Fig. 7. Signal peptide of PRAP is cleaved in HeLa cells. A. HeLa cells were transfected with *PRAP*, encoding full-length PRAP (Lane 1); *PRAP*/SP, encoding PRAP without signal peptide (Lane 2); and *PRAP*/29, encoding PRAP with 29 amino acid truncated from the NH<sub>2</sub>-terminal (Lane 3). B. Western blot showing the specificity of polyclonal antibody against PRAP. Five µg of liver lysate was loaded in each lane. The blot was incubated with anti-PRAP antibody 0.2 µg/ml (Lane 1), anti-PRAP antibody preincubated with 2 µg/ml GST-PRAP (Lane 2), anti-PRAP antibody preincubated with 2 µg/ml GST (Lane 3), and 0.2 µg/ml IgG from preimmune serum (Lane 4). The membranes were stripped and reprobed with anti-GAPDH monoclonal antibodies.

PRAP was cleaved. *PRAP*/29-transfected cells expressed a protein that was evidently smaller than *PRAP* and *PRAP*/SP. Fig. 7B shows the specificity of the *PRAP* polyclonal antibody. Transfection of *PRAP* into HepG2 cells resulted in the secretion of *PRAP* into the supernatant (Table 3).

Identification of PRAP Variants in Colon and Liver. *PRAP* was amplified from normal colon and liver to identify *PRAP* isoforms present in these tissues. We randomly selected 10 clones each from colon and liver. The results are summarized in Table 4. About 45% of the 20 clones were identified as *PRAP*, 15% *PRAP*<sub>V1</sub>, 25% *PRAP*<sub>V2</sub>, and 15% others. *PRAP*<sub>V1</sub> had an extra 3 bp inserted at position 201 bp of the *PRAP* coding sequence. The 3 bp insertion was predicted to result in a change in amino acid at position 42 from lysine to arginine (42K to NR substitution/insertion). The 3 bp insertion resulted from a variation in the splicing between exon 3 and 4 (alternative exon EA7 to EA8, according to nomenclature in GenBank) of the *PRAP* genome (GenBank Locus ID 118471, National Center for Biotechnology Information Acession). *PRAP*<sub>V2</sub> is a splice variant that had 27 bp deleted from bp 309 to 335 of the coding region, also because of a variation in splicing between exon 3 and 4 (alternative EA7 to EA9). The deletion is predicted to generate a 9 amino acid deletion in *PRAP*. The other variants identified were single base differences that resulted in an amino acid change and likely represent polymorphisms among individuals. Fig. 8 shows a comparison of *PRAP* and the variants.

Table 3. Detection of PRAP in culture medium of PRAP-transfected HeLa cells by ELISA

<table>
<thead>
<tr>
<th>Volume of culture medium</th>
<th>20 µl</th>
<th>50 µl</th>
<th>100 µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector</td>
<td>0.0713</td>
<td>0.0633</td>
<td>0.0533</td>
</tr>
<tr>
<td>PRAP</td>
<td>0.5273</td>
<td>0.6513</td>
<td>1.2313</td>
</tr>
</tbody>
</table>

Table 4. Number of transcripts of PRAP and its variants in human normal colon mucosa and liver

<table>
<thead>
<tr>
<th></th>
<th>Colon</th>
<th>Liver</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>PRAP</em></td>
<td>4</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td><em>PRAP</em>&lt;sub&gt;V1&lt;/sub&gt;</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td><em>PRAP</em>&lt;sub&gt;V2&lt;/sub&gt;</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Others</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

DISCUSSION

We have cloned the human homologue of the *PRAP* gene. Like the mouse and rat genes, it encodes a protein that has 151 amino acids. There is a high degree of conservation in the NH<sub>2</sub>-terminal 20 amino acids among the three species. The NH<sub>2</sub>-terminal 20 amino acids are predicted to encode a signal peptide, suggesting that *PRAP* is a secreted protein. This was supported in two separate studies. In the first study, we transfected full-length *PRAP*, *PRAP* without signal peptide (*PRAP*/SP), and *PRAP* without the first 29 amino acids (*PRAP*/29) into HeLa cells. *PRAP*/SP encodes a protein similar to *PRAP*. *PRAP* V2 encodes full-length *PRAP* (*PRAP*), encoding full-length *PRAP* (*PRAP*<sub>V1</sub>, 15% *PRAP*<sub>V2</sub>, and 25% *PRAP*/SP). The membranes were stripped and reprobed with anti-GAPDH monoclonal antibodies.

Overexpression of PRAP and Variants Decreased Cell Numbers and Colony Formation. The overexpression of *PRAP* in HeLa cells decreased significantly cell numbers 48 and 72 h after transfection (Fig. 9A). The number of cells was reduced by 55% at 72 h compared with vector-transfected controls. Interestingly, transfection of truncated *PRAP*/SP did not result in a reduction in cell numbers after 72 h. We also determined the effect of overexpressing PRAP and PRAP variants on cell numbers in both HeLa and HepG2 after 72 h (Fig. 9, B and C, respectively). Overexpression of PRAP and PRAP variants resulted in significant reductions in cell numbers of between 51–67% and 23–40% in HeLa and HepG2 cells, respectively, compared with vector-transfected controls. There was no significant difference between PRAP and the variants in cell numbers. The difference in response between HeLa and HepG2 likely represents the difference in transfection efficiencies. The overexpression of PRAP and PRAP variants in HeLa cells was confirmed by Western blot analysis (Fig. 9D).

The effect of PRAP on the establishment and growth of colonies in HeLa cells was studied over a period of 3 weeks of selection in 1200 µg/ml of G418. The number of visible colonies on the plate was counted, and the results of four separate transfections are summarized in Fig. 10. Transfection of *PRAP* resulted in a significant 50% reduction in the number of colonies. We also noted a reduction in the size of the *PRAP*-transfected colonies compared with controls.

In a separate experiment, HT29 cells transfected with pCDNA3.1/PRAP or pCDNA3.1 vector were subjected to a similar selection. We selected 22 clones after 3 weeks and expanded them. Two clones were successfully expanded and retained overexpression of PRAP (Fig. 11B). We monitored the growth rate of these two clones in parallel with two stably transfected vector controls. Both of the PRAP stably transfected clones showed a significantly lower rate of cell growth (*P* < 0.01; Fig. 11A).

DISCUSSION

We have cloned the human homologue of the *PRAP* gene. Like the mouse and rat genes, it encodes a protein that has 151 amino acids. There is a high degree of conservation in the NH<sub>2</sub>-terminal 20 amino acids among the three species. The NH<sub>2</sub>-terminal 20 amino acids are predicted to encode a signal peptide, suggesting that *PRAP* is a secreted protein. This was supported in two separate studies. In the first study, we transfected full-length *PRAP*, *PRAP* without signal peptide (*PRAP*/SP), and *PRAP* without the first 29 amino acids (*PRAP*/29) into HeLa cells. *PRAP*/SP encodes a protein similar to *PRAP*. *PRAP*/29 encodes full-length *PRAP* (*PRAP*), encoding full-length *PRAP* (*PRAP*<sub>V1</sub>, 15% *PRAP*<sub>V2</sub>, and 25% *PRAP*<sub>/SP</sub>). The membranes were stripped and reprobed with anti-GAPDH monoclonal antibodies.

Overexpression of PRAP and Variants Decreased Cell Numbers and Colony Formation. The overexpression of *PRAP* in HeLa cells decreased significantly cell numbers 48 and 72 h after transfection (Fig. 9A). The number of cells was reduced by 55% at 72 h compared with vector-transfected controls. Interestingly, transfection of truncated *PRAP*/SP did not result in a reduction in cell numbers after 72 h. We also determined the effect of overexpressing PRAP and PRAP variants on cell numbers in both HeLa and HepG2 after 72 h (Fig. 9, B and C, respectively). Overexpression of PRAP and PRAP variants resulted in significant reductions in cell numbers of between 51–67% and 23–40% in HeLa and HepG2 cells, respectively, compared with vector-transfected controls. There was no significant difference between PRAP and the variants in cell numbers. The difference in response between HeLa and HepG2 likely represents the difference in transfection efficiencies. The overexpression of PRAP and PRAP variants in HeLa cells confirmed by Western blot analysis (Fig. 9D).

The effect of PRAP on the establishment and growth of colonies in HeLa cells was studied over a period of 3 weeks of selection in 1200 µg/ml of G418. The number of visible colonies on the plate was counted, and the results of four separate transfections are summarized in Fig. 10. Transfection of *PRAP* resulted in a significant 50% reduction in the number of colonies. We also noted a reduction in the size of the *PRAP*-transfected colonies compared with controls.

In a separate experiment, HT29 cells transfected with pCDNA3.1/PRAP or pCDNA3.1 vector were subjected to a similar selection. We selected 22 clones after 3 weeks and expanded them. Two clones were successfully expanded and retained overexpression of PRAP (Fig. 11B). We monitored the growth rate of these two clones in parallel with two stably transfected vector controls. Both of the PRAP stably transfected clones showed a significantly lower rate of cell growth (*P* < 0.01; Fig. 11A).
PRAP except without the first 20 amino acids, the putative signal peptide. PRAP/-29 encodes a protein similar to PRAP except that it starts from the second ATG and is 29 amino acids shorter than full-length PRAP. Using polyclonal antibodies specific for the PRAP protein, we observed that both transfections of PRAP and PRAP/-SP resulted in the expression of PRAP proteins that are similar in size. This suggests that full-length PRAP is processed in the cell to generate a protein that is 20 amino acids shorter. The results also indicated that full-length PRAP was translated from the first ATG start site. The second experiment was a more direct demonstration of the secretion of PRAP into the medium in HepG2 cells. HepG2 cells were transfected with either full-length PRAP or vector control. PRAP was

<table>
<thead>
<tr>
<th>PRAP</th>
<th>M R L L L V T S L V U V L L W E A G V P A P K V P I K M</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRAPV1</td>
<td>M R L L L V T S L V U V L L W E A G V P A P K V P I K M</td>
</tr>
<tr>
<td>PRAPV2</td>
<td>M R L L L V T S L V U V L L W E A G V P A P K V P I K M</td>
</tr>
<tr>
<td>PRAPV3</td>
<td>Q V K H W P S E Q D F E K - A W G A R V E V P E D E K D D Q L</td>
</tr>
<tr>
<td>PRAPV4</td>
<td>Q V K H W P S E Q D F E K - A W G A R V E V P E D E K D D Q L</td>
</tr>
</tbody>
</table>

Fig. 8. Amino acid sequence of PRAP and its variants. The protein sequences are represented in one-letter code. Hyphens (−) have been introduced to optimize alignment. Identical amino acids are shaded.

Fig. 9. Overexpression of PRAP and its variants in cell lines. A, HeLa cells transfected with Vector, PRAP, and PRAP/-SP at 48 and 72 h. Column, cell numbers; bar, ±SE. Four transfections were done for each plasmid. *, * P < 0.01, relative to vector control. B and C, HeLa and HepG2 cells transfected with Vector, PRAP/-SP, PRAP, PRAPV1, and PRAPV2, respectively for 72 h. D, Lanes 1–5, Western blot showing the transfections of Vector, PRAP/-SP, PRAP, PRAPV1, and PRAPV2 in HeLa cells. GAPDH was used as an internal control.
detected in the supernatant of PRAP-transfected but not vector-transfected controls.

Although there were similarities in the pattern of PRAP expression between human and rodent, there were differences as well. Unlike the human, PRAP was not expressed in the mouse and rat livers and kidneys. The significance of the species-specific expression of PRAP is unclear but indicates that it may have different functions in different species. We were not able to obtain sufficient lengths of human small intestine to study the expression of PRAP along the human small intestine, but there appears to be a proximal-distal gradient of PRAP expression in the human colon, similar to the pattern in rodent small intestine (20).

We studied the expression of PRAP in 17 proximal colon (caecum and ascending colon) cancers and compared the expression with matched normal mucosa. PRAP expression was significantly down-regulated in the tumors compared with matched normal mucosa. We noted that in some samples, PRAP expression was no longer detectable by Northern blot suggesting that the gene may be completely silenced in some cases. PRAP expression was also reduced in 8 of the 9 liver adenocarcinoma samples. To determine whether the down-regulation of PRAP in colon cancer was epigenetically regulated, HT29 colon cancer cells were treated with either butyrate or TSA.

PRAP is expressed in different cell types and species. We were not able to obtain sufficient lengths of human small intestine to study the expression of PRAP along the human small intestine, but there appears to be a proximal-distal gradient of PRAP expression in the human colon, similar to the pattern in rodent small intestine (20). We next studied the functional significance of the epigenetic down-regulation of the PRAP gene in cancers. HeLa cells were transfected with PRAP or a truncated version of PRAP that did not contain the signal peptide. Vector-transfected cells were used as controls. Overexpression of full-length PRAP in HeLa cells decreased cell numbers within 48 h after transfection. This was more evident after 72 h. The overexpression PRAP without the putative signal peptide did not cause a similar decrease in cell numbers, suggesting that PRAP needs to be secreted to exert its effect. The experiment also demonstrated that the effect of PRAP on cell numbers is not a nonspecific effect of overexpressing a protein in the cell. The levels of PRAP and PRAP/-SP were comparable in the transfected cell lines. We extended the study to PRAP variants in a subsequent experiment using a different cell line, HepG2. Consistent with the previous finding in HeLa cells, overexpression of full-length PRAP resulted in a significant reduction in cell numbers in HepG2 as well. This indicates that the effect of PRAP on cell numbers was not cell-line specific. Both variants were also able to reduce cell numbers when transfected into HeLa and HepG2 cells. There was no significant difference among PRAP and PRAP variants transfected cells. The functional significance of the variants is currently unknown. How PRAP acts to reduce

---

Fig. 10. Overexpression of PRAP on colony formation. Summary of colony formation assay in HeLa cells transfected with Vector or PRAP. Results shown are means of four separate wells; bars, ±SE. *, P < 0.01 relative to vector controls using unpaired t test.

---

Fig. 11. Proliferation of HT29 stable clones. A, proliferation of PRAP stable clones as measured by MTT colorimetric assay. ---, cell number of PRAP clones; ---, cell number of vector clones. Bars, ±SE of six wells. *, P < 0.01 relative to two vector controls using unpaired t test. B, representative Western blot showing expression of stable clones. Lanes 1 and 3, vector controls; Lanes 2 and 4, clones stably overexpressing PRAP. Bottom panel, GAPDH control.
cell numbers is also unclear. It is possible that PRAP causes cell differentiation and cycle arrest, thereby reducing cell proliferation. Alternatively, PRAP may increase cell death by apoptosis. The possibilities are not mutually exclusive. The effect of PRAP in transient transfection assays was reinforced by the stable expression of PRAP.

There was a significant reduction in colony formation with the PRAP-expressing vector compared with vector control, both of which confer G418 resistance to HeLa cells. Both the number and the size of colonies were affected. It was very difficult to expand the stably transfected PRAP-expressing cells. Most of them grew for a while and then lost the overexpression or stopped growing altogether. We managed to expand two colonies that maintained high levels of PRAP expression. Both showed robust overexpression of PRAP.

Growth rates of these cells were significantly lower compared with vector controls. There was no significant increase in apoptosis detected in the cells, suggesting that the lower growth rates may result from cellular differentiation rather than cell death.

In summary, we have characterized a novel gene that is downregulated in cancers by methylation and deacetylation. Overexpression of this gene reduced cell numbers in HepG2 and HeLa cells suggesting that it may play an important role in the activation of differentiation switches in the cell.

ACKNOWLEDGMENTS

We thank Bor Luen Tang for help with purification of the PRAP antibodies.

REFERENCES


Downloaded from cancerres.aacrjournals.org on January 27, 2018. © 2003 American Association for Cancer Research.
The Proline-Rich Acidic Protein Is Epigenetically Regulated and Inhibits Growth of Cancer Cell Lines

Jinqiu Zhang, Hangyee Wong, Sriram Ramanan, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/63/20/6658

Cited articles
This article cites 34 articles, 8 of which you can access for free at:
http://cancerres.aacrjournals.org/content/63/20/6658.full#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/63/20/6658.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/63/20/6658.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.