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Astroprincin (FAM171A1, C10orf38): A regulator of human cell shape and invasive growth

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Abstract

Our group originally found and cloned cDNA for a 98 kDa type 1 transmembrane glycoprotein of unknown function. Due to its abundant expression in astrocytes it was called the protein astroprincin (APCN). Two thirds of the evolutionarily conserved protein is intracytoplasmic whereas the extracellular domain carries two N-glycosidic side chains. APCN is physiologically expressed in placental trophoblasts, skeletal and hearth muscle, and kidney and pancreas. Overexpression of APCN (cDNA) in various cell lines induced sprouting of slender projections whereas knockdown of APCN expression by siRNA caused disappearance of actin stress fibres. Immunohistochemical staining of human cancers for endogenous APCN showed elevated expression in invasive tumor cells compared to intratumoral cells. Human melanoma cells (SK-MEL-28) transfected with APCN cDNA acquired ability of invasive growth in semisolid medium (Matrigel) not seen with control cells. A conserved carboxyterminal stretch of 21 amino acids was found to be essential for APCN to induce cell sprouting and invasive growth. Yeast-2-hybrid screening revealed several interactive partners of which ornithine decarboxylase (ODC) antizyme-1, NEEP21 (NSG1), and ADAM10 were validated by co-immunoprecipitation. This is the first functional description of APCN. These data show that APCN regulates the dynamics of the actin cytoskeletal and thereby the cell shape and invasive growth potential of tumor cells.
Introduction

We originally obtained evidence for a previously uncharacterized protein, when a human brain expression library was screened with a polyclonal antibody made in sheep against human brain homogenate. The full-length cDNA was cloned and its mRNA was initially deposited on July 11th 2004 in the GenBank (ID: AY683003.1, https://www.ncbi.nlm.nih.gov/nuccore/AY683003.1). A peptide antibody to the protein was generated and a high expression was found in brain astrocytes by immunohistochemistry; hence the protein was named astroprincin (APCN).

With the clone mapping and sequencing of human chromosome 10 the APCN gene was identified and the coding sequence annotated as C10orf38 subsequently called FAM171A1 [1]. Based on the coding sequence APCN was found to be a protein of 98kDa size containing 890 amino acids (NCBI Accession: NP_001010924.1, https://www.ncbi.nlm.nih.gov/protein/NP_001010924.1).

There is scanty previous information on APCN in the literature. APCN/FAM171A1 has occurred in different settings of screening for gene expression or proteomics. Simmen et al investigated the impact of the transcription regulator Krüppel-like factor 9 (KLF9) in human HEC-1-A endometrial carcinoma cells [2]. Among the genes showing enhanced mRNA expression upon overexpression of KLF9 was C10orf38 annotated as “putative membrane-associated protein”. Liao et al searched for proteins carrying ZU5 motifs and found the presence of extracellular ZU5-like domains in the FAM171 proteins [3]. The ZU5 domains are versatile protein-protein interaction modules mediating, for example, bridging between ankyrin and β-spectrin [4].

Prunotto et al performed proteomic analysis of podocyte exome–enriched fractions from human urine and found FAM171A1 among the 1,195 identified proteins [5]. In a study by temporal proteomics during NGF-induced neural outgrowth in SH-SY5Y neuroblastoma cells FAM171A1 was among the 1,923 proteins showing transiently up-regulated expression [6].
St-Denis et al employed complementary affinity purification and proximity-based interaction proteomics to screen for interactomes of 140 human proteins with phosphatase catalytic domains\[^7\]. Among the 1,335 identified proteins, FAM171A1 was found to interact with the protein tyrosine phosphatase receptor Type F (PTPRF) or leukocyte antigen-related (LAR) tyrosine phosphatase\[^7\]. This transmembrane tyrosine phosphatase has been functionally linked to adherent junctions between epithelial cells and involved in regulation of β-catenin signaling. Huttlin et al investigated HEK293T cells by affinity purification-mass spectrometry proteomics and found evidence for interaction between FAM171A1 and pro-cadherin gamma subfamily B1, a calcium-dependent cell adhesion protein that has been implicated in the establishment and maintenance of brain neuronal connections\[^8, 9\].

In a genome-wide transcriptome analysis of human epidermal melanocytes Haltaufderhyde and Oancea found a 12-fold up-regulated expression of FAM171A1 in lightly pigmented melanocytes compared with darkly pigmented cells\[^10\]. Santuario-Facio et al analyzed genetic signatures of high-grade breast cancer and found FAM171A1 among the nine tumor-associated genes displaying overexpressed expression in triple-negative aggressive tumors\[^11\].

There is however no previous information about the molecular function(s) of APCN/FAM171A1. This study reports the first functional characterization of the APCN protein. It shows that APCN is an evolutionarily conserved 98 kDa transmembrane type I glycoprotein expressed in various normal and malignant cells. Data demonstrate that APCN is involved in regulation of the cytoskeletal dynamics and thereby the cell shape and invasive growth of tumor cells.

**Materials and methods**

**Bioinformatics**
Online-databases and tools (at ExPASy website, https://www.expasy.org/) were used to identify known motifs from the APCN-sequence. Online-alignment services BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), MAFFT (http://align.bmr.kyush-u.ac.jp/mafft/online/server/), and ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) were used to compare APCN sequences between different species.

To determine the tissue transcription profile of APCN, a radioactive probe was prepared for the region spanning nucleotides 86 to 417 of the ORF and hybridized to a Multiple Tissue Expression (MTE) Array and Multiple Tissue Northern (MTN) Blot (Clontech, Palo Alto, CA) as described previously.[12]

**In situ hybridization**

Tissue sections from early (sixth week of gestation) placenta were hybridized with antisense and sense cRNA probes corresponding to the region 808 to 1237 of APCN cDNA. The cRNA probes were labeled with digoxigenin-UTP by *in vitro* transcription with T7 polymerase using a DIG RNA Labelling Kit (Roche Diagnostics GmbH, Mannheim, Germany), and 200 ng of the probe was used for each hybridization. A Ventana Discovery Staining Platform was used for automated hybridization. Detection of the DIG label was performed with monoclonal biotinylated anti-digoxin antibody (Jackson Immuno Research Laboratories Inc., PA) at 1:2000 dilution using a BlueMap kit (Ventana Medical Systems Inc. Tucson, AZ). The slides were counterstained with Nucleofast Red (Ventana).

**Preparation of peptide antibodies to APCN in rabbits**

A synthetic peptide (SVTSHGRPEAPGTKELM) corresponding to amino acids 378 to 394 of APCN was synthesized on a four-branch lysine core as multiple antigen presentation peptide
(MAP$_4$) with Applied Biosystems 433A automated peptide synthesizer using Fmoc chemistry. The peptide was purified by reverse phase high performance liquid chromatography and verified by matrix-assisted laser adsorption time of light spectroscopy.

Two female rabbit were immunized with 400 µg of the peptide polymer in Freund’s complete adjuvant. After four weeks three booster injections with 200 µg peptide polymer in Freud’s incomplete adjuvant were given with three-week intervals and 10 days after the last immunization, blood was collected and the sera were isolated. The antibody was produced at the Viikki Laboratory Animal Centre, University of Helsinki, Finland. All animals were handled in strict accordance with good animal practice as defined by the relevant Finnish animal welfare bodies, and the European Communities Council directive (86/609/EEC).

**Immunostaining**

Formalin-fixed and paraffin-embedded anonymous tissue specimens were collected from the archives of the HUSLAB and the Department of Pathology, Haartman Institute, University of Helsinki, in accordance with the Finnish legislation and local ethical guidelines. Four micron thick sections were deparaffinized in xylene and rehydrated. Antigen was retrieved by microwaving in 10 mM citric acid monohydrate for 3 x 5 min at 650W. Endogenous peroxidase activity was blocked by treatment with 0.5% H$_2$O$_2$. The slides were incubated overnight in a refrigerator at + 4 °C with the primary antibody in PBS containing 0.5% normal human serum. The same procedure was used for negative controls, except that the incubation overnight took place in phosphate-buffered saline (PBS) diluent without antibody. The reaction was visualized with 3-amino-9-ethylcarbazole (Vectastain, Vector Laboratories, Burlingame, CA). Immunohistochemical staining of some sections was performed in an Autostainer 480 (Lab Vision Corp., Fremont, CA) by the Dako REAL EnVision Detection system, Peroxidase/DAB+, Rabbit/Mouse (Dako, Glostrup, Denmark).
Cells grown on coverslips were rinsed once with cold PBS and fixed in ice-cold methanol for 10 minutes at +4 °C. Alternatively, cells were fixed with 3.5 % PFA for 15 minutes at RT and permeabilized with 0.02 % IGEPAL CO-630 (Sigma Aldrich, St. Louis, MO) for 20 minutes at RT. Blocking was done with human serum diluted 1:10 in 1xPBS or with Background Blocker (Enzo Life Sciences, NY) for 30 minutes at RT. Cells were first incubated with primary antibody for 1 h, washed three times with 1xPBS and then incubated with species-appropriate secondary antibodies conjugated with either Alexa Fluor-488 or Alexa Fluor-555 (both at 1:1000 dilution, Thermo Fisher Scientific, MA) or with FITC goat anti-mouse (at 1:50 dilution, Dako) or TRITC swine anti-rabbit (at 1:30 dilution, Dako).

Wheat germ agglutinin (WGA)-TRITC (20 µg/mL, EY Laboratories Inc. San Mateo, CA) was used to visualize cell surface membrane. WGA-TRITC was added to cells grown on coverslips after initial rinsing, incubated for 30 minutes at +4 °C in the dark, and fixed.

**Cell cultures and transient transfections**

COS-7, HEK293, MCF-7, NIH3T3, and SK-MEL-28 cell lines were obtained from ATCC. The U373MG astrocytoma line was provided by Professor Bengt Westermark, University of Uppsala, Sweden and SK-MEL-103 (NRAS Q61R) and SK-MEL-147 (NRAS Q61R) (both originating from Dr. Alan Houghton, Memorial Sloan-Kettering Cancer Center, New York, NY; provided by Dr. Maria Soengas, Spanish National Cancer Research Center, Madrid, Spain). The cell lines, except for COS-7, were grown in RPMI-1640 medium supplemented with 10 % (v/v) Fetal Bovine Serum (FBS, Sigma-Aldrich), 1 mM L-glutamine, 50 mg/mL penicillin, and 50 mg/mL streptomycin at +37 °C in a humidified atmosphere of 5 % CO₂ in air. Cos-7 cells were grown under similar conditions in Dulbecco's modified Eagle's medium.
Cells were transfected using Lipofectamine™ 2000 transfection reagent (Invitrogen, Carlsbad, CA) or with FuGENE6 transfection reagent (Roche) according to the manufacturer’s directions. Lipofectamine was also used when cells were transfected with 6-Carboxyfluorescein–labelled siRNA against FAM171A1 (Sigma-Aldrich) and control siRNA (Ambion, Thermo Fisher Scientific). Lines of SK-MEL-28 cells stability overexpressing APCN or pcDNA3 empty vector were selected by cultivation in the presence of 1000 µg/mL G418.

RNA interference

SK-MEL-147 and U373MG cells were seeded on coverslips (18 mm) at 30,000 cells per well on a 12-well scale or 100,000 cells per 6-well scale 24 h before siRNA transfection. Cells were transfected with 20 nM APCN siRNA 6-FAM (sense: 5’-CUGAUGAGUGAGGUCCAUU[dT][dT][6FAM]-3’; antisense: 5’-AAUGGACUCCACUCAUCAG-3’) or 20 nM MISSION® siRNA Fluorescent Universal Negative Control #1, 6-FAM (Sigma-Aldrich). Cells were transfected using MISSION® siRNA Transfection Reagent (Sigma-Aldrich) according to manufacturer’s protocol.

Reverse transcription-PCR, RT-qPCR, and primers

To examine the transcription of the APCN gene in cells, total RNA was extracted using the TRI REAGENT® -RNA/DNA/Protein isolation reagent (Molecular Research Centre, Inc., Cincinnati, OH) according to the manufacturer’s instructions. Total RNA (1 µg) was reverse transcribed with the High Capacity RNA-to cDNA Kit (Applied Biosystems, Foster City, CA), cDNA was used as a template for quantitative real-time PCR analysis using a Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher) and a LightCycler II instrument (Roche). Primers used for real-time analysis included: APCN (forward) 5’-TTACCACGTATCACACGGTG-3’ and (reverse) 5’-
TTTGAACTCTCCAGTCTG-3'; GAPDH (forward) 5’-GGTGAGGTCGGGTCAAC-3’ and (reverse) 5’-CAAATGAGCCCGAGCTC-3’.

**Antibodies**

The following antibodies were used in this study (including dilutions/amounts used for immunofluorescence, immunohistochemistry, western blot, or immunoprecipitation): MAP346 (1:70 immunofluorescence; 1:400 immunohistochemistry, 1:200 western blot), FLAG (F3165; Sigma-Aldrich; 10µg/mL immunofluorescence, 3µg/mL western blot, 5 µg or 7.5 µg immunoprecipitation), Myc (562; MBL; 1:3000 western blot and M4439; Sigma-Aldrich; 1:7000 western blot, 7.5 µg immunoprecipitation), NSG1 (HPA035775, Sigma-Aldrich; 1:100 western blot and bs-840R; Bioss Antibodies, Woburn, MA), GFP (632375, Clontech, Mountain View, CA; 3 µg/mL immunofluorescence), ADAM10 (MAB1427; R&D Systems; 1:50 immunohistochemistry and sc-48400; Santa Cruz Biotechnology, CA; 1:20 immunofluorescence), AZ1 (HPA009291; Sigma-Aldrich; 1:2000 western blot).

**Imaging**

Microphotographs were taken with an Olympus BX51 microscope (Olympus Corporation, Shinjuku, Japan) equipped with Nikon Digital Sight DS-U1 camera system (Nikon Corporation, Shinjuku, Japan), a Zeiss Axioplan 2 Imaging fluorescence microscope (Carl Zeiss AG, Oberkoschen, Germany) and a Leica Confocal laser scanning microscope (Leica TCS SP2, Leica Microsystems GmbH, Wetzlar, Germany)

**Yeast two-hybrid screening**

Yeast two-hybrid screening was performed using Matchmaker™ GAL4 Two-hybrid System 3 (Clontech). The full-length APCN cDNA was cloned in-frame with the GAL4 DNA-binding domain of pGBKKT7 vector and then used as a bait construct. The bait construct was then
transformed into yeast strain AH109. The yeast strain Y187 was pre-transformed with a human placenta Matchmaker™ cDNA library (Clontech) in the pACT2 vector.

cDNA constructs

The full-length Astroprincin-cDNA (flAPCN, amino acids 1-890) from ZM6-a10 C10orf38 Image Clone (Geneservice, Cambridge, UK) was cloned into CMV14-vector (Sigma-Aldrich) containing 3xFLAG-tag with PCR oligonucleotides (all PCR oligonucleotides were purchased from Oligomer, Helsinki, Finland) employing HindIII and BamHI sites. The oligonucleotides are described in Table 1. Standard DNA techniques were applied as described [13]. Different N- and C-terminally truncated (cDNA) APCN constructs (Supplemental Figure S1) were amplified from a flAPCN-CMV14 construct using specific primers that introduced HindIII and BamHI sites, followed by cloning into CMV14.

Internal deletion variants of (cDNA) APCN were constructed with two-step overlap extension PCR (OE-PCR) [14]. Firstly, two PCR products representing flanking regions of the APCN sequence to be deleted were prepared by using one non-chimeric and one chimeric primer (Table 1). Secondly, two PCR products were used as template for a ligation PCR that with the outmost primer pair containing HindIII and BamHI sites.

Four point mutations (S136A, N159A, N190A, N194A) in the extracellular portion were made as described by Edelheit et al [15]. In brief, the point mutation is generated with a reverse-complementary primer-pair containing the desired mutation. Using flAPCN in a CMV14-plasmid as a template, two PCR reactions were performed with only one primer in each and the two PCR-products were mixed, denatured, and randomly re-annealed. The methylated template strands were digested with DpnI leaving only the pairs with fresh PCR-strand intact. The DpnI reaction was subsequently stopped by heat inactivation (20 minutes at + 80 °C). The double point mutant
N159A/N194A was similarly made by using N194A\textsubscript{APCN-FLAG} as template and N159A as mutation primers. fl\textit{APCN} was also cloned in the pEGFP-N1–vector (Clontech) to produce APCN-EGFP fusion protein.

For construction of Flag-NEEP21, the corresponding DNA fragments were amplified from Human Universal QUICK-Clone\textsuperscript{TM} cDNA (Clontech) using specific primers (Table 1) introducing \textit{HindIII} and \textit{BamHI} sites and cloned into CMV14 (Sigma-Aldrich). The Myc-NEEP21 expression construct was generated by amplification of \textit{Neepl1} from Flag-Neep21 expression vector using specific primers that introduce \textit{EcoRI} and \textit{SalI} sites (Table 1), followed by cloning into pAMC. pCI-Neo (Promega, Madison, WI) containing N-terminal c-Myc\textsuperscript{[16]} was a gift from Dr. Tomi Mäkelä (University of Helsinki). All constructs were verified by sequencing.

Ornithine decarboxylase antizyme-1 (AZ1) cDNA was kindly provided by Prof. Shin-ichi Hayashi, Jikei University School of Medicine, Tokyo Japan. The Myc-\textit{ADAM10} expression construct (Addgene plasmid # 31717)\textsuperscript{[17]} was a gift from Dr. Rik Derynck, Dept. of Cell and Tissue Biology, UCSF, San Francisco, CA.

\textbf{Cell invasion assay}\n
To investigate invasive cell growth, spheroids of cells were generated by cultivation overnight on agarose-coated trays or on bacterial plates. Spheroids were manually collected and transferred to 96-well tissue trays pre-coated with Matrigel\textsuperscript{TM} (BD Biosciences San Jose, CA) and overlaid with a second layer of Matrigel.

\textbf{Immunoprecipitation}\n
COS-7 cells were transfected, collected after 24 h in PBS and lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.5 % sodium deoxycholate, protease inhibitor cocktail (Roche)). For preclearing, protein-G-agarose beads (Roche) were added to cleared cell lysates for 1 h at +4 ºC. Supernatants were subjected to immunoprecipitation using the indicated antibodies (5 to 7.5 µg) for 3 h at +4 ºC, and protein-G-agarose beads were added overnight for immune-precipitation. The beads were washed and suspended into 2× Laemmli sample buffer. Samples were separated on SDS-PAGE and analyzed by Western blotting.

**SDS-PAGE and Western blotting**

For immunoblot analysis, the cells were sonicated in 2× Laemmli sample buffer and heated to +100 ºC for 5 minutes. The amount of proteins present in total cell lysates was quantified by the Bradford assay (Bio-Rad, Hercules, CA). Proteins were resolved by 10% or 12% SDS-PAGE and transferred to hydrophobic Immobilon PVDF membranes (Millipore, MA). The membranes were blocked with Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE), incubated with primary antibodies, followed by Alexa Fluor-680 goat anti-rabbit (Thermo Fisher) and IRDye 800CW donkey anti-mouse (LI-COR Biosciences) antibodies (both diluted 1:10,000). An Odyssey Infrared Imager (LI-COR Biosciences) was used for visualization.

**Results**

**Cloning and sequence**

Human APCN is a 98 kDa protein consisting of 890 amino acids. The coding gene is located on chromosome 10p13. The APCN sequence is also annotated *FAM171A1* or C10orf38.

*In silico* database search shows that APCN has no homology to previously functionally characterized human proteins. APCN is evolutionarily highly conserved. The homologous
Fam171a1 protein of mouse (GenBank identification number NP_001074630, https://www.ncbi.nlm.nih.gov/protein/NP_001074630) shows 92 percent, of chicken (XP_418631) 81 percent, of frog (NP_001088656, https://www.ncbi.nlm.nih.gov/protein/NP_001088656) 67 percent, and of zebrafish (XP_001335931) 48 percent identities with several large conserved regions in common with human APCN (Figure 1).

The molecular structure of APCN

Different online-tools (ExPASy website) were used to predict the APCN protein structure. These suggested that APCN is a type I transmembrane protein with the hydrophobic amino acids from 304 to 324 in the transmembrane region. The orientation of APCN was confirmed by expression of an APCN-GFP cDNA construct in COS-7 cells that revealed intracellular localization of GFP-labeled C-terminus (Supplemental Figure 2 A-F). The extracellular part of APCN is encoded by the seven short exons and contains several evolutionarily conserved stretches. The region immediately outside and inside the transmembrane stretch, encoded by exon seven, is virtually identical from zebrafish to human suggesting some important function. The long intracytoplasmic portion, representing roughly two thirds of the protein, is encoded almost exclusively by the large exon 8 and contains several evolutionarily conserved regions (Figure 1).

A database search revealed a putative glycosaminoglycan-binding site (VSGF) and three potential N-glycosylation sites (N159, N190, and N194) in the extracellular portion as well as several putative phosphorylation sites throughout the sequence.

APCN is glycosylated

The size of the APCN apoprotein is 98 kDa. Western blotting with antibodies to FLAG of lysates from COS-7 and MCF-7 cells transfected with (cDNA)APCN-flag revealed a major, slightly diffuse
band of 130 kDa apparent MW. Western blotting of lysates from transfected cells treated with Tunicamycin yielded a band of about 100 to 115 kDa (Figure 2A). This reduction in apparent MW is indicative of N-linked glycosylation. The experiment was performed in MCF-7 and COS-7 cells (data not shown) with identical results in both cell lines.

To map the glycosylation pattern, the three predicted N-glycosylation sites were subjected to N-A point mutations (N159A, N190A, N194A). N190A_APCN gave a western blotting identical to intact flAPCN. N159A_APCN and N194_APCN yielded reduced expression of the higher MW band with accentuation of the 100 to 115 kDa band. A double mutant N159/194A-APCN lead to disappearance of the high MW band (Figure 2B) Mutation of the putative glycosaminoglycan binding site (S136A) did not change the apparent MW of APCN (Figure 2B). These findings indicated that N159 and N194 are the N-glycosylation sites. The glycan structures of the N-glycoside side chains remain to be determined.

**Tissue expression of APCN**

A radiolabeled fragment of the cDNA, corresponding to base pairs 86 to 417 of the coding region, was used as a probe to investigate the tissue distribution of APCN mRNA. Dot blot analysis revealed expression in various tissues (Supplemental Figure S3A, Supplemental Table 1). High expression levels were observed in the different parts of the brain. Abundant expression of APCN message was also seen in fetal tissues, especially in the brain, heart, and kidney (Supplemental Figure S3 B).

To localize the tissue distribution of APCN protein expression an antibody was raised in rabbits to a synthetic peptide representing amino acids 378 to 394 (called MAP346). The specificity of MAP346 was validated by double immunofluorescence (Supplemental Figure S2G-I). Since brain
tissue showed strong signals in northern blotting, sections from normal human brain were stained by immunohistochemistry. An intense reticular staining was seen in *glia limitans* in a distribution corresponding to the abundant astrocyte processes and in pyramidal neurons (Figure 3A). Due to the robust appearance of the protein in astrocytes (Figure 3B) the protein was called Astroprincin.

**Transfection of APCN cDNA changes cell shape**

Transient transfection with (cDNA)fl*APCN-FLAG* induced dramatic changes in the cell shape. Overexpressed APCN induced sprouting of slender, dendrite-like and branching projections. This effect was consistently seen in all cells tested (MCF-7, HEK293, COS-7, NIH3T3, and SK-MEL-28). MCF-7 cells are shown in Figure 4 A-C.

To investigate which part(s) of APCN is needed to induce sprouting of dendrites, a panel of truncated, C-terminally FLAG-tagged cDNA constructs were made (Supplemental Figure S1) and transfected into cells. The integrity of the different C-terminally FLAG-tagged truncation clones was affirmed by sequencing.

When MCF-7 were transfected with FLAG-tagged intracytoplasmic portion of (cDNA)*APCN* with deleted extracellular and the transmembrane parts (cyt*APCN*) no sprouting was induced. The cells retained the shape of untransfected cells with cytoplasmic accumulation of the truncated protein (Figure 4 D-F).

**Down-regulation of endogenous APCN expression by siRNA inhibits actin stress fiber formation**

To investigate the functional interaction between APCN and the actin cytoskeleton SK-MEL-147 cells grown on coverslips were transfected with 6-Carboxyfluorescein–labeled siRNA to *APCN* or with control siRNA. Staining of the cells after 72 hrs with TRITC-phalloidin showed that expression of *APCN* siRNA induced redistribution of the cytoskeletal actin with disappearance of
stress fibers (Figure 5 A-C) whereas cells transfected with control siRNA displayed well organized stress fibers. (Figure 5 D-F). APCN siRNA transfection of U393MG astrocytoma cells also induced disappearance of stress fibers and accumulation of actin in donuts-like protrusions at the cell edges (Supplemental Figure S4).

**The conserved carboxyterminal 21 amino acids of APCN are needed for cell sprouting and invasive growth**

Analysis by qPCR and Western blotting of human melanoma cell lines revealed higher expression of endogenous APCN transcript and protein in SK-MEL-103 and in SK-MEL-147 than in SK-MEL-28 melanoma cells (Figure 6 A). SK-MEL-28 cells did not invade Matrigel (Fig. 6 B) whereas SK-MEL-103 (Figure 6 C) and SK-Mel-147 (Figure 6 D) cells displayed invasive growth in Matrigel. Overexpression of full length (cDNA)APCN in SK-MEL-28 cells induced sprouting of slender projections and ability to grow invasively in Matrigel (Figure 6 F and I) not seen with SK-MEL-28 cells transfected with the empty vector (Figure 6 E and H).

To investigate which part(s) of the APCN molecule was of importance for induction of invasive cell growth, mutated constructs of (cDNA)APCN were expressed in SK-MEL 28 cells. Expression of (cDNA)T3-APCN (Supplementary Figure S1) with deletion of the ultimate 21 carboxyterminal amino acids (869 to 890) in SK-MEL-28 cells did not induce sprouting in monolayer cultures or invasive growth in Matrigel (Figure 6 G and J).

**High expression of endogenous APCN in placental trophoblasts and invasive cancer**

Since trophoblasts are physiologically invasively growing cells IHC staining was performed with MAP346 of early (H5) and full-term placenta. The results showed robust trophoblast expression of
APCN (Figure 7 A and B). In situ hybridization with antisense and sense RNA probes to APCN revealed presence of APCN mRNA in the trophoblast layers of early placenta (Figure 7 C and D). IHC with MAP346 also discerned enhanced expression of APCN at the invasive front of nodular melanomas and in particular in individual infiltrating tumor cells (data not shown). In sections of lobular breast cancer containing both non-invasive, in situ lesions and infiltrating tumor cells a robustly up-regulated expression of APCN was found in the invasively growing cancer cells. Normal lobular epithelium remained negative for APCN (Figure 7 E and F).

Co-localization of APCN with integrin β1 and ADAM10 in U373MG astrocytoma cells

Confocal microscopy of immunofluorescence co-staining of U373MG cells transiently transfected with (cDNA)APCN with rabbit antibodies to APCN and mouse monoclonal antibodies to endogenous β1 integrin revealed an overlapping distribution. (Figure 8A-C). Co-staining of U373 cells with antibodies to ADAM10 and MAP346 to endogenous APCN revealed co-distribution at the leading edge of the cells (Figure 8 D-F).

APCN interacts with ADAM10, ornithine decarboxylase antizyme-1, and NEEP21 (NSG1)

Yeast-2-hybrid screening revealed several putative binding partners of APCN among which ADAM10, ornithine decarboxylase antizyme-1 (AZ-1), and NEEP21 (NSG1) were selected for initial validation. Immunoprecipitation with antibodies to ADAM10 from lysates of COS-7 cells co-transfected with (cDNA)ADAM10-Myc and (cDNA)APCN-FLAG pulled down APCN (Figure 9 A) confirming the interaction between APCN and ADAM10.

Binding of AZ-1 to APCN was studied by co-expression of (cDNA)AZ-1 with a 5’myc tag and FLAG-tagged (cDNA)flAPCN in COS-7 cells. Immunoprecipitation from lysates of transfected
cells with anti-FLAG followed by western blotting with antibodies to AZ-1 showed co-precipitation of AZ-1 (Figure 9 B). APCN with deletion of the conserved portion between amino acids 541 to 746 (Δ3-APCN, Supplemental Figure S1) did not pull down AZ-1 (Supplemental Figure S5).

Immunoprecipitation of lysates from COS-7 cells co-transfected with NEEP21-Myc and APCN-Flag cDNA constructs with antibodies to APCN also brought down NEEP21 indicating formation of detergent-resistant protein complexes (Figure 9 C).

Discussion

In this paper we report the first functional characterization of the APCN protein. Human APCN alias FAM171A1 or C10orf38 is a type I transmembrane glycoprotein and a member of the UPF0560 protein family that also includes FAM171A2, encoded by a gene on chromosome 17q21.31 and FAM171B on chromosome 2q32.1. FAM171A2 shows 39 percent and FAM171B 32 percent identity with APCN. The APCN gene is located on chromosome 10p13, a region that is deleted in DiGeorge type 2 and velocardiofacial syndromes [18].

Hybridization experiments and immunohistochemistry revealed physiological expression of APCN in various tissues, particularly in the brain where the astrocytes exhibit abundant APCN.

Yeast-2-hybrid screening indicated ODC AZ1 binding to APCN. This was validated by co-immunoprecipitation and the binding site was localized to a domain between aa 541 and 746 including the region between aa 541 and 569 that is highly conserved in the three members of the FAM171 proteins and in Fam171a1 proteins of different species. The activity of ODC, the rate limiting enzyme of polyamine synthesis, is intimately coupled to cell activation, transformation, and proliferation [19].
ODC translocates to the plasma membrane during cell activation \cite{20}. ODC activity is required for microvascular sprouting and remodeling of the actin cytoskeleton in endothelial cells \cite{21}. Moreover, ODC activity or local polyamine synthesis regulates the activity and traffic of RhoA, which is a main regulator of the dynamics of the actin cytoskeleton \cite{22}. A sizeable portion of cellular ODC sequestered in catalytically inactive form to AZ-1 and gets released and activated by competitive binding of antizyme inhibitor. The interaction between AZ-1 and APCN may provide a mechanism by which ODC is targeted to the membrane where the activity is of importance for cytoskeletal reorganization occurring during formation of cellular sprouts and acquisition of invasive phenotype.

Overexpression of full-length (cDNA)APCN induced outgrowth of slender and frequently branched extensions in both epithelial and mesenchymal cell lines. This was not seen with (cDNA)APCN lacking the extracellular portion. These findings suggested the APCN is involved in signaling between the cytoskeleton and the external environment.

A role for APCN in the maintenance of an organized actin cytoskeleton is further supported by these findings showing that down-regulation of endogenous APCN by siRNA interference provoked disappearance of organized actin stress fibers. Our attempts to delete endogenous APCN expression by CRISPR/Cas9 technology failed since viable cells could not be selected with total knockout of both alleles. Occasional polykaryotic cells with over 20 nuclei per cell were found (data not shown). This suggests that total absence of endogenous APCN causes disturbance in the actin dynamics needed for cytokinesis.

APCN is not known to mediate cell adhesion but by confocal microscopy a co-localization with integrin β1 was observed. Whether there is a direct interaction between integrin β1 and APCN or
whether the observed co-localization is due to formation of a larger complex with other proteins remains to be investigated.

ADAM10, like APCN, is a transmembrane type I glycoprotein with a complex N-glycoside side chain. The short intracytoplasmic portion contains two SH3 motifs and a consensus binding site for calmodulin. It is a member of transmembrane zinc-dependent metalloproteinase or “sheddase” acting as amyloid precursor protein cleaving α-secretase. More than 20 membrane-bound proteins have been identified as ADAM10 substrates, including Notch, proEGF, ErbB2, E-cadherin, CD44, and inflammatory cytokines [23]. ADAM10 is also essential for migration of neuronal precursors during embryonic brain morphogenesis [24]. Double immunofluorescence showed co-localization of endogenous ADAM10 and APCN in the leading edge of U373MG astrocytoma cells. It is tempting to speculate that interaction between ADAM10 and APCN is of relevance for brain morphogenesis and in particular for the arborization of astrocytes. Elevated expression of ADAM10 has also been reported to enhance growth and metastatic dissemination of neoplasms including melanomas, breast cancer, and liver cancers [25, 26]. Release of Nlgn3 that promotes glioma growth and differentiation was recently shown to be mediated by ADAM10 [27].

There is limited information in the literature about neuron-enriched endosomal protein of 21 kDa, NSG1 (NEEP21). NEEP21 has been found functionally involved in regulation of endosomal vesicle trafficking and membrane receptor recycling [28]. NEEP21 is involved in sorting of the neuron-glial adhesion molecule L1/NgCAM that regulates outgrowth of neurites [29]. Elevated expression of L1/NgCAM on the other hand has been found in a variety of cancers where it correlates with aggressive behavior of the neoplasm [30].
By expression of deletion mutants of APCN cDNA it was found that the 21 C-terminal amino acids are required for induction of invasive growth and sprouting of SK-MEL-28 melanoma cells. This sequence is also present in the two other members of the FAM171 protein family and is conserved in APCN of different species. A motif search revealed domains of homology in MAP1A, a structural protein that is involved in cross-bridging between microtubules and other cytoskeletal elements [31, 32]. These include Ephexin-2 that is a guanine nucleotide exchange factor for RhoA GTPase and in PHIP (pleckstrin homology domain interacting protein) that is involved in regulation of cell morphology and cytoskeletal organization [33, 34]. Moreover, BCAS1 that is amplified in a variety of cancers and associates with more aggressive tumor types also carries a homologous motif. We are presently investigating the molecular details of the interactions of the N-terminal domain of APCN.

BLAST searches of the conserved extracellular regions of APCN revealed a NCAM-homologous motif in the domain between aa 85 to 113. NCAM mediates neuron-neuron adhesion and is involved in neurite outgrowths. A region present in APCN 137 to 160 is also found in Septin5, which is encoded by a gene located on 22q11.2, a chromosomal region that is deleted in diseases including DiGeorge and velocardiofacial syndromes [18].

Taken together, we report the first functional characterization of APCN. Further investigations are required to unravel the molecular details of APCN, but the emerging picture is an evolutionarily highly conserved type I transmembrane glycoprotein that is involved in the regulation of the cytoskeletal dynamics and thereby the cell shape and invasive growth behavior of tumor cells.
Acknowledgements

We thank Mrs. Tiiu Arumäe for technical help. The U373MG astrocytoma line was provided by Professor Bengt Westermark, University of Uppsala, Sweden and SK-MEL-103 and SK-MEL-147 (both originating from Dr. Alan Houghton, Memorial Sloan-Kettering Cancer Center, New York, NY) were provided by Dr. Maria Soengas, Spanish National Cancer Research Center, Madrid, Spain. pCI-Neo containing N-terminal c-Myc was a gift from Dr. Tomi Mäkelä, University of Helsinki, Helsinki, Finland. AZ1 cDNA was provided by Prof. Shin-ichi Hayashi, Jikei University School of Medicine, Tokyo Japan. The Myc-ADAM10 expression construct (Addgene plasmid # 31717) was a gift from Dr. Rik Derynck, Dept. of Cell and Tissue Biology, UCSF, San Francisco, CA.
References


Figure legends

Figure 1. Alignment of APCN protein sequences in human (Homo sapiens), mouse (Mus musculus), chicken (Gallus gallus), African clawed frog (Xenopus laevis), and zebra fish (Danio rerio). Conserved regions are marked in yellow. The transmembrane region is marked in red. Sequence missing in aquatic species (amphibian and fish) is marked in green. Lower panel: Organization and nucleotide lengths of the APCN exons. The transmembrane region (in red) is encoded by exon 7 and the intracellular portion is encoded almost exclusively by exon 8.

Figure 2. APCN is N-glycosylated. A: Western blotting with antibodies to FLAG of lysates from COS-7 cells transfected with (cDNA) flAPCN-Flag and cultured with (lane 1) and without (lane 2) Tunicamycin. B: Point mutations showing that N159 and N194 are the major glycosylation sites. Western blotting of lysates from Cos-7 cells transfected with the following cDNA constructs: flAPCN-Flag (lane 1) APCN-Flag with mutation of the putative glycosaminoglycan-binding site (VSGF) (lane 2) APCN-Flag mutated at N159A (lane 3), APCN-Flag mutated at N194A (lane 4), and double mutated N159A/N194A APCN-Flag (lane 5).

Figure 3. Immunohistochemistry showing APCN expression in glia limitans of human brain and in pyramidal neurons (A) and astrocytes (B). Scale bars: A= 100 µM, B= 20 µM.

Figure 4. Impact of APCN on cell shape. Expression of (cDNA)flAPCN-Flag in MCF-7 cells induces membrane sprouting (A) not seen with cytAPCN-Flag (Supplementary Figure S1) (D) as visualized with mouse monoclonal antibody to Flag and goat anti-mouse-FITC antibodies. The cell membranes are visualized with WGA-TRITC (B and E). C and F: Merged pictures.
Figure 5. Transfection of SK-MEL-147 cells with APCN siRNA induces loss of stress fibers (A). Cells transfected with control siRNA display robust stress fibers (E). B and F: Visualization of 6-carboxyfluorescein–labeld siRNAs. C and G: Merged pictures. D: Western blotting showing the efficiency of siRNA-mediated down-regulation of APCN expression (siRNA) compared with control siRNA (CsiRNA).

Figure 6. Relative expression levels of endogenous APCN mRNA measured by qPCR in the melanoma cell lines SK-MEL-28, SK-MEL-103, and SK-MEL-147 (A). SK-MEL-28 does not invade Matrigel (B) like SK-MEL-103 (C) and SK-MEL-147 (D) cells. Transfection of SK-MEL-28 cells with the empty vector (control) (E and H), with (cDNA)flAPCN (F and I) or with (cDNA)APCN lacking the 21 C-terminal amino acids (G and J) showed that APCN induced sprouting (F) and ability to invade Matrigel (I) and that the C-terminal 21 amino acids are needed for the APCN activity (G and J).

Figure 7. Immunohistochemical (IHC) staining with MAP346 antibodies shows trophoblastic expression of APCN in early (A) and full term (B) placenta. (Scale bars 100 µM). In situ hybridization with APCN cRNA anti-sense (C) and sense (D) probe showing APCN mRNA in trophoblasts of early placenta (Scale bar 50 µM). IHC with MAP346 of sections from lobular breast cancer (E, F) shows elevated expression of APCN in infiltrating cancer cells compared with cells in the in situ (IS) lesions. Scale bars: E= 200 µM, F= 50 µM.

Figure 8. Confocal microscopy of U373MG cells transiently transfected with (cDNA)APCN-Flag and co-stained with MoAb antibodies to human β1-integrin (A) and with anti-FLAG (B). C: Merged picture. Staining of U373MG astrocytoma cells with antibodies to ADAM10 (D) and with MAP346 to endogenous APCN (E). F: Merged picture.
Figure 9. Interaction of APCN with ADAM10 (A), AZ-1 (B), and Neep21 (C) shown by co-immunoprecipitation and western blotting of lysates from COS-7 cells transiently co-transfected with (cDNA)ADAM10 and (cDNA)flAPCN-Flag (A), (cDNA) AZ-1 with a 5’myc tag and (cDNA) flAPCN-Flag (B), and (cDNA) NEEP21-Flag and (cDNA)flAPCN (C).
Table 1. The list of oligonucleotides used in this study.

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