Identity between the *PCPH* proto-oncogene and the *CD39L4* (*ENTPD5*) ectonucleoside triphosphate diphosphohydrolase gene

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Abstract. *PCPH* was initially defined as a proto-oncogene on the basis of its frequent detection as an activated oncogene in tumorigenic Syrian hamster embryo fibroblast cell lines converted to the neoplastic state by a single treatment with the carcinogen 3-methylcholanthrene (MC). Further studies identified the translation product of the *PCPH* gene as a ribonucleotide-binding protein with special affinity for ribonucleoside diphosphates. Later, we showed that the PCPH protein was homologous to the product of the yeast *GDA1* gene and demonstrated that it had intrinsic guanosine diphosphatase activity, although it did not complement the disrupted phenotype when expressed in *gda1* null *Saccharomyces cerevisiae* strains. These results indicated that the primary function of PCPH was unlikely to be related to the ribonucleotide recycling function that its yeast counterpart performs in the Golgi during the process of protein glycosylation. However, taken together, our data strongly suggested that the normal cellular function of PCPH was related to ribonucleotide metabolism. We now report that PCPH is structurally and functionally identical to the mammalian ectonucleoside triphosphate diphosphohydrolase CD39L4 (ENTPD5), recently described as a member of the lymphoid activation antigen ('cluster of differentiation') CD39 protein family. These results may help to establish the normal cellular function of the PCPH proto-oncogene product and its role in neoplastic development during carcinogenesis.

Introduction

Exposure of primary 13-day old Syrian hamster embryo cells, sparsely seeded in culture, to a single dose of a variety of chemical carcinogens resulted in the rapid and frequent appearance of colonies of cells exhibiting a transformed morphology. In numerous cases, these transformed cells, which were clonal in origin, progressed farther along in the carcinogenic process and acquired a fully malignant phenotype, causing the formation of tumors when injected into newborn Syrian hamsters or into nude mice (1-4). Molecular analysis coupled with gene transfer experiments using DNA of tumorigenic cells initiated with MC showed that they contained novel transforming sequences (5,6). These sequences were later cloned, characterized and identified as a novel oncogene that was initially termed *cph* (7) and later renamed *PCPH* (8). Further experiments established that carcinogen exposure caused the oncogenic activation of the *PCPH* gene by inducing a single base-pair deletion within its coding sequences, which resulted in a shift of the normal open reading frame (ORF). This mutated ORF encoded a truncated oncoprotein (9) which encompassed the first 213 N-terminal amino acids of the normal PCPH protein and had an additional hydrophobic C-terminal tail of 33 amino acids, not present in the normal protein.

The cDNAs for the normal mouse and human *PCPH* proto-oncogenes were isolated and characterized in our laboratory, and we showed that these proto-oncogenes were located in syntenic regions of chromosomes 12 and 14, respectively (8,10). Gene expression analyses showed that *PCPH* was widely expressed in Syrian hamster, mouse, rat and human tissues, suggesting that it plays a central role in cellular physiology (8,10-12). Transfection assays with *PCPH* expression constructs demonstrated that PCPH interacts with mitogenic and stress signaling pathways and participates in the cellular response to stress (9,13). We identified the *PCPH* gene product as a ribonucleotide-binding protein with high affinity for ribonucleoside diphosphates (9). We also demonstrated the homology between PCPH and the yeast GDA1 gene product and showed that PCPH had intrinsic guanosine diphosphatase activity (13). Nevertheless, PCPH did not complement the disrupted phenotype when expressed in *gda1* null *Saccharomyces cerevisiae* strains, indicating that the primary function of PCPH was most likely unrelated to the ribonucleotide recycling function that its yeast counterpart performs in the Golgi during the process of protein glycosylation (14). Although these results suggested a close relationship to ribonucleotide metabolism, the normal function of PCPH remains to be elucidated. To gain some insight into the possible function of PCPH we have taken

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Abbreviations: MC, 3-methylcholanthrene; ORF, open reading frame; TLC, thin-layer chromatography; UT, untranslated

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advantage of the availability of the draft human genome sequence and performed several searches with the hamster, mouse and human *PCPH* sequences. Sequence analyses pointed to a structural near-identity of *PCPH* with *CD39L4* (*ENTPD5*) which was later functionally confirmed by demonstrating that *in vitro* synthesized hamster, mouse and human PCPH proteins indeed had adenosine triphosphate diphosphohydrolase activity. *CD39L4* (later renamed *ENTPD5*) was first described (15) as a gene encoding a variant of the lymphoid cell activation antigen CD39 (16,17), and its product was further characterized as a secreted human apyrase, preferentially active on nucleoside diphosphates (18,19).

These results indicate that PCPH may participate in the catabolism of extracellular ATP and ADP and, consequently, in maintaining the size of the intracellular ribonucleotide pools. It is known that alterations in ribonucleotide pools are key determinants for the regulation of important cellular functions such as cell cycle arrest, commitment to apoptosis, proliferation or differentiation (20,21). Our data make it possible to hypothesize that, when expressed as a mutated product, the *PCPH* oncogene may create an imbalance in the cellular ATP pools and promote its oncogenic effects by interfering with one or more of those cellular processes.

Materials and methods

Nucleotide sequence determination and analysis. Determinations of nucleotide sequences were carried out at the Macromolecular Analysis Core Facility of the V.T. Lombardi Cancer Center using an ABI PRISM 377 DNA Sequencer system (Applied Biosynthesis, Foster City, CA). Most DNA and protein sequence analyses were performed using DNASTAR software (DNASTAR Inc., Madison, WI). Homology searches in DNA and protein databases were performed using BLAST (22). Analyses for the possible presence of introns and frameshift errors were performed, respectively, using either the Wise2 software from The Sanger Centre (http://www.sanger.ac.uk) or the FSED software (23). Multiple sequence alignments were carried out using the CLUSTAL W (24) or the Multalin (25) programs.

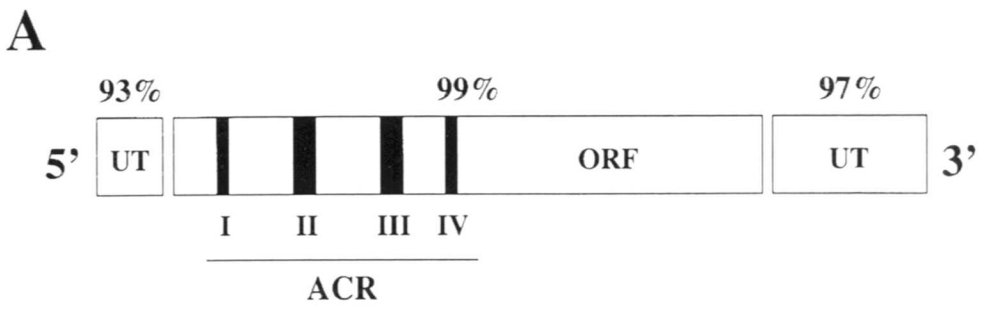
Generation of a fully spliced human PCPH cDNA. The original human *PCPH* cDNA clone had retained sequences from intron 4 (10). To make sure that this clone could be used to express a full length, functional PCPH protein we used an inverse PCR approach to eliminate the intron sequences and reconstitute the normal ORF. PCR primers (21-mers) were designed according to the 3'-most sequence of exon 3 and the 5'-most sequence of exon 4, in addition to the commercial T7 and SP6 primers, specific for sequences flanking the cDNA insert in the pcDNA-3 vector. Amplification was performed essentially as described (26), but using the thermostable *Tfl* DNA polymerase (Epicentre Technologies, Madison, WI) which does not add any extra nucleotides at the ends of amplified fragments (27). Sequences amplified from both sides of the intron were cleaved with either EcoRI (5' cDNA fragment) or XhoI (3' fragment), gel-purified and re-ligated into the same vector to reconstitute the intron-less, full-length human cDNA. The integrity of the reconstituted ORF was ascertained by nucleotide sequencing.

In vitro coupled transcription/translation. The TnT T7 Quick one-reaction system (Promega, Madison, WI) was used for simultaneous transcription and translation of the ORFs present in the original (10) and reconstituted *PCPH* cDNAs and for the *in vitro* synthesis of the full-length PCPH protein for determinations of its ribonucleotide phosphohydrolase activity. Plasmid DNAs were added to the rabbit reticulocyte lysate in the presence of L-[^{35}S] methionine. Reaction conditions were as recommended by the manufacturers. Conditions for gel purification of *in vitro* translated proteins were described previously (9).

Ribonucleotide phosphohydrolase activity assay. Assays were performed in a buffer containing 20 mM HEPES-Tris, pH 7.4, 120 mM NaCl, 5 mM KCl, 1 mM EGTA, 0.3 mM ATP or ADP (Sigma, St. Louis, MO) and 0.8 μM [α-^{32}P]-ATP (3000 Ci/mmol) or [2,8-^{3}H]-ADP (40 Ci/mmol) (Perkin-Elmer, Boston, MA), in a final volume of 100 μl. Reactions were started by addition of PCPH protein preparations and the assay mixtures were incubated at 37°C for 20 min. Control assays were carried out under identical conditions but with PCPH preparations that had been heated at 95°C for 5 min. Additionally, blank reactions without enzyme were included in every assay to control for spontaneous denaturation of the radioactive substrates. At the completion of the assays, one tenth of the reaction volume was loaded onto MN300 polyethyleneimine cellulose thin-layer chromatography (TLC) plates impregnated with fluorescence indicator (Macherey-Nagel, Gmbh & Co., Düren, Germany) and phosphonucleotides were separated by ascending chromatography in 0.75 M KH_2PO_4, pH 3.5. TLC plates containing samples from reactions in which ADP was the substrate were sprayed with EN^{3}HANCE (NEN-DuPont, Wilmington, DE) and exposed to X-OMAT AR film (Eastman Kodak, Rochester, NY). TLC plates containing [α-^{32}P]-labeled ribonucleotides were exposed directly to X-ray film. The chromatographic mobility of the radioactive reaction products was confirmed by that of cold ribonucleotide standards run in parallel in the TLC separations.

Results and Discussion

A BLAST search of the draft human genome sequence with the full-length nucleotide sequence of our human *PCPH* cDNA resulted in the identification, in addition to the sequences of our mouse (AF136571) and Syrian hamster (AF084568 and AF084569) *PCPH* sequences, of three other entries with alignment scores above 200 over the entire length of our query. Two of these entries (RefSeq NM_001249 and XM_007435) corresponded to mRNA of the human ectonucleoside triphosphate diphosphohydrolase 5 (*ENTPD5*), whereas the third one (GenBank AF039918) was identified as the full length cDNA sequence of the *CD39L4* gene (15). In fact, these three sequences correspond to the same gene. The symbol *ENTPD* was recently adopted to designate members of the CD39 diphosphohydrolase family (28), and *ENTPD5* was assigned to *CD39L4* (15,28). The overall level of



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CD39L4  ---------- ---------- -------GCG C--GCGCGTT TTCCTTGTT- CCTGGT--CA
PCPH    AGGCCCCAGG AAATTTCGGC AAGGAGG||| |CG||||||| |||||||||T |||T||TCA|

CD39L4  ACAAAGAAA- TGTGGAGTGT CTTGGCTGAA TCCT-CATAC AGACAAGATC ATTATGGTGC
PCPH    |||||||||A ||C|||||C| TC|||||||| ||||T||||| |||||||||| ||||||||||

CD39L4  TGTTAGGTAG GACTTGTATC CAGATGTAAG GTTGAAAAAG TGATATAATA AAGGAACCAA
PCPH    |||C|||||| -|||||||||| |||||||||| ||G||||||| |||||||||| ||||||||||

CD39L4  GGAGAAAATT CAGAAGGAAA GAAAAAATTG C---CTCTGC AGGTGTGCGA GCAGGATTGC
PCPH    |||||||||| |||||||||| |||||||||| CTGC|||||| |||||||||| |||||||||T

CD39L4  TTCTGCAACA AAAGCCTCCA CCCAGCCACA TCTTGGGAAA AGA
PCPH    |||||||||| |||||T|||| |||||||||| |||||||||| |||
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Figure 1. Homology between *PCPH* and *CD39L4* (*ENTPD5*). Panel A depicts the level of homology of their non-coding (UT) and coding (ORF) regions, and the position of the identical apyrase conserved regions (ACR I through IV) present in both genes. Panel B details the extensive homology at the level of the 5' UT, the least homologous regions between the two sequences.

identity between the *ENTPD5/CD39L4* entries and *PCPH* was 98%, strongly suggesting that *PCPH* was the same gene as *ENTPD5/CD39L4*. This possibility appeared even more likely taking into consideration that both *PCPH* and *ENTPD5* had been initially described as single copy genes in the human genome, located in the same region of chromosome 14 (10,15). However, there were still at least two other possibilities that could explain their near identity, although *PCPH* and *ENTPD5* might still be two different genes: i) that both genes represented two independent isolates of a duplicated portion within the chromosome 14q24.3 region, or ii) the existence of related pseudogene(s).

To address these possibilities, and reasoning that if both *PCPH* and *ENTPD5* were indeed independent clones of the same unique human gene their untranslated (UT) sequences should also be highly similar, if not identical, we performed separate homology analyses of the 5'-UT region, the ORF, and the 3'-UT region between our *PCPH* sequence and those of the three *ENTPD5* entries in the human genome database. Results from these analyses (Fig. 1) showed that the high level of overall identity was indeed a reflection of the high degree of homology of the various regions of the two genes (Fig. 1A). In particular, the ORFs were nearly one hundred percent identical, with only one mismatch identified in one of the three entries (XM_007435) from the draft human genome. Interestingly, the single base pair difference in XM_007435 is located four base pairs downstream from the mutation that caused the oncogenic activation of *PCPH* (9), and predicts that its translation product will be a truncated protein very similar to the PCPH oncoprotein. The similarities observed in the 5'-UT (93%) and the 3'-UT (97%) regions were also within the general homology range described for UT regions in mammalian genomes (29-31), and could be attributed to the fact that the various *PCPH* and *ENTPD5* cDNA clones were isolated from libraries of different origins, or to the use of more or less error-prone polymerases in the preparation of the cDNA libraries themselves. These results provided almost conclusive structural evidence in support of the identity between *PCPH* and *ENTPD5*. This conclusion was further strengthened by the confirmation of our previous mapping of PCPH as a unique gene in the human genome (10) with cytogenetic techniques (data not shown).

A close comparison between data published on the characterization of *CD39L4* (15,18,19) and our own results on *PCPH* (10) provided additional support to our finding: i) the protein products of both genes have four apyrase conserved regions (ACRs, Fig. 1A); ii) the pattern of mRNA expression of both genes is essentially the same, not only with regard to the tissue specificity but also with the size of their major transcripts in human cells; iii) both genes mapped to the same chromosomal region; iv) electrophoretic mobility

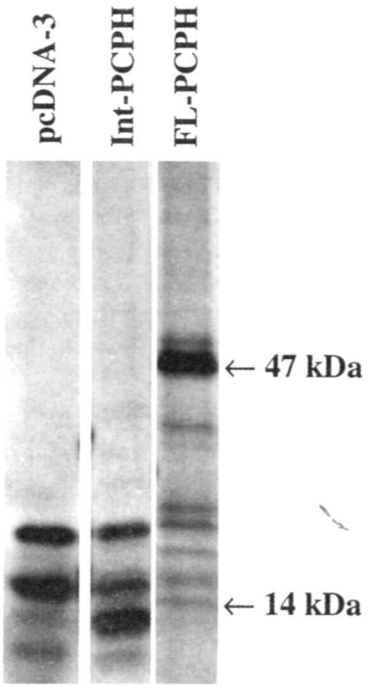

Figure 2. *In vitro* transcription/translation analysis. The ORFs from the intron-containing (Int-PCPH) and reconstituted, full-length (FL-PCPH) were inserted into the EcoRI and XhoI of the pcDNA-3 plasmid vector. Purified, circular DNAs from the two constructs or the vector alone were used for the ITT reactions in the presence of [L-^{35}S]-methionine. Auto-radiography of the translation products confirmed that the reconstituted human ORF encoded a product of the expected size (47 kDa), whereas the intron-containing ORF encoded a polypeptide of about 11 kDa. Other detectable polypeptides correspond to plasmid-derived ORFs or to internal translation-initiation events (35).

indicated a similar molecular mass for their protein products. Even more importantly, this close parallelism was also observed when we compared (data not shown) our results on the characterization of the mouse *PCPH* proto-oncogene (8) and published information on the murine *CD39L4* (15,32-34). These results were also consistent with our data on the characterization of the Syrian hamster PCPH product (7,9,11,13). The only apparent contradiction was related to the reported enzymatic activities of the protein products of *CD39L4* and *PCPH*. The CD39L4 protein was described as an apyrase with specificity for nucleoside diphosphates, being able to cleave ADP about 20-fold more efficiently than ATP (18). On the contrary, experiments performed in our laboratory indicated that purified bacterial recombinant Syrian hamster PCPH protein bound ADP or ATP with very low affinity (9) and that total extracts of various cell types transfected with recombinant constructs for the expression of Syrian hamster *PCPH* showed increased levels of guanosine diphosphatase activity but rather negligible levels of activity against ADP or ATP (13). Assuming that these differences could be attributed to kinetic differences between the Syrian hamster and human enzymes, in order to resolve this inconsistency we performed experiments to establish whether *in vitro* synthesized human PCPH was able to cleave ADP and/or ATP, using radiolabeled substrates to maximize the sensitivity of our assays.

We reported that our human *PCPH* cDNA clone was obtained from a partially spliced germinal center B cell transcript that retained most of intron 4 (10). Translation of the cDNA sequences would generate a short polypeptide encompassing the first 73 amino acids of the normal PCPH protein followed by 18 more residues resulting from the

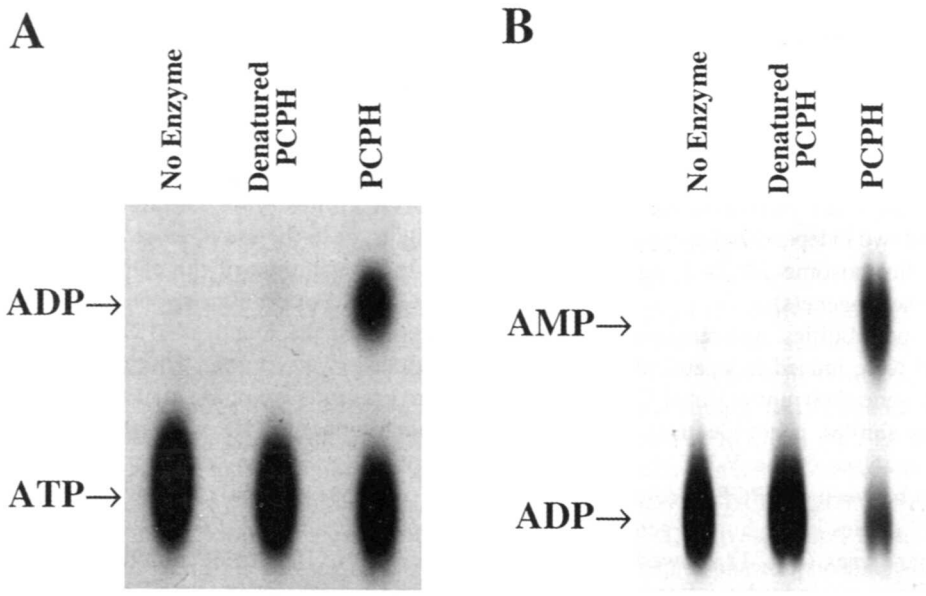

Figure 3. Nucleoside triphosphate diphosphohydrolase activity of human PCPH. *In vitro* translated, gel purified, normal PCPH protein was used in activity assays using ATP (panel A) or ADP (panel B) as substrates. Reactions contained either [α-^{32}P]-ATP or [2,8-^{3}H]-ADP in addition to the corresponding cold nucleoside. TLC was used to separate the reaction products, the mobility of which is indicated. Control assays included reactions without enzyme and reactions carried out with preparations of heat-denatured PCPH protein.

translation of the in-frame intron-derived sequences up to the first UAG termination codon, 57 bp downstream from the 5' splice junction. So, the intron sequences had to be precisely removed in order to synthesize the correct PCPH protein. We generated an intronless human *PCPH* cDNA as described above, confirmed its correct structure by nucleotide sequencing and its coding capacity by performing *in vitro* coupled transcription-translation (ITT) assays. Results (Fig. 2) showed that the unspliced *PCPH* cDNA encoded a protein of about 11 kDa whereas the reconstructed ORF encoded a protein of about 47 kDa, the expected size of the normal PCPH protein.

We carried out preparative-scale ITT reactions, gel-purified the human PCPH protein as described (9), and used the electroeluted protein to determine its hydrolytic activity against ADP and ATP. TLC analysis of the reaction products showed (Fig. 3) that the PCPH protein is indeed a nucleoside triphosphate diphosphohydrolase, capable of cleaving both ADP and ATP, and that such activity is sensitive to heat denaturation. Furthermore, triplicate in-tube activity assays (data not shown) confirmed that the mouse PCPH protein also had activity against ADP and ATP, and that the human PCPH protein was about 11.5-fold more active on ADP than on ATP, under our experimental conditions. The truncated PCPH polypeptide synthesized by the ORF from the intron-containing cDNA clone did not have any detectable activity against ADP or ATP (data not shown) as it could be expected from the fact that it only retained the first ACR.

In summary, our results provide conclusive structural and functional evidence to demonstrate the identity between the *PCPH* proto-oncogene and the *ENTPD5* ectonucleoside triphosphate diphosphohydrolase gene. Our finding provides very important information on the biochemical activity of the PCPH protein, that may be instrumental in establishing its normal cellular function and the mechanism of action of the activated PCPH oncoprotein during the process of carcinogenesis. Our finding that PCPH cleaves ATP and ADP provides grounds to hypothesize that the carcinogenic action of the *PCPH* oncogene may be mediated by its ability to imbalance the cellular ATP pools, which in turn would interfere with cellular processes such as cell cycle arrest, commitment to apoptosis, proliferation or differentiation.

Acknowledgments

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