INTRODUCTION

Armadillo-like (Arm) proteins are characterized by a series of repeats of 42-45 amino acid residues (aa), originally described in the Drosophila protein Armadillo (Riggleman et al., 1989). These Arm repeats have meanwhile been detected in a wide variety of proteins in a number of organisms, even including plants (Lo and Frasch, 1998; Peifer et al., 1994; Vithalani et al., 1998; Wang et al., 1998).

β-catenin is probably the most extensively studied Arm protein at the moment. The protein acts as a mediator of cell-cell adhesion through the E-cadherin/catenin complex in epithelial cells (Aberle et al., 1994; Ozawa et al., 1989). However, β-catenin is also a regulator of gene transcription via its interaction with LEF-1/TCF transcription factors (Behrens et al., 1996; Molenaar et al., 1996). In the E-cadherin/catenin cell adhesion complex, either β-catenin or γ-catenin (also known as plakoglobin) links the cytoplasmic domain of E-cadherin to the actin cytoskeleton via αE-catenin (Herrenknecht et al., 1991; Knudsen et al., 1995; McCrea and Gumbiner, 1991). Another Arm protein present in this cell-cell adhesion complex is p120ctn, which interacts with the cytoplasmic domain of E-cadherin but not with αE-catenin (Daniel and Reynolds, 1995; Reynolds et al., 1994). The p120ctn protein comprises 10 Arm repeats, while β-catenin and plakoglobin each comprise 12.5 Arm repeats. It serves as a prototype of a novel subfamily of Arm proteins (Reynolds and Daniel, 1997). Five proteins have already been described to share the p120ctn Arm repeat organization, namely plakophilin-1 (also known as ‘band 6 protein’ of bovine muzzle desmosome fractions; Heid et al., 1994; Schmidt et al., 1997), plakophilin-2 (Mertens et al., 1996), the ARVCF (Armadillo repeat gene deleted in velo cardio-facial syndrome) gene product (Sirotkin et al., 1997), p0071 (Hatzfeld and Nachtsheim, 1996), and δ-catenin, which is also known as the neural plakophilin-related arm-protein (NPRAP) (Paffenholz and Franke, 1997; Zhou et al., 1997). These proteins are generally expressed in a wide variety of cell types, except for δ-catenin/NPRAP, which is so far the only protein of this family displaying a restricted expression pattern, in this case neural tissue (Paffenholz and Franke, 1997).

For several members of the p120ctn/plakophilin family a desmosomal localization has been shown. Desmosomes (maculae adhaerentes) are epithelial adhering junctions involved in cell-cell adhesion, differentiation and signal transduction (Bornslaeger et al., 1997; Hatzfeld, 1997). They are assembled on a scaffold of transmembrane glycoproteins...
of the cadherin superfamily, i.e. the desmogleins and desmocollins (King et al., 1997; Koch et al., 1992). Both types of desmosomal cadherins are essential for cell-cell adhesion (Chitaev and Trowanovsky, 1997). Desmosomal cadherins are linked to the intermediate filament cytoskeleton through desmosomal plaque proteins, which commonly include plakoglobin and desmoplakin-I. Desmoplakin-II, encoded by the same gene as desmoplakin-I but generated by alternative splicing, exhibits a more cell-type-specific expression pattern (Bornsma et al., 1997). Additional desmosomal plaque components are continuously being detected and include plakophilin-1, plakophilin-2 and p0071, which share with plakoglobin membership of the superfamily of Arm proteins (Hatfield and Nachtsheim, 1996; Heid et al., 1994; Mertens et al., 1996; Moll et al., 1997). Despite their initial identification as desmosomal plaque proteins, the plakophilins were shown to be generally expressed as nuclear proteins translocated to desmosomes only in certain stages of differentiation (Mertens et al., 1996; Schmidt et al., 1997). This was a surprising observation, especially in the case of plakophilin-1, which had been assumed for a long time to be restricted to desmosomal cell-cell contacts of stratified and complex epithelia (Kapprell et al., 1988). These results suggest, besides a mechanical function for plakophilins in desmosomes of certain cell types, their involvement in signal transduction pathways between the plasma membrane and the cell nucleus. In cells deprived of desmosomes, both plakophilins may display an exclusively nuclear function. Originally, the p0071 protein has been localized to desmosomes in cultured cell lines (Hatfield and Nachtsheim, 1996). However, it has also been detected in cell nuclei, which suggests that p0071 might exert a role in signaling pathways (Hatfield, 1997).

In the present study we describe the novel plakophilin-3 protein, for which the protein expression pattern seems to be largely restricted to epithelial cell types. We could localize this protein in the desmosomal plaque and in the cell nucleus, and therefore it is likely to be involved in plasma membrane/cell nucleus signal transduction pathways. Sequence alignment with other p120ctn/plakophilin subfamily members suggests that plakophilin-3 might exert some specific functions, or a combination of different functions displayed by other family members.

MATERIALS AND METHODS

Database searches and DNA sequencing

BLASTP searches (Altschul et al., 1990) were performed at GenomeNet in Japan (http://www.blast.genome.ad.jp/). EST clones encoding unknown Arm-like proteins were ordered from the UK HGMP Resource Centre (Hinxton, UK) and from Genome Systems (St Louis, MO). The sequences of the full-size inserts of these clones and all other DNA sequences were determined by an ABI 377 automated DNA sequencer (Applied Biosystems, Foster City, CA). Sequence data were processed using the DNAstar software (DNAStar, Madison, WI) and the Staden Package (Bonfield et al., 1995; see also the Staden Package WWW site at http://www.mrc-lmb.cam.ac.uk/pubseq/).

Reverse transcription (RT)-PCR

Total RNA from human uterus was purchased from Clontech Laboratories (Palo Alto, CA). cDNA synthesis was performed as described (Keirsebilck et al., 1998). PCR primers were designed using the Oligo 5.0 Primer Analysis software (NBI, Plymouth, MN) and were purchased from Life Technologies (Paisley, UK). PCR was performed with the Advantage GC KlenTaq Polymerase Mix (Clontech) on a PTC-200 Peltier Thermal Cycler PCR system (MJ Research, Watertown, MA). The PCR reaction mixture contained template cDNA, 25 pmol of each primer, 10 μl KlenTaq 5× PCR buffer, 10 μl GC Melt, 4 μl 5 mM dXTPs and 1 μl KlenTaq in a final volume of 50 μl. Cycling conditions were 3 minutes at 94°C (initial denaturation), followed by 35 cycles of 30 seconds at 94°C, 45 seconds at 60°C and 2 minutes at 72°C. Final extension was for 10 minutes at 72°C. A plakophilin-3 specific RT-PCR fragment, used for northern blot hybridization and cDNA library screening, was generated accordingly. Forward primer sequence (5′→3′): ccgctggacctcttac; reverse primer sequence (5′→3′): ttactctgaggttgtgt. The predicted 774 bp PCR product was checked on a 1% agarose gel for the presence of aspecific reaction products, followed by purification with the Qiaquick PCR purification kit (Qiagen, Chatsworth, CA) and sequencing.

Northern blot analysis

Total RNA was prepared with the RNeasy kit (Qiagen) following the manufacturer’s protocol. Total RNA (25 μg) was glyoxylated, size-fractionated on a 1% agarose gel and transferred onto a Hybond-N+ membrane (Amersham Pharmacia Biotech, Raimham, UK). Hybridizations were performed as described before (Bussemakers et al., 1991). A 774 bp RT-PCR product (see above) was 32P-labeled using the RadPrime DNA labeling system (Life Technologies). For data collection PhosphorImager 425 equipment was used (Molecular Dynamics, Sunnyvale, CA).

5′ rapid amplification of cDNA ends (5′ RACE)

5′ RACE experiments were performed using either the Marathon cDNA amplification kit (Clontech) or a 5′ RACE system (Life Technologies) following the manufacturer’s instructions and using different human messenger RNA (mRNA) sources. None of these experiments resulted in completion of the human plakophilin-3 mRNA sequence. Using the Marathon cDNA amplification kit only aspecific products were amplified, while the Gibco 5′ RACE kit yielded specific, though very short products. The latter observation indicated that reverse transcription was suboptimal, probably due to the formation of secondary structures in the G/C-rich 5′ end of the mRNA.

cDNA library screening

A human fetal kidney 5′ stretch cDNA library in vector λDR2 (Clontech) was screened with a 32P-labeled 774 bp RT-PCR product as mentioned above. Five positive plaques were identified upon screening of approximately 800,000 plaques. After a second screening cycle, plaques were cut out and converted in vivo to pDR2-derived plasmids according to the manufacturer’s instructions. Restriction digestion and sequence analysis revealed only one clone (1315B) containing a full-length cDNA insert. To facilitate sequencing of the unknown 5′ region of the cDNA, a BamHII restriction fragment of about 1,330 bp was subcloned into the pGEM-11Zf(+) vector (Promega, Madison, WI) and sequenced.

The fetal mouse Rapid-Screen cDNA library (Origene, Rockville, MD) was screened by PCR according to the manufacturer’s instructions. Forward primer sequence (5′→3′): ttccagccacctcaacct; reverse primer sequence (5′→3′): ttgctgatcatactctgg. Reaction conditions of the PCR, which generates a 813 bp product, were as follows: 2 minutes at 95°C, followed by 35 cycles of 40 seconds at 94°C, 45 seconds at 60°C and 1 minute at 72°C. Final extension was for 5 minutes at 72°C.

In vitro transcription/translation assay

The in vitro transcription/translation assay was performed using the TNT Coupled Reticulocyte Lysate System kit (Promega) according to
the manufacturer’s instructions. The full-length cDNA was obtained by HindIII digestion of clone pDR2 13H5B and ligated into the pGEM-11Zf(+) vector (Promega). Depending on the orientation of the insert, either T7 or SP6 RNA polymerase was used for transcription. The translation products were [35S]methionine-labeled and separated by SDS-PAGE on an 8% gel followed by drying. The labeled proteins were detected using a PhosphorImager 425 (Molecular Dynamics).

Human genomic DNA library screening

A BAC (Bacterial Artificial Chromosome) human genomic DNA library (Genome Systems) was screened by PCR as recommended by the supplier. The PCR reaction was performed with the Taq PCR Core kit (Qiagen) supplemented with GC Melt (Clontech) on a PTC-200 Peltier Thermal Cycler PCR system (MJ Research). The reaction mixture contained 100 ng template DNA, 25 pmol of each primer, 5 μl 10× PCR buffer, 2 μl 25 mM MgCl₂, 7 μl GC Melt, 1 μl 10 mM dXTPs and 0.5 μl (2.5 units) Taq DNA polymerase in a final volume of 50 μl. Forward primer sequence (5’→3’): gcgtggttgccctctatctaca; reverse primer sequence (5’→3’): tegtggaggggtgaggtcaga. PCR reaction conditions as follows: 5 minutes at 94°C, followed by 35 cycles of 30 seconds at 94°C, 45 seconds at 60°C and 1 minute at 72°C. Final extension was for 5 minutes at 72°C. This reaction yielded a 192 bp product, containing an 81 bp intron sequence. Products were analyzed on a 2% agarose gel which revealed one BAC clone (245A8) containing the screened region. DNA of this clone was purified using KB-100 columns (Magnus, Genome Systems).

Chromosomal localization of the human plakophilin-3 gene by fluorescent in situ hybridization (FISH)

FISH analysis using BAC clone 245A8 containing the human plakophilin-3 gene was performed according to standard procedures (Kievits et al., 1990) with some minor modifications. DNA of the BAC clone was biotinylated using the BioNick-kit (Life Technologies) (Kievits et al., 1990) with some minor modifications. DNA of the BAC clone HCT8/E8 and HCT8/R1 were obtained by subcloning of cell line HCT8 (CRL-224), where E stands for epitheloid and R for round cell variants (Vermeulen et al., 1995). Similarly, DLD1/R2/7 was a round cell variant subcloned from DLD1 (CCL-221) (Vermeulen et al., 1995; Watabe-Uchida et al., 1998). GLC34 is derived from a small cell lung carcinoma (De Leij et al., 1985). Cell lines LIRC-HN2, LIRC-HN3 and LIRC-HN6 are derived from head and neck squamous cell carcinomas (Easty, 1981). MKN45 is a gastric carcinoma cell line (Motoyama and Watanabe, 1983); PC AA/C1, abbreviated below as PC, is a colon adenocarcinoma-derived cell line (Paraskeva et al., 1984). MCF-7/AMC and MCF-7/AMC cell lines are derived from the MCF-7 (HTB-22) human mammary carcinoma cell line (Bracke et al., 1991). HaCaT is a human keratinocyte cell line (Boukamp et al., 1988). FS4 is a human foreskin fibroblast cell line, and HEK293 is a human embryonic kidney fibroblast cell line.

Construction and transfection of a plasmid encoding plakophilin-3

Plasmid DNA from clone pDR2 13H5B was used as template in two PCR reactions, generating the appropriate cDNA fragments for in-frame ligation in the pEFHOBES eukaryotic expression vector downstream of an E-tag-encoding cDNA fragment. The pEFHOBES vector was a kind gift from M. Van de Craen (DMB, University of Gent, Belgium), consisting of the expression vector pEF-BOS (Mizushima and Nagata, 1990), in which the E-tag from plasmid pCANTAB5E (Pharmacia) is inserted. In this construct, transcription of the cDNA is under control of the human EF1-α-promoter. Transfection was performed using Lipofectamin Reagent (Life Technologies) according to the manufacturer’s instructions with some minor modifications.

Antibodies, antibody production and purification

Antibodies specific for both the human and mouse plakophilin-3 protein were raised by immunization of rabbits with 200 μg of a synthetic peptide with sequence NH₂-KLHRDFRAGKYRKED-COOH. This peptide was coupled to keyhole limpet hemocyanin via an additional cysteine residue at the NH₂-terminal end. Immunization was followed by boost injections after 2 weeks. After another 2 weeks, antisera were collected on a biweekly basis for 1 month and tested in ELISA assays, using the synthetic peptide. Antibodies were affinity-purified using the synthetic peptide covalently bound to p-hydroxymercuribenzoate-agarose (Sigma, St Louis, MO). Purified antibodies were then tested on in vitro transcribed and translated products separated by SDS-PAGE and blotted as described below. Recognition of plakophilin-3 by the antibodies was inhibited by incubation of the polyclonal antibody with the antigenic peptide for 1 hour prior to use. This procedure also abrogated detection of plakophilin-3 when tested on western blots.

The following mouse monoclonal antibodies were used: anti-
desmoglein antibody DG3.10 (Cymbus Bioscience, Southampton, UK), human keratin 18 antibody RGE.53 (Euro-Diagnostics, The Netherlands) and anti-plakophilin-1 and -2 antibodies (Progen, Heidelberg, Germany).

Secondary antibodies used in immunofluorescence microscopy were coupled to either FITC (Amersham Life Science, Buckinghamshire, UK), Alexa594 or Alexa488 (Molecular Probes, Eugene, OR) anti-rabbit Ig, anti-rat Ig or anti-mouse Ig antibodies.

**Western blot analysis**

Total protein lysates were prepared by washing subconfluent cell cultures twice with 1x PBS, followed by scraping of the cells in 1x Laemmli sample buffer (Laemmli, 1970) and sonication. Protein concentration was measured using the DC protein assay kit (Biorad, Richmond, CA). 40 ng of total protein were boiled in 5% 2-mercaptoethanol and separated by 8% SDS-PAGE. Proteins were transferred onto Immobilon-P membranes (Millipore, Bedford, MA) and blocked with 5% nonfat dry milk, 0.1% Tween-20 in PBS (pH 7.4) prior to incubation with the primary antibody. Secondary anti-rabbit Ig and anti-mouse Ig antibodies coupled to horseradish peroxidase (Amerham Pharmacia Biotech) were used for detection of proteins on western blots. The ECL western blotting detection system (Amerham Pharmacia Biotech) was used for detection of the secondary antibodies after extensive washing of the blots with PBS (pH 7.4).

**Immunofluorescence assays of cultured cell lines**

Cell cultures were grown on glass coverslips and briefly washed in PBS, 2 mM MgCl₂, 2 mM CaCl₂ (complete PBS) followed by fixation with either methanol or paraformaldehyde. In the first case, cells were briefly rinsed with ice-cold 100% methanol, followed by a 10-minute treatment with ice-cold 100% methanol. After brief air-drying, coverslips were incubated for 5 minutes in complete PBS, 0.2% Triton X-100. The cells were then incubated for 1 hour at room temperature with primary antibodies diluted in 0.4% gelatin in complete PBS. Cells were washed 3 times for 10 minutes with complete PBS, followed by incubation of the appropriate secondary antibody diluted in 0.4% gelatin in complete PBS. Finally, cells were washed again and incubated with DAPI solution. Specimens were subsequently mounted with Vectashield (Vector Laboratories) to prevent photobleaching.

Alternatively, cells were fixed for 20 minutes with 3% paraformaldehyde in complete PBS at room temperature. Fixation was followed by quenching in a 50 mM NH₄Cl solution in complete PBS for 5 minutes and by permeabilization in complete PBS, 0.2% Triton X-100 for 5 minutes at room temperature. All antibodies were diluted in 0.3% skimmed milk in complete PBS and incubated for 1 hour at room temperature. After incubation with secondary antibodies, cells were washed again and mounted as described above.

Fluorescent image results were captured by a Photometrics Image Point CCD camera (Photometrics-GmbH, Germany) mounted on a Zeiss Axiosph microscope, or by a Zeiss LSM 410 confocal laser-scanning immunofluorescence microscope. Image processing was performed using the MacProbe v3.4.1 software (Perceptive Scientific International LTD.).

**RESULTS**

**Discovery and cloning of the human plakophilin-3 mRNA**

In our search for novel Arm proteins, BLASTP searches (Altschul et al., 1990) were performed in non-redundant databases using the sequences of various known Arm proteins. These searches revealed a number of human expressed sequence tags (EST) displaying significant sequence similarity with human plakophilin-1 and -2 proteins. Complete sequencing of the inserts of these cDNA clones allowed the assembly of a contig representing a 1,970 bp cDNA fragment, which may encode a protein fragment highly related to plakophilin-1 and -2. This sequence, still incomplete at the 5′ end, appeared to be derived from a genuine mRNA with one long open reading frame (ORF) and a 3′ untranslated region (UTR), containing a polyadenylation signal (AAATAA) 18 bp in front of a poly(A) tail. To determine the total length of the corresponding mRNA, a northern blot hybridization experiment was performed on total RNA extracted from various cell lines, some of which originated from the same tissue type as the EST clones used to construct the contig, for instance colon. This indicated the corresponding full-length mRNA to be approximately 3 kb in size (Fig. 1). However, despite several attempts, we were unable to clone the missing 5′ end of the mRNA by RACE experiments (see Materials and methods). This is probably due to the relative high GC content of the 5′ end of the mRNA (up to 85%, while its overall GC content is 67%), which stimulates the formation of secondary structures and thereby inhibits efficient reverse transcription (Zhang and Frohman, 1998). Therefore, a human fetal kidney cDNA library was screened using a 770 bp RT-PCR product as probe. This resulted in the identification of five specific cDNA clones, of which one (clone 13H5B) contained a full-length insert. The completed 2,786 bp plakophilin-3 cDNA sequence (GenBank accession number: AF053719) contains an ORF of 2,391 bp, a very short 5′ UTR (54 bp) and a 3′ UTR of 341 bp. The ORF encodes a 797 aa protein, with a calculated Mᵉ of 87×10³. The ATG codon at position 55 is very likely the correct translation initiation site, as it lies in a sequence context favoring such initiation of translation and because no upstream ATG codons are present (Kozak, 1996, 1997). This was consolidated by an in vitro transcription/translation assay, using this putatively full-length plakophilin-3 cDNA as template. Only one major polypeptide was produced and this showed the correct predicted size (results not shown).

**Cloning of the mouse plakophilin-3 mRNA**

Starting from mouse EST clones, we sequenced and assembled a partial mouse plakophilin-3 cDNA, which was about 2,320 bp in size but still lacked the 5′ end. In order to complete this sequence, a fetal mouse cDNA library was screened by PCR. This resulted in the identification of two clones containing a full-length mouse plakophilin-3 cDNA insert. The 2,829 bp murine sequence (GenBank accession number: AF136719) encodes a 797 aa protein which is 94% identical and 96% similar to the human plakophilin-3 protein. The overall nucleotide identity between human and mouse plakophilin-3 mRNA was found to be 81%.

**Fig. 1.** Detection of plakophilin-3 mRNA by northern blot hybridization of total RNA extracted from various human cell lines.
Mapping of the human plakophilin-3 gene to chromosomal region 11p15

We mapped the human plakophilin-3 gene (proposed gene symbol: PKP3) by FISH to chromosomal region 11p15 (Figs 2A,B). The assignment to chromosome 11 was confirmed by PCR performed on a human monochromosomal cell-hybrid-mapping panel (Fig. 2C). The 192 bp PKP3-specific fragment was detected only in the lanes containing the positive control and human chromosome 11. The PKP-3 gene was not found in GeneMap'98 (http://www.ncbi.nlm.nih.gov/genemap98) using ‘plakophilin’ as search string (most of the plakophilin-3-specific EST clones present in public databases are annotated with ‘similar to plakophilin’). According to GeneMap’98 several EST clones annotated ‘plakophilin-2’ were mapped to different chromosomes, but none to chromosome 11. However, the similarity of these EST clones to plakoglobin-2 is probably based on an Alu repeat present in an exon, specific for the plakoglobin-2b isoform (our unpublished observations).

Plakophilin-3, a novel member of the p120ctn/plakophilin subfamily of Armadillo proteins

The plakophilin-3 protein contains a central Armadillo domain composed of 10 repeats preceded by a 293 aa amino-terminal region and a short (27 aa) carboxy-terminal region (Fig. 3). The aa sequence and the organization of these repeats (4+1+1+4) are very similar to these of the proteins belonging to the p120ctn/plakophilin Arm subfamily (Reynolds and Daniel, 1997). Multiple alignment of plakophilin-3 with the previously reported proteins of this subfamily, namely p120ctn, the ARVCF protein, p0071, δ-catenin/NPRAP, and plakophilin-1 and -2 was performed using the CLUSTAL W program (Higgins and Sharp, 1989; Thompson et al., 1994) (Figs 3, 4A). A phylogenetic tree generated by the CLUSTAL W alignment of the Arm-repeats of the p120ctn/plakophilin family members, β-catenin, plakoglobin and the Drosophila protein Armadillo is presented as Fig. 4B. As evident from Fig. 4A, the plakophilins form a somewhat distinct subgroup within the p120ctn/plakophilin protein family. The interprotein similarities of the full-size proteins and of the central Arm repeat regions are presented in, respectively, Tables 1 and 2. The plakophilin-3 protein shows equal similarity to both plakophilin-1 and -2 (44-45%), but also substantial similarity to the other Arm proteins (41%). The same observation holds true when identities instead of similarities were calculated (data not shown). However, markedly higher intermolecular resemblance is obvious for the p120ctn and ARVCF proteins, plakophilin-1 and -2, and p0071 and δ-catenin/NPRAP (Table 1, bold numbers). When the sequences of the central Arm repeat regions are compared to each other (Table 2), the former observations were largely corroborated. Together, these data indicate that three pairs of very similar proteins can be distinguished, i.e. the p120ctn and the ARVCF proteins, which are also quite similar to the p0071 plus δ-catenin/NPRAP pair, and finally the more

Table 1. Interprotein aa similarities of the full-length proteins of the p120ctn/plakophilin subfamily

<table>
<thead>
<tr>
<th></th>
<th>ARVCF</th>
<th>p120ctn</th>
<th>NPRAP</th>
<th>p0071</th>
<th>pp-1</th>
<th>pp-2</th>
<th>pp-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARVCF</td>
<td>100.0%</td>
<td>65.7%</td>
<td>51.8%</td>
<td>50.2%</td>
<td>40.8%</td>
<td>40.4%</td>
<td>42.7%</td>
</tr>
<tr>
<td>p120ctn</td>
<td>100.0%</td>
<td>100.0%</td>
<td>50.7%</td>
<td>48.4%</td>
<td>40.4%</td>
<td>38.7%</td>
<td>41.1%</td>
</tr>
<tr>
<td>NPRAP</td>
<td>100.0%</td>
<td>100.0%</td>
<td>63.3%</td>
<td>39.6%</td>
<td>40.7%</td>
<td>41.3%</td>
<td></td>
</tr>
<tr>
<td>p0071</td>
<td>100.0%</td>
<td>100.0%</td>
<td>41.1%</td>
<td>39.9%</td>
<td>41.6%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pp-1</td>
<td>100.0%</td>
<td>100.0%</td>
<td>53.5%</td>
<td>44.3%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pp-2</td>
<td>100.0%</td>
<td>100.0%</td>
<td>100.0%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pp-3</td>
<td>100.0%</td>
<td>100.0%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

pp-1, -2, -3: plakophilin-1, -2, -3.
Western blot analysis of plakophilin-3

A rabbit polyclonal antibody was raised against a carboxy-terminal plakophilin-3-specific peptide and affinity-purified. The sequence of this 15 aa peptide is poorly conserved in either plakophilin-1 or -2 (Fig. 3) which makes cross-reactivity of the antibody with the latter plakophilins very unlikely. This was confirmed by a western blot analysis using mouse monoclonal antibodies against plakophilin-1 and -2 and the rabbit polyclonal anti-plakophilin-3 antibody (Fig. 5A). In order to confirm that our plakophilin-3 cDNA was complete, HEK293 cells were transfected with a cDNA encoding a tagged plakophilin-3 protein with a predicted mass of 89 kDa. Untransfected HEK293 cells do not express endogenous plakophilin-3 protein (Fig. 5B, lanes 2 and 3). This result indicated our plakophilin-3 cDNA was complete, HEK293 cells were transfected with a cDNA encoding a tagged plakophilin-3 protein. These include human embryonic kidney cells (HEK293), FS4 fibroblasts and the SV-40 transformed fibroblastoid cell line VA13. The SK-LMS1 leiomyosarcoma, SW872 sarcoma and HOS osteosarcoma cell lines also did not express plakophilin-3 (Fig. 5C).

Immunodetection of nuclear and desmosome-associated plakophilin-3

In order to determine the intracellular location of plakophilin-3, immunostainings were performed on cultured human cell lines using the same rabbit polyclonal antibody mentioned above. The staining results varied dramatically depending on the fixation and permeabilization protocols used.

In methanol-fixed human ileocecal adenocarcinoma cells HCT8/E8, plakophilin-3 was detected along cell-cell borders in a punctate staining pattern typical for desmosomal proteins (Fig. 6A). A desmosome-like immunostaining was also detected in methanol-fixed HaCaT cells (result not shown). Colocalization of the plakophilin-3 protein with the desmosomal protein desmoglein-2 was demonstrated in HCT8/E8 cells, transfected with a cDNA plasmid encoding a full-length plakophilin-3 protein (Fig. 6B-D). When the plakophilin-3 antibody was pre-incubated prior to use with the peptide against it was raised, the punctuate staining along cell-cell borders was no longer detectable (result not shown).

In addition to the desmosomal localization, immunostaining was observed as bright nuclear speckles in HCT8/E8 cells, especially upon appropriate focusing (Figs 6A, 7A). The plakophilin-3 specificity of this nuclear staining was shown by its complete inhibition upon pre-incubation of our antibody with the immunogenic peptide (Fig. 7B). Such plakophilin-3 detection in HCT8/E8 nuclei was insensitive to the fixation protocol used, whereas desmosomal staining was abolished by the paraformaldehyde fixation protocol used. In contrast, it was easily detectable in paraformaldehyde-fixed HaCaT cells (Fig. 7C,D), but poorly detectable in methanol-fixed HaCaT cells. As can be seen in Fig. 7C, nucleioli are not stained. The nuclear presence of plakophilin-3 was confirmed by confocal microscopy on A431 cells, showing the presence of plakophilin-3 as nuclear spheres (Fig. 8).

DISCUSSION

In the present study, we describe a novel Armadillo protein, designated plakophilin-3, as it shows significant similarity to

Table 2. Interprotein aa similarities of the Arm repeat regions of the proteins belonging to the p120ctn/plakophilin subfamily

<table>
<thead>
<tr>
<th></th>
<th>ARVCF</th>
<th>p120ctn</th>
<th>NPRA</th>
<th>p0071</th>
<th>pp-1</th>
<th>pp-2</th>
<th>pp-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARVCF</td>
<td>100.0%</td>
<td>75.3%</td>
<td>68.1%</td>
<td>65.0%</td>
<td>48.1%</td>
<td>51.1%</td>
<td>52.0%</td>
</tr>
<tr>
<td>p120ctn</td>
<td>100.0%</td>
<td>100.0%</td>
<td>68.6%</td>
<td>65.7%</td>
<td>48.1%</td>
<td>50.6%</td>
<td>51.3%</td>
</tr>
<tr>
<td>NPRA</td>
<td>100.0%</td>
<td>100.0%</td>
<td>100.0</td>
<td>81.5%</td>
<td>50.1%</td>
<td>53.0%</td>
<td>53.8%</td>
</tr>
<tr>
<td>p0071</td>
<td></td>
<td></td>
<td></td>
<td>100.0%</td>
<td>51.7%</td>
<td>51.5%</td>
<td>52.8%</td>
</tr>
<tr>
<td>pp-1</td>
<td></td>
<td></td>
<td></td>
<td>100.0%</td>
<td>60.9%</td>
<td>51.9%</td>
<td>51.0%</td>
</tr>
<tr>
<td>pp-2</td>
<td></td>
<td></td>
<td></td>
<td>100.0%</td>
<td>51.0%</td>
<td>51.0%</td>
<td>100.0%</td>
</tr>
<tr>
<td>pp-3</td>
<td></td>
<td></td>
<td></td>
<td>100.0%</td>
<td>51.0%</td>
<td>51.0%</td>
<td>100.0%</td>
</tr>
</tbody>
</table>

pp-1, -2, -3: plakophilin-1, -2, -3.
Fig. 3. CLUSTAL W alignment of the protein sequences of plakophilin-1a, -2a and -3. The high interprotein similarity in the Arm repeat domain (B) is obvious, while the amino-terminal domains (A) and the very short carboxy-terminal regions (C) are less well conserved. The underlined sequence in the carboxy-terminal region of plakophilin-3 was used to synthesize a peptide for raising a polyclonal antibody.
It is composed of a central Arm repeat domain, containing 10 Arm repeats in the configuration (4+1+1+4), typical of the p120\textsuperscript{ctn}/plakophilin subfamily of Arm proteins (Reynolds and Daniel, 1997). The very short C-terminal domain (27 aa) is typical of all plakophilins reported so far. Unlike the situation for plakophilin-1 and -2, we were unable to demonstrate that alternative splicing occurs in the plakophilin-3 mRNA. Nonetheless, a thorough RT-PCR and western blot screening of putative plakophilin-3 isoforms still has to be performed in different cell lines and tissues. One plakophilin-3-specific EST clone present in public databases (GenBank-ID AA515691; origin: colon) contains an 81 bp insert with respect to our cDNA sequence. In silico translation results in an additional 27 aa fragment which displays no similarity to any known protein. We believe, however, this fragment to be intronic, as it displays typical intron features such as the gt/ag splice donor/acceptor sites, a branch site and a C/T stretch at its 3\textsuperscript{′} end. Moreover, western blot experiments on colon-derived cell lines did not reveal evidence of alternative splicing, and at least in small intestine mRNA this additional fragment was not detectable by an appropriate RT-PCR reaction.

The human plakophilin-3 gene (PKP3) was mapped to the chromosomal region 11p15 by FISH. The gene is not linked to either the plakophilin-1 gene (PKP1), mapped on 1q32, or the plakophilin-2 gene (PKP2) mapped on 12p13, or any other gene encoding a p120\textsuperscript{ctn}/plakophilin family member; p120\textsuperscript{ctn}, ARVCF, p0071 and \(\delta\)-catenin/NPRAP (Bonné et al., 1998). Hence, these mapping data do not reveal particular evolutionary links between these various gene family members. So far, the Online Mendelian Inheritance in Man Morbid Map (http://www.ncbi.nlm.nih.gov/Omim/searchmorbid.html), which presents the reported cytogenetic map locations of disease genes does not reveal any disease in which plakophilin-3 might be involved on the basis of our mapping. There is also no evidence that unknown genes localized in the chromosomal region 11p15 are affected in human cancers (Mitelman et al., 1997).

Multiple sequence alignments of either the full-size protein sequences or Armadillo repeat regions revealed (1) that plakophilin-3 belongs to the p120\textsuperscript{ctn}/plakophilin subfamily of Armadillo proteins, and (2) that the plakophilins themselves form a distinct subgroup within this subfamily. Although the overall sequence of plakophilin-3 is most related to those of plakophilin-1 and -2, in the Arm repeat region the protein shows equal similarity to each of the other p120\textsuperscript{ctn}/plakophilin family members. This might indicate that plakophilin-3 combines functions from these different proteins of this family, mirrored by several locally conserved protein regions. Alternatively, the non-conserved sequences of plakophilin-3 may specify particular functions of this protein not shown by the other p120\textsuperscript{ctn}/plakophilin family members. Discrimination between these two possibilities has to await detailed functional analysis of this largely enigmatic protein family.

Near their amino terminus domain, the plakophilins contain a well-conserved sequence stretch, which we denoted the plakophilin HR2 or PKP-HR2 domain. For p120\textsuperscript{ctn}, the ARVCF gene product, p0071 and \(\delta\)-catenin/NPRAP, a short conserved amino-terminal domain was previously denoted Homology Region 2 (HR2; Fig. 3) (Reynolds and Daniel, 1997). This HR2 was suggested to have a common function in the different proteins or to correspond with a common binding motif (Reynolds and Daniel, 1997). However, no
evidence for either one of these hypotheses has been presented so far. Thus, an analogous but hardly homologous domain is also present in the plakophilins. The function of these HR2 regions in the otherwise poorly conserved amino-terminal domain is unknown. The PKP2-HR2 sequence might, however, mediate an important biological function, as it is even conserved in sequence data we obtained from a *Xenopus laevis* plakophilin cDNA clone (our unpublished data).

Using an anti-peptide rabbit polyclonal antibody, the plakophilin-3 expression pattern was investigated in a variety of cell lines. Several cell lines originating from epithelial tissues expressed plakophilin-3. We were, however, unable to detect expression of this new plakophilin in fibroblast-derived cell lines, including different sarcoma cell lines. This is apparently different from the expression patterns reported for plakophilin-1 and -2, which are widespread nuclear proteins, also detectable in nuclei of fibroblast-derived cell lines, including different sarcoma cell lines. This is apparently different from the expression patterns reported for plakophilin-1 and -2, which are widespread nuclear proteins, also detectable in nuclei of fibroblast-derived cell lines (Mertens et al., 1996; Schmidt et al., 1997). The p120ctn protein is also known to be widely expressed (Keirsebilck et al., 1998; Mo and Reynolds, 1996). Mutations in the desmosomal plaque protein plakophilin-1 have recently been described too (McGrath et al., 1997). A patient with two independent mutations in the plakophilin-1 alleles, resulting in a functional knockout, displayed features of skin fragility and congenital ectodermal dysplasia, affecting skin, hair and nails. Remarkably, no significant abnormalities were detected in other epithelia or tissues. The phenotype described is apparently due to the loss of desmosome-associated plakophilin-1, known to be present in stratified and complex epithelia (Schmidt et al., 1997). No defect in this patient could, however, be linked to the absence of the plakophilin-1 fraction, which normally is present in the nuclei of a wide variety of cell types and tissues (Schmidt et al., 1997). One may speculate that this is due to a functional redundancy of plakophilins-1, -2 and -3 in the nucleus but not in desmosomes, although no evidence for this has been presented yet.

Nuclear plakophilin-3 was detected as bright speckles, dispersed throughout the nuclear volume but excluding the nucleoli. The presence of plakophilin-3 in the cell nucleus was confirmed by confocal microscopy. A similar nuclear appearance has been described for plakophilin-1 (Klymkowsky, cell-cell contacts. Mutations in or complete loss of desmosomal components, such as desmosomal cadherins or proteins of the intracellular desmosomal plaque, are held responsible for loosened cell-cell adhesion. The desmosomal cadherins desmogleins and desmocollins are both necessary for proper desmosomal functionality (Chitaev and Troyanovsky, 1997).

Decreased functionality of either desmoglein-1 or -3 can be caused by autoimmune diseases, resulting in affected keratinocyte cell-cell adhesion and ultimately skin blistering (Emery et al., 1995; Karpati et al., 1993; Koch et al., 1997).
Fig. 6. Immunofluorescence microscopy of endogenously and exogenously expressed plakophilin-3 in human epithelial HCT8/E8 cells, using a polyclonal plakophilin-3-specific antibody. The plakophilin-3 protein colocalizes with the desmosomal cadherin desmoglein-2. (A) Endogenously expressed plakophilin-3 is detected as a linear punctate pattern along cell-cell contacts, which is reminiscent of the typical expression pattern of desmosomal components. Note the bright fluorescent speckles in the nucleus, which are plakophilin-3-specific. (B-D) Double immunofluorescence for desmoglein-2 (B) and overexpressed plakophilin-3 (C) in HCT8/E8 cells transfected with a plasmid encoding plakophilin-3. (D) Merged image.

1999; Schmidt et al., 1997). The mechanism whereby the plakophilins are transported into the nucleus is still unclear, as these proteins lack obvious nuclear localization signals. Using green fluorescence protein-tagged human plakophilin-1 and fragments thereof, Klymkowsky (1999) investigated the nuclear accumulation of these proteins. Full-size plakophilin-1 accumulated almost exclusively in cell nuclei, both in Xenopus embryos and in epithelial Xenopus A6 cells, and was often

Fig. 7. Nuclear localization of plakophilin-3 by immunofluorescence in methanol-fixed HCT8/E8 cells (A,B) or paraformaldehyde-fixed HaCaT cells (C,D). Cell nuclei were stained with DAPI. (A) Detection of plakophilin-3 as spherical nuclear particles. Due to focusing at the nuclear level, desmosome-associated plakophilin-3 is not visible. (B) Pre-incubation of the antibody with the antigenic peptide abrogates completely the detection of nuclear plakophilin-3. (C) Immunofluorescence detection of nuclear plakophilin-3, clearly leaving the nucleoli unstained. (D) After incubation with secondary antibodies only, no specific signals were detected.
Plakophilin-3, a novel Armadillo-like protein

Onderzoek in de Industrie. J.v.H. and F.N. are postdoctoral fellows with the Fund for Scientific Research-Flanders, and F.v.R. is a Research Director with the Fund for Scientific Research-Flanders.

REFERENCES


Herrenknecht, K., Ozawa, M., Eckerskorn, C., Lottspeich, F. and Lenter, M.

We are grateful to B. Gilbert and E. Vanden Eynde for technical assistance. We thank Dr C. Buys and Dr L. de Leij (University of Groningen, The Netherlands) for providing the GLC34 cell line, and Dr J. Vilcek for providing the FS4 cell line. Dr J. Vandeckervroote is acknowledged for providing the plakophilin-3 specific peptide. We are grateful to M. Klymkowsky for sharing unpublished data. This work was supported by the Geconcerteerde Onderzoeksacties, University of Gent; the Fund for Scientific Research-Flanders; and Algemene Spaar- en Lijfrenteekas, Belgium. S.B. was supported by the Vlaams Instituut voor de bevordering van het Wetenschappelijk-Technologisch

present in similar nuclear structures, as reported here for plakophilin-3. Also according to Klymkowsky (1999), both the amino-terminal head domain and the carboxy-terminal Armadillo repeat domain can enter nuclei, though the amino-terminal domain and the full-size polypeptide were present mainly in the nucleus of Xenopus embryos, while the Armadillo domain also localized in the cytoplasm with enrichment at the cell cortex and cell-cell contacts.

The occurrence of the novel plakophilin-3 in both desmosomes and nuclei of epithelial cells may suggest that this Armadillo protein is involved in signal transduction pathways between the plasma membrane and the nucleus, besides a mechanical role in cell-cell attachment by desmosomes. Such a role is reminiscent to the one well-documented for beta-catenin, an Arm protein present in adherens junctions but also involved in the Wnt signaling pathway (reviewed by Willert and Nusse, 1998). In the nucleus, beta-catenin associates with LEF-1/TCF transcription factors and modulates gene transcription (Behrens et al., 1996; Huber et al., 1996; Molenaar et al., 1996). Nevertheless, injection of full-size plakophilin-1 mRNA in Xenopus embryos had no obvious effect on early embryonic development, indicating that at least plakophilin-1 is not involved in the Wnt-like pathway in the early Xenopus embryo (Klymkowsky, 1999). The injection of mouse pl120cat isoform 1B into ventral Xenopus blastomeres was also reported not to mimic the Wnt pathway (Geis et al., 1998). Therefore, it is still unclear in which signaling pathways the plakophils are involved, if any.

We are grateful to B. Gilbert and E. Vanden Eynde for technical assistance. We thank Dr C. Buys and Dr L. de Leij (University of Groningen, The Netherlands) for providing the GLC34 cell line, and Dr J. Vilcek for providing the FS4 cell line. Dr J. Vandeckervroote is acknowledged for providing the plakophilin-3 specific peptide. We are grateful to M. Klymkowsky for sharing unpublished data. This work was supported by the Geconcerteerde Onderzoeksacties, University of Gent; the Fund for Scientific Research-Flanders; and Algemene Spaar- en Lijfrenteekas, Belgium. S.B. was supported by the Vlaams Instituut voor de bevordering van het Wetenschappelijk-Technologisch.
Kozak, M. (1997). Recognition of AUG and alternative initiator codons is augmented by G in position +4 but is not generally affected by the nucleotides in positions +5 and +6. EMBO J. 16, 2482-2492.