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Cloning and characterization of a new *armadillo* family member, p0071, associated with the junctional plaque: evidence for a subfamily of closely related proteins

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SUMMARY

Cell contacts of the adherens type are organized around transmembrane proteins of the cadherin family. Whereas the extracellular domains mediate homophilic interactions between cadherins of neighbouring cells the cytoplasmic domains organize a set of proteins into the junctional plaque. Among these junctional plaque proteins are members of the *armadillo* gene family, β-catenin, plakoglobin (γ-catenin), B6P/plakophilin and p120. These proteins are assumed to play a key role in cell cell signalling through intercellular junctions.

Here we report cloning of a cDNA encoding a new *armadillo* family member, p0071, closely related to p120 and B6P/plakophilin and more distantly related to armadillo, plakoglobin, β-catenin and other members of the gene family. The deduced amino acid sequence encodes a basic protein of 1,211 amino acids with a central armadillo repeat region which is conserved in sequence and organization of its ten individual motifs between p120, B6P/plakophilin and p0071. In contrast the end domains of the three proteins are variable in size and sequence. The RNA coding for p0071 is expressed in all tissues examined. Using antibodies generated against the *armadillo* repeat region of the protein we show that p0071 is localized at cell-cell borders and is expressed in the desmosomal plaque of some cultured epithelial cells. The protein seems to be an accessory component of the desmosomal plaque as well as of other adhesion plaques and might be involved in regulating junctional plaque organization and cadherin function.

Our data provide evidence for a subfamily of *armadillo* related proteins that share not only structural features but also have in common their localisation in the junctional plaque. We therefore suggest that family members exert similar functions and might be involved in cell signalling through cell contacts.

Key words: Armadillo, p120, B6P/plakophilin, Desmosome, Adherens junction

INTRODUCTION

Many of the adhesive interactions that connect adjacent cells and their cytoskeletal elements go far beyond simple mechanical linkage. Proteins that were identified originally as linker molecules at the sites of cellular contacts have newly recognized functions in signal transduction pathways and control cell behaviour during development.

Two main categories of adhering junctions have been distinguished primarily based on the filament system to which they attach. Cell-cell junctions that anchor actin filaments include the zonula, fascia and punctum adhaerens summarized as adherens junctions whereas desmosomes (maculae adhaerentes) are linked to the intermediate filament network (for review see Schmidt et al., 1994). Both types of junctions are similar at the ultrastructural level and are composed of distinct but related sets of proteins (for review see e.g. Garrod, 1993; Magee and Buxton, 1991; Schmidt et al., 1994; Schwarz et al., 1990).

Cell adhesion is mediated by members of the cadherin family of proteins which are Ca^{2+} dependent homophilic adhesion molecules. Whereas adherens junctions contain the classical E- and N-cadherins the desmosomal cadherins comprise two protein families, the desmogleins and the desmocollins that are related in sequence to the classical cadherins. Insight into the cadherin mediated adhesive interaction has been recently provided by crystal structure analysis of the extracellular amino-terminal repeat of N-cadherin (Shapiro et al., 1995). A model deduced from this study suggests that cadherins form dimers in the plane of the membrane. Dimers from one cell bind to those from opposing cells by relatively weak interactions. By forming a zipper the strength of the intercellular interactions is increased strongly.

The intracellular domains of the cadherins interact with a set of cytoplasmic proteins called α-, β-, and γ-catenins in adherens junctions (Kemler, 1993) and desmoplakin and plakoglobin in desmosomes (for review see Magee and Buxton, 1991; Schmidt et al., 1994). These proteins represent obligatory constituents of the junctional plaques. Interaction of cadherins with these proteins is essential for the adhesive interactions (Kintner, 1992). Based on immunological data it has

been suggested that γ-catenin and plakoglobin are identical. α-Catenin is related to vinculin and has been implicated in linking the actin cytoskeleton to the adherens junctions. Desmoplakin shares sequence homology in its carboxy terminal domain to plectin and BPAG1 and seems to be involved in linking the intermediate filament network to desmosomes (Hatzfeld et al., 1994; Kouklis et al., 1994; Stappenbeck et al., 1993; Stappenbeck and Green, 1992).

β-Catenin and plakoglobin are closely related to the *Drosophila* segment polarity gene product armadillo (McCrea et al., 1991; Peifer et al., 1992) which is a component of the wingless signal transduction pathway. Armadillo resembles its vertebrate homologs in that it is a component of *Drosophila* adherens junctions (Peifer, 1993; Peifer et al., 1993). Based on these findings a model was proposed which combines armadillo's localization at the junctional plaque with its signalling activity (Peifer et al., 1994b,c; Peifer, 1995). According to this model the junctional form of armadillo is inactive in signal transduction whereas the cytoplasmic form activates a so far unknown effector. The intracellular localization of armadillo is regulated through wingless.

In vertebrates some developmental signals of wnt-1 are transduced through plakoglobin and β-catenin (Funayama et al., 1995; Karnovsky and Klymkowsky, 1995; McCrea et al., 1993) and this signalling activity is regulated by cadherins (Fagotto et al., 1996; Karnovsky and Klymkowsky, 1995). An effect of wnt-1 on plakoglobin, β-catenin and cell adhesion has also been described in cultured epithelial cells (Bradley et al., 1993; Hinck et al., 1994a,b).

Additional members of the *armadillo* multigene family include p120 (Reynolds et al., 1992) and the recently cloned desmosomal band 6 protein (B6P) (Hatzfeld et al., 1994), also named plakophilin1 (Heid et al., 1994). B6P was described as a component of desmosomes in stratified and complex epithelia (Kapprell et al., 1988), that strongly interacts with keratins in vitro (Hatzfeld et al., 1994; Kapprell et al., 1988). It had been suggested that the protein might be involved in ordering keratin filaments and regulating their interactions with the desmosomal plaque (Hatzfeld et al., 1994). p120 was originally identified as a major tyrosine phosphorylation substrate of the src kinase (Kanner et al., 1991; Reynolds et al., 1992) which is enriched in adherens junctions (Tsukita et al., 1991). Since tyrosine phosphorylation of p120 correlates with a transformed phenotype it was assumed to play a role in growth control. In addition to being tyrosine phosphorylated by src, p120 is phosphorylated upon stimulation of growth factor receptor tyrosine kinases (PDGF, EGF, CSF-1) and may thus be a component of mitogen stimulated and receptor tyrosine kinase signalling pathways (Downing and Reynolds, 1991; Kanner et al., 1991). More recently it has been shown that p120 is a component of adherens junctions that directly binds to E-cadherin (Aghib and McCrea, 1995; Daniel and Reynolds, 1995; Reynolds et al., 1994; Shibamoto et al., 1995; Staddon et al., 1995).

Sequence characteristics of *armadillo* family members are a series of imperfect 45 amino acid repeats (Peifer et al., 1994a; Peifer and Wieschaus, 1990). Based on this modular composition of the *armadillo* family members it has been suggested that the individual motifs or couples of these motifs function as protein modules in signalling cascades establishing protein-protein interactions (Pawson, 1995).

Several findings suggested the existence of additional members of the *armadillo* multigene family. Thus, several human expressed sequence tag (EST) sequences contained short stretches of armadillo related motifs with the closest homology to p120 (Peifer et al., 1994a) and B6P/plakophilin1. In addition western blot experiments with antibodies against p120 (Reynolds et al., 1994; Staddon et al., 1995) and B6P/plakophilin1 (Heid et al., 1994) revealed the presence of several immunologically related proteins. Peptide map analysis (Reynolds et al., 1994) or partial amino acid sequencing (Staddon et al., 1995) of p120 related proteins showed that these proteins are either splice variants of p120 or closely related proteins.

Based on these observations we were interested in identifying and characterizing additional members of the *armadillo* multigene family closely related to p120/B6P/plakophilin1. Here we report cloning of a cDNA based on EST number 00071 encoding a novel member of the gene family. The cDNA sequence predicts a protein of 1,211 amino acids with a molecular mass of 135 kDa. It shares the highest degree of sequence conservation over its central armadillo repeat region with p120 and B6P/plakophilin1 and is more distantly related to other members of the gene family. The protein was predominantly detected in the epithelial compartment of tissues where it localized at cell-cell borders. In some cultured epithelial cells colocalization with desmoplakin in the junctional plaque of desmosomes was observed. We conclude that p0071, p120 and B6P/plakophilin1 are members of a subfamily of armadillo related proteins.

MATERIALS AND METHODS

Preparation of RNA and rt-PCR

RNA from A431, HeLa cells and mouse muscle was prepared by the LiCl/urea extraction method (Auffrey and Rougeon, 1980). Total RNA from all other mouse tissues was prepared using the RNeasy kit (Qiagen GmbH, Hilden, Germany).

First strand cDNA synthesis for rt-PCR was performed from total RNA using the first strand synthesis kit from Pharmacia (Pharmacia Biotech, Uppsala, Sweden); 1.5 μg of RNA were used in each assay.

cDNA cloning and sequence analysis

A partial cDNA clone of p0071 was obtained by PCR with the following oligonucleotides deduced from the cDNA sequence of EST 00071 (accession no. M62015): 0071/1: CTG AGT TGC CTG AGG TCA TTC ACA TGC; 0071/2: GGT TGA GAG AGC ATC TCG AAT GAA TGT CAT. An oligo dT- and random primed cDNA library in λZapII from human brain mRNA (Stratagen, La Jolla, CA) was used as template. After confirming that the cDNA was represented in the library we used the specific primers given above in combination with T3 and T7 primers to amplify cDNA ends. Two sets of amplification were necessary to obtain the complete coding sequence. Primers used in the second set of PCRs were as follows: 0071/3: CCA GTG TCT TGT CTG ATG AGA CCA TGG C; 0071/4: TTG CCT GAA CAG ATG GGA ACT GGT GC. PCR conditions were: 5 minutes denaturation at 98°C followed by 35 cycles of 1 minute denaturation at 94°C, 2 minutes annealing at 55°C, 2 minutes elongation at 72°C. Taq polymerase (MBI Fermentas GmbH, St Leon-Roth, Germany) was added after the initial denaturation step. PCR products were analysed on agarose gels, relevant products eluted using the Qiaquick gel extraction kit (Qiagen GmbH, Hilden, Germany) and cloned into the TA PCR™II vector (Invitrogen BV, NV Leek, The Netherlands) for sequence analysis.

To obtain a partial mouse cDNA clone the following oligonucleotides deduced from the human sequence were used in an rt-PCR on total RNA isolated from mouse tissues: PCR 1: 0071/5: CTC TAT TCA CCA GAA CAG ACA TCT CTC CAT G and 0071/2 (see above); PCR 2: 0071/6: GTA AGG GAG CTT GTT ACA GGA GTT CTT TGG and 0071/7: CAC CTC AAT CTG AAC AAG GTT AG.

5′-RACE was performed using a 5′/3′ RACE kit (Boehringer Mannheim, Germany) according to the attached protocol. Oligonucleotides used in the RACE-protocol were as follows: (1) 0071/8: CAT GGA GAG ATG TCT GTT CTG GTG AAT AGA G; (2) 0071/9: GTA TTT GGC ACG TCT GTT GAT CTC CAA GG; (3) 0071/10: CCA AGC CTA CAT CTT TCT AGC TGA CTG G; 0.95 μg of poly(A) RNA was used as template.

Sequencing of both strands was performed using the T7 sequencing kit (Pharmacia Biotech). Critical regions were analysed on 40% formamide gels.

Southern blot analysis

PCR products separated on 1% agarose gels were transferred to a nylon membrane by capillary blotting and cross-linked to the membrane by exposure to UV (Stratalinker, Stratagene, La Jolla, CA). Filters were prehybridized and hybridized in DIG easy hyb (Boehringer-Mannheim, Germany). A DIG labeled probe was generated by PCR. Washing and immunological detection procedures with DIG alkaline phosphatase conjugated antibody were as described by the manufacturer. Membranes were developed in NBT/BCIP substrate solution.

Northern analysis

Total RNA (30 μg) from A431 and HeLa cells was separated by electrophoresis in formamide gels, transferred to positively charged nylon membranes by capillary blotting and cross-linked to the membrane by baking for 30 minutes at 120°C. The membrane was prehybridized and hybridized in 50% formamide, 5× SSC, 10 mM Tris-HCl, pH 7.5, 1% SDS, 1× Denhardt's, 10% dextransulfate, 1 mM EDTA and 100 μg/ml lax sperm DNA. A 720 bp fragment was ^{32}P labeled using the multi prime DNA labelling system (Amersham Buchler, Braunschweig, Germany). Hybridization was carried out overnight at 42°C. The membrane was washed in 2× SSC, 0.1% SDS for 15 minutes at room temperature, twice in 2× SSC, 0.1% SDS at 50°C and twice in 0.2× SSC, 0.1% SDS at 65°C, and exposed to Kodak XAR-5 X-ray films.

Cloning and expression of 0071 repeat domain

Oligonucleotides containing a *Bam*HI (GCA CCA CAG GAT CCC CAT CAA TAG ACA GC) and *Hin*dIII restriction site and a stop codon (CGA TAT TGC CAT AAG CTT TAC AAG ACC TGG GCT GC) were used in an rt-PCR on total RNA from A431 cells. PCR products were purified and cloned into PCR™II-vector, the relevant fragment generated by restriction digestion with *Bam*HI and *Hin*dIII, gel purified and cloned into the pINDU (Hatzfeld and Weber, 1990) and the pRSET A (Invitrogen BV, NV Leek, The Netherlands) expression vectors.

Antibodies

An inclusion body preparation (Hatzfeld and Weber, 1990) of p0071 repeat domain expressed in pINDU was used to immunize a rabbit. For affinity purification of the antibody p0071 expressed in pRSET was partially purified on Ni-NTA resin under denaturing conditions according to the manufacturer's protocol (Qiagen GmbH, Hilden, Germany), separated on SDS gels, transferred to nitrocellulose and the relevant band excised. After blocking in 5% BSA in TBST (10 mM Tris-HCl, 140 mM NaCl, 0.1% Tween-20) the filter strips were incubated overnight at 4°C with the antiserum, washed twice in TBST containing 1% Triton X-100 and bound antibody eluted in 0.2 M glycine-HCl at pH 2.7. After immediate neutralization, BSA was added to 0.5% and the antibody stored at 4°C. In some cases nitrocellulose with epidermal keratins or a cross-reacting band from HaCaT-cell lysates were subsequently used to remove cross-reacting antibodies.

A monoclonal antibody against desmoplakin (Osborn and Weber, 1985) was kindly provided by Dr Mary Osborn. An antibody against E-cadherin was from Transduction Laboratories (Affiniti, UK).

Secondary antibodies were: FITC-conjugated rabbit anti-mouse immunoglobulins (Dako GmbH, Hamburg, Germany), rhodamine-conjugated affinipure goat anti-rabbit immunoglobulins (Dianova, Hamburg, Germany) for immunofluorescence analysis and affinity isolated alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma Aldrich Chemie GmbH, Deisenhofen, Germany) for western blot analysis.

Cells and immunocytochemistry

The bovine kidney epithelial cell line MDBK, human vulval carcinoma cell line A431, human cervical carcinoma cell line HeLa SS6 and the spontaneously transformed keratinocytes of the HaCaT cell line (Boukamp et al., 1988) were maintained in DMEM containing 10% foetal calf serum. Cells grown on coverslips were fixed in 100% methanol at −10°C for 5 minutes and air dried. After a short wash in PBS containing 0.05% Tween-20 cells were overlaid with the primary antibody for one hour at 37°C. Culture supernatant containing desmoplakin antibody was used undiluted, affinity purified p0071 antibody was diluted 1+1 in PBS containing 1% BSA. After three 5 minute washes in PBS the FITC- or rhodamine-conjugated secondary antibodies were applied for one hour at 37°C at a 1:60 and 1:50 dilution, respectively. After three 5 minute washes coverslips were mounted in Mowiol and viewed using a Zeiss Axiophot microscope.

Gel electrophoresis and western blot analysis

Whole cell lysates were prepared by rapidly replacing the medium with hot sample buffer. Triton soluble and insoluble fractions were prepared as described by Shibamoto et al. (1995). SDS gel electrophoresis was performed according to standard protocols. Proteins were transferred to nitrocellulose using the semi-dry transfer procedure (Khyse-Anderson, 1984). Filters were blocked in TBST containing 5% BSA or non fat dry milk, incubated for two hours at room temperature or at 4°C overnight with a 1:1,000 or a 1:250 dilution of the affinity purified antibody, washed in TBST, TBST containing 0.5% Triton X-100 and TBST. The secondary antibody was applied for 1-2 hours followed by the same washing procedure. Filters were developed by incubation in BCIP/NBT or for chemiluminescence detection in CDP-Star (Tropix, Bedford, MA). For competition assays purified p0071 repeat domain was added together with the primary antibody.

RESULTS

Cloning and sequence analysis of p0071

EST 00071 (accession no. M62015) shows sequence similarity to p120 and B6P/plakophilin1 and contains two complete armadillo related repeats. To clone the entire coding sequence of this protein we used oligonucleotides deduced from the EST-sequence combined with library specific primers (T3 and T7) to amplify the 5′ and 3′-ends from a human brain cDNA library. In the first set of PCRs two fragments of 1,200 bp (primers 0071/1 + T7) and 1,500 bp (primers 0071/2 + T3) were obtained. Since these fragments did not include the entire coding sequence a second set of PCR reactions was performed using primers deduced from sequence information obtained in the first set of PCRs. In order to exclude the possibility that the clones contained library artefacts due to random ligation of unrelated fragments we performed 5′-RACE on RNA isolated from A431

cells to confirm and extend the sequence information of the 5′-end. We also used internal primers on RNA isolated from A431 and HeLa cells and confirmed the entire coding sequence except for a GTA codon in position 1,480 which was not detected in several independent clones derived from the brain library. In addition, two splice variants were detected in A431 and HeLa cells: the larger variant which was the only form amplified from the brain cDNA library represented the minor isoform in both epithelial cell lines. Here a smaller splice variant lacking nucleotides 3,268 to 3,396 was predominantly detected. Fig. 1 gives the nucleotide and deduced amino acid sequence of the human p0071 clone. The cDNA sequence spans 3,907 nucleotides, the presumptive methionine initiator codon is embedded in a sequence favourable for translation initiation (Kozak, 1989). The deduced amino acid sequence predicts a protein of 1,211 amino acids (1,158 amino acids for the smaller splice variant) with a calculated molecular mass of 135 kDa (130 kDa) and an isoelectric point of 9.7 (9.72).

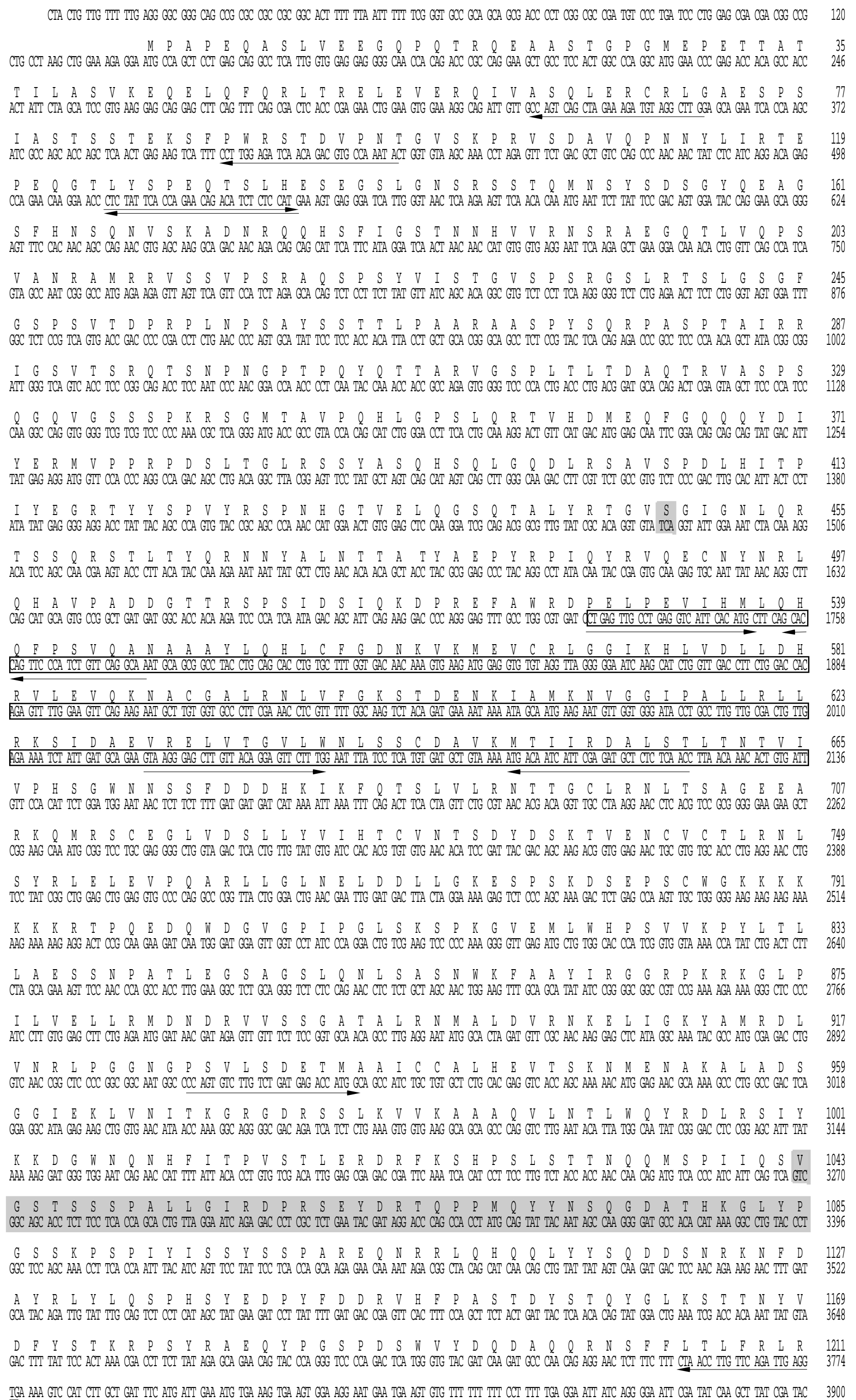

Fig. 1. Complete nucleotide and deduced amino acid sequence of human p0071. Valine in position 448 is detected in clones derived from HeLa and A431 cells but not in clones derived from the human brain cDNA library. Shaded amino acids are missing in a shorter splice variant from HeLa and A431 cells. The boxed region comprises the original EST-sequence. Arrows indicate the positions of cloning primers. These sequence data are available from EMBL GenBank under accession number X81889.

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101 SKPRVSDAVQPNNYLIRTEPEQGTLYSPEQTSLHESEGSLGNSRSSTQMNSYSDSGYQEAGSFHNSQNVSKADNRQQHSFIGSTNNHVVRNSRAEGQTLV
|||||||||||||||||||||||||||||||| | ||| | || | ||| ||||| |||||||||
1 SEGSLGNSRSSTQMNSYSDSGYQEAGSFHNSQTVNKADSR.QHPFTGSTSNHVVRTSRAEGQTLV

201 QPSVANRAMRRVSSVPSRAQSPSYVISTGVSPSRGSLRTSLGSGFGSPSVTDPRPLNPSAYSSTTLPAARAASPYSQRPASPTAIRRIGSVTSRQTSNPN
|||||||||||||||||||||||||.||||||||||||||||||||||||||.||||||||||.||||.|||||||||||||||:||:||||||||||||
65 QPSVANRAMRRVSSVPSRAQSPSYVTSTGVSPSRGSLRTSLGSGFGSPSVTDSRPLNPSAYSSSTLPAQRAASPYSQRPASPTAVRRVGSVTSRQTSNPN

301 GPTPQYQTTARVGSPLTLTDAQTRVASPSQGQVGSSSPKRSGMTAVPQHLGPSLQRTVHDMEQFGQQQYDIYERMVPPRPDSLTGLRSSYASQHSQLGQD
||.||||||.|||||||||||||||||||||||||||||||||||||||||||||||||||:|||||||||||||||||||||||||||||||||||||:
165 GPVPQYQTTTRVGSPLTLTDAQTRVASPSQGQVGSSSPKRSGMTAVPQHLGPSLQRTVHDMDQFGQQQYDIYERMVPPRPDSLTGLRSSYASQHSQLGQE

401 LRSAVSPDLHITPIYEGRTYYSPVYRSPNHGTVELQGSQTALYRTGVSGIGNLQRTSSQRSTLTYQRNNYALNTTATYAEPYRPIQYRVQECNYNRLQHA
||||||||||||||||||||||||||||||||||||||||||||||  |||||||||||| .||||||||||||.||||::|||:|||||||.||||||.
265 LRSAVSPDLHITPIYEGRTYYSPVYRSPNHGTVELQGSQTALYRTGSVGIGNLQRTSSQRQALTYQRNNYALNTAATYADAYRPVQYRVQECSYNRLQHT

501 VPADDGTTRSPSIDSIQKDPREFAWRDPELPEVIHMLEHQFPSVQANAAAYLQHLCFGDNKVKMEVCRLGGIKHLVDLLDHRVLEVQKNACGALRNLVFG
.|||||.|||||||||||||    |||||||||||||:||||||||||||||||||||||||||||:|||||||||||||||||||||||||||||||||
365 GPADDGATRSPSIDSIQKDP....WRDPELPEVIHMLQHQFPSVQANAAAYLQHLCFGDNKVKMEVYRLGGIKHLVDLLDHRVLEVQKNACGALRNLVFG

601 KSTDENKIAMKNVGGIPALLRLLRKSIDAEVRELVTGVLWNLSSCDAVKMTIIRDALSTLTNTVIVPHSGWNNSSFDDDHKIKFQTSLVLRNTTGCLRNL
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
461 KSTDENKIAMKNVGGIPALLRLLRKSIDAEVRELVTGVLWNLSSCDAVKMTIIRDALSTLTNTVIVPHSGWNNSSFDDDHKIKFQTSLVLRNTTGCLRNL

701 TSAGEEARKQMRSCEGLVDSLLYVIHTCVNTSDYDSKTVENCVCTLRNLSYRLELEVPQARLLGLNELDDLLGKESPSKDSEPSCWGKKKKKKKRTPQED
.|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||.|||||||||||||||||||||||||||||||
561 SSAGEEARKQMRSCEGLVDSLLYVIHTCVNTSDYDSKTVENCVCTLRNLSYRLELEVPQARLLGLNELHDLLGKESPSKDSEPSCWGKKKKKKKRTPQED

801 QWDGVGPIPGLSKSPKGVEMLWHPSVVKPYLTLLAESSNPATLEGSAGSLQNLSASNWKFAAYIRGGRPKRKGLPILVELLRMDNDRVVSSGATALRNMA
|||||||||||||||||||||||||||||||||||||||||||||||||||||||:|||||||||:: .| ||||||||||||||||||||.||||||||
661 QWDGVGPIPGLSKSPKGVEMLWHPSVVKPYLTLLAESSNPATLEGSAGSLQNLSAGNWKFAAYIRAAVRKEKGLPILVELLRMDNDRVVSSVATALRNMA

901 LDVRNKELIGKYAMRDLVNRLPGGNGPSVLSDETMAAICCALHEVTSKNMENAKALADSGGIEKLVNITKGRGDRSSLKVVKAAAQVLNTLWQYRDLRSI
||||||||||||||||||||||||||||:|||||:|||||||:.||||||||||||||||||.|||||||||||||||||||||||||||||||||||||
761 LDVRNKELIGKYAMRDLVNRLPGGNGPSILSDETVAAICCALRKVTSKNMENAKALADSGGIKKLVNITKGRGDRSSLKVVKAAAQVLNTLWQYRDLRSI

1001 YKKDGWNQNHFITPVSTLERDRFKSHPSLSTTNQQMSPIIQSGSSKPSPIYISSYSSPAREQNRRLQHQQLYYSQDDSNRKNFDAYRLYLQSPHSYEDPY
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||.||.:||||||||||:||||||
861 YKKDGWNQNHFITPVSTLERDRFKSHPSLSTTNQQMSPIIQSGSSKPSPIYISSYSSPAREQNRRLQHQQLYY.QDDSTRKTLDAYRLYLQSPRSYEDPY

1101 FDDRVHFPASTDYSTQYGLKSTTNYVDFYSTKRPSYRAEQYPGSPDSWVYDQDA.QQRNSFFLTLFRLR*. 1150
|||||||||||||||||||||||||||||||||||||||||||||||||||: |..|:|||| ||
960 CDDRVHFPASTDYSTQYGLKSTTNYVDFYSTKRPSYRAEQYPGSPDSWVYDGDGCLLRGAFLLTLFGLRGSPSC* 1009
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Fig. 2. Amino acid sequence alignment of the partial mouse p0071 clone with the corresponding region of human p0071. The human sequence is on the top lines. Vertical lines indicate identical amino acids, colons indicate conservative replacements. The sequence identity is 93.7% over 1,034 amino acid residues.

Secondary structure analysis shows for the central region a number of short α-helical stretches alternating with β-turns. Several putative phosphorylation sites on serine, threonine and tyrosine are predicted in the p0071 sequence.

To facilitate the analysis of p0071 expression we used our oligonucleotides on RNA isolated from mouse tissues to clone two partial mouse cDNA fragments. One fragment spans nucleotides 514 to 2,117 of the human sequence, the other fragment includes 1,647 base pairs corresponding to nucleotides 2,032 to 3,679 of the human clone. Fig. 2 shows that p0071 is highly conserved between man and mouse (93.7% identity over 3,164 residues).

p0071 is a novel member of the *armadillo* multigene family

The p0071 sequence covers three distinct domains: The amino and carboxy-terminal domains (amino acid residues 1 to 508 and 988 to 1,211, respectively) lack significant homology to any protein in the data base. The central domain of 480 amino acids shows strong homology to p120 and B6P/plakophilin1 and is more distantly related to other members of the *armadillo* multigene family such as armadillo, plakoglobin, β-catenin, pendulin/importin (Küssel and Frasch, 1995; Weis et al., 1995) and APC (Rubinfeld et al., 1993; Su et al., 1993). Fig. 3 aligns the armadillo repeat regions of p0071, p120 and B6P. Individual repeats are defined according to the criteria of Peifer et al. (1994a). Sequence comparison reveals that the three proteins share an identical organisation of their armadillo related motifs. All three proteins contain ten complete repeats according to the current definition of individual repeats (Peifer et al., 1994a). The repeat domain contains three short insertions that are conserved between the three proteins suggesting that these inserts might represent protein binding sites that have been conserved during evolution.

Fig. 4 gives a schematic presentation of the number and organisation of armadillo motifs in various members of the gene family. Comparison of the domain organisation of these proteins reveals that p0071, p120 and B6P/plakophilin share an identical subunit organisation that differs from the organisation of other members of the protein family suggesting that these three proteins constitute a subfamily of closely related proteins.

Computer analysis of sequence conservation between the repeat regions of various members of the gene family places

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0071    510   SPSIDS-IQKD-PR-EFAWR-DPEL--PEVIHMLEH--Q-FPSV---QANAAAY
p120    347   LASLDS-LRKG-MPPPSNWR-QPEL--PEVIAMLGF--RLDA-V---KSNAAAY
B6P     223   LSFGHS-RASS--KICSEDIECSGLTIPKAVQYLSS--Q-DEKY---QAIGAYY

0071    552   LQHLCF-GDNK-VK-MEVCR-LGGI--KHLVDLLDH--RVLEV----QKNACGA
p120    390   LQHLCY-RNDK-VK-TDVRK-LKGI--PILVGLLDH--PKKEV----HLGACGA
B6P     268   IQHTCF-QDES-AK-QQVYQ-LGGI--CKLVDLLRS--PNQNV----QQAAAGA

0071    594   LRNLVFGKSTDENK-IAMKN-VGGI--PALLRLLRK--SIDAEV---RELVTGV
p120    432   LKNISFGRDQD-NK-IAIKN-CDGV--PALVRLLRK--ARDMDL---TEVITGT
B6P     310   LRNLVF-RSTT-NK-LETRR-QNGI--REAVSLLRR--TGNAEI---QKQLTGL

0071    639   LWNLSS-CDA--VK-MTII--RDAL--STLTNTVIV--PHSGWN---NSSFDDD---HKIKFQTSLVLRNTTGC
p120    476   LWNLSS-HDS--IK-MEIV--DHAL--HALTDEVII--PHSGWE---REPNEDC---KPRHIEWESVLTNTAGC
B6P     353   LWNLSS-TDE--LK-EELI--ADAL--PVLADRVII--PFSGWC----DGNSNM---SREVVDPEVFF-NATGC

0071    690   LRNLTSAGEEA-RKQMRS---CEGL--VDSLLYVIH--TCV------NTSDYDS---KTVENCVCT
p120    534   LRNVSSERSEA-RRKLRE---CDGL--VDALIFIVQ--AEI------GQKDSDS---KLVENCVCL
B6P     409   LRNLSSA--DAGRQTMRN---YSGL--IDSLMAYVQ--NCE------AASRCDD---KSVENCMCV

0071    746   LRNLSY-RLELEVPQAR----LLGL--NELDDLLGKESPSKDSEP-SCWGKKKK---KKKRTPQEDQWDGVGPI
p120    583   LRNLSY-QVHREIPQA-----ERYQ--EALPTVANSTGP-HAA---SCFGAKKG---KGKKPT-EDPANDTVDF
B6P     457   LHNLSY-RLDAEVPT-------RYR--KLEYNARNAY-PEKSST--GCFSNKSD---K------MMNNNYDCPL

0071    809   PGLSKSP-KGVEMLWH-----PSVVK--PYLTLLAES--SNPAT---LEGSAGS
p120    641   PKR-TSPARGYELLFQ-----PEVVR--IYISLLKES--KTPAI---LEASAGA
B6P     509   PEEETNP-KGSG--WLYH---SDAIR--TYSNLMGKS--KKDAT---LEACAGA

0071    850   LQNLSASNWKFAAYIRGG-RPKRKGL-PILVELLRM--DNDRV----VSSGATA
p120    682   IQNLCAGRWTYGRYIRSA-LRQEKAL-SAIAELLTS--EHERV----VKAASGA
B6P     550   LQNLTASKGLMSSGMSQLIGLKEKGL-PQIARLLQS--GNSDV----VRSGASL

0071    896   LRNMALDVR--NKELIG----KYAM--RDLVNRLPGGNGPSVL-----SDETMAAICC-
p120    728   LRNLAVDAR--NKELIG----KHAI--PNLVKNLPGGQQNSSWNF---SEDTVVSILN-
B6P     597   LSNMSRHPL--LHRVMG----NQVF--PEVTRLLTSHTGNTSN-----SEDILSSACYT

0071    941   ALHEVTSKNMENAKALAD---SGGI--EKLVNITKGRGDRS-SLK---VVKAAAQVL
p120    775   TINEVIAENLEAAKKLRE---TQGI--EKLVLINKS-GNR--SEK---EVRAAALVL
B6P     643   VRNLMASQP-QLAKQYFS---SSML--NNIINLCRS-SA---SPK---AAEAAR-LL
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Fig. 3. Amino acid sequence alignment of p0071 homologs over their armadillo repeat regions. The sequences of p0071, p120 (EMBL GenBank accession number Z17804) and B6P (accession number X79293) are aligned. Each lane shows an individual repeat, the three short inserts between repeats 4 and 5, 5 and 6 and 6 and 7 are on the right side. Identical amino acids are shaded.

p0071, p120 and B6P/plakophilin 1 in a common branch separated from other members of the gene family (Fig. 5) supporting the conclusion that these members constitute a subfamily of the *armadillo* gene family.

RNA expression and tissue distribution of p0071

To assess the length of the human transcripts northern blot analysis was performed on total RNA isolated from A431 and HeLa cells. A single band of ~4.0 kb was detected (Fig. 6), suggesting that size differences between the two splice variants described above are too small to be detected. Since our human cDNA clone spans 3,907 nucleotides we conclude that this clone represents the entire or almost the entire length of the transcript.

To determine the tissue distribution of p0071 we prepared RNA from several murine tissues and tested RNA expression by rt-PCR analysis. The fragment amplified spans 959 bp corresponding to nucleotides 1,084 to 2,123 of the human cDNA sequence. This region includes part of the head domain and the first three armadillo repeats. Two fragments were detected after gel electrophoresis of the PCR products (Fig. 7A): in skin, heart, skeletal muscle, lung, spleen, brain and uterus the smaller fragment was exclusively amplified whereas an additional, slightly larger fragment was isolated from liver, kidney, stomach and intestine RNA. To analyse whether or not both fragments represented p0071 cDNA fragments we performed Southern blot analysis with a p0071 fragment (Fig. 7B). This experiment showed a positive reaction with both fragments indicative of the presence of two splice variants (Fig. 7B). Sequence analysis revealed that the larger clone contained a 20 amino acid insert in the first armadillo repeat (Fig. 7C). Thus splice variants of p0071 were detected in all tissues examined and these isoforms revealed a tissue specific expression pattern.

p0071 is a component of cell contacts

In order to analyse the protein expression and the intracellular localization of p0071 we prepared an antiserum against the armadillo repeat region of p0071. The repeat domain was amplified from A431 RNA and cloned into prokaryotic expression vectors. Protein expressed in pINDU without a tag sequence was used to immunize a rabbit. The antiserum was affinity purified and its specificity was tested on various related protein domains expressed in *E. coli*. Fig. 8 shows the result of an immunoblot analysis with the affinity purified antibody on total extracts of bacteria expressing B6P repeat domain (Fig. 8, lane 2), plakoglobin repeat domain (Fig. 8, lane 3), and p0071 repeat domain in pRSET A or pINDU respectively (Fig. 8, lanes 4 and 5). The antibody gave a strong signal with p0071

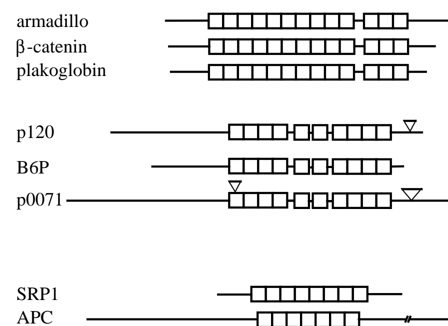

Fig. 4. Schematic representation of the structural composition of armadillo family members. Boxes represent individual armadillo repeats, non repeat regions are shown as lines. Inserts found in splice variants of p120 and p0071 are indicated by ▽ on top of the proteins. Repeats are defined according to the criteria of Peifer et al. 1994a.

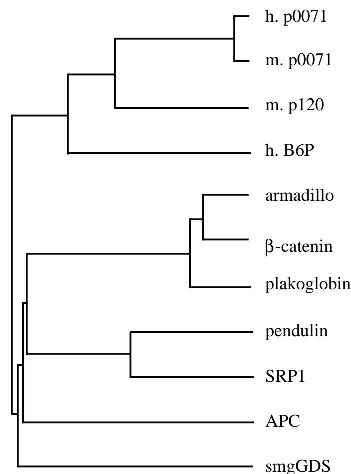

Fig. 5. Dendrogram of the sequence similarity of various armadillo family members. The length of the horizontal connections correlates with the degree of sequence conservation. This alignment was created using the PILEUP TREE algorithm of the HUSAR package (EMBL).

expressed in either the pINDU or the pRSET expression system and did not react with the armadillo repeat domain of any of the related proteins. In addition, the antibody did not recognize p120 which was enriched in desmosomal preparations as shown by immunoblotting with a p120 antibody (data not shown). These findings suggest that this antibody is specific to p0071 and does not cross react with related proteins including p120 and B6P/plakophilin.

The affinity purified p0071 antibody stained predominantly the epithelial component of tissues such as tongue (Fig. 9A-C), skin and intestine in indirect immunofluorescence. The staining was maximal at cell-cell borders although cytoplasmic staining was also present. Strong staining was observed in the biosynthetically active basal and lower suprabasal layers of these tissues and was diminished in the upper strata where the cells undergo terminal differentiation. This staining pattern would be consistent with localization to regions of cell-cell contacts. We therefore explored the intracellular localization of p0071 in cultured epithelial cells such as HeLa and A431 known to express the p0071 RNA (see above). Fig. 10A shows that the antibody gives a punctate staining pattern along the plasma membrane of HeLa cells. Double fluorescence analysis with an antibody against desmoplakin revealed colocalization of p0071 with desmoplakin indicative of a desmosomal localization of p0071 in HeLa cells (Fig. 10A and B) and A431 cells (not shown). The same subcellular distribution of p0071 was observed in the HaCaT keratinocyte cell line (Fig. 10C,D). In contrast in MDBK cells the p0071 antibody stained along most of the cell-cell borders, a staining pattern that resembles E-cadherin staining. Double labeling with an antibody against E-cadherin (Fig. 10E and F) revealed an overlapping localisation of both proteins suggesting that p0071 might be a direct or indirect binding partner of E-cadherin and a component of adherens junctions in this cell line. In all cell lines examined a punctate cytoplasmic staining concentrated around the nucleus was also observed.

Immunoblot analysis of total cellular extracts and Triton soluble and insoluble protein fractions from HeLa cells showed that the p0071 antibody reacts with a protein of 145-150 kDa that was not labelled by the preimmune serum (Fig. 11). This 150 kDa band was not detected when purified p0071 was added in a competition assay although a high background staining was observed here. This probably arises from unspecific binding of the highly charged basic p0071 protein to acidic proteins in the lysate. A molecular mass of 150 kDa is slightly above the calculated molecular mass of 135 and 130 kDa for the two p0071 splice variants detected in HeLa and A431 cells at the RNA level. This small discrepancy could be due either to posttranslational modifications that lead to an increase in the molecular mass or to anomalous migration of the proteins in SDS-gels. The Triton insoluble protein fraction revealed an additional 140 kDa band, not detected in whole cell lysates presumably representing a degradation product of p0071.

DISCUSSION

p0071, p120 and B6P/plakophilin1 constitute a subfamily of the armadillo multigene family

In this study we describe cloning and characterization of a cDNA encoding an additional member of the *armadillo* multigene family. Sequence characteristics of all members of this gene family are a series of imperfect ~45 amino acid repeats that were first detected in armadillo (Peifer and Wieschaus, 1990) and more recently in a number of additional proteins such as the junctional plaque proteins plakoglobin, β-catenin (McCrea et al., 1991; Peifer et al., 1992), p120 (Reynolds et al., 1992) and B6P/plakophilin (Hatzfeld et al., 1994; Heid et al., 1994), the tumor suppressor gene APC (Rubinfeld et al., 1993; Su et al., 1993), a suppressor of RNA polymerase 1 mutations in yeast (SRP1) (Yano et al., 1994) and related proteins in vertebrates that include the receptor for the nuclear localisation signal, human SRP1α

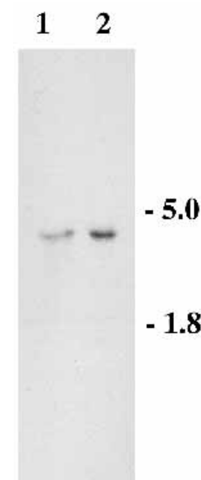

Fig. 6. Northern blot hybridization of p0071 RNA. Total RNA (~30 μg) from HeLa (lane 1) and A431 (lane 2) cells was separated on an agarose-formamide gel and probed as described in Materials and Methods. A single transcript of ~4 kb was detected. Positions of rRNAs are given at the right (in kb).

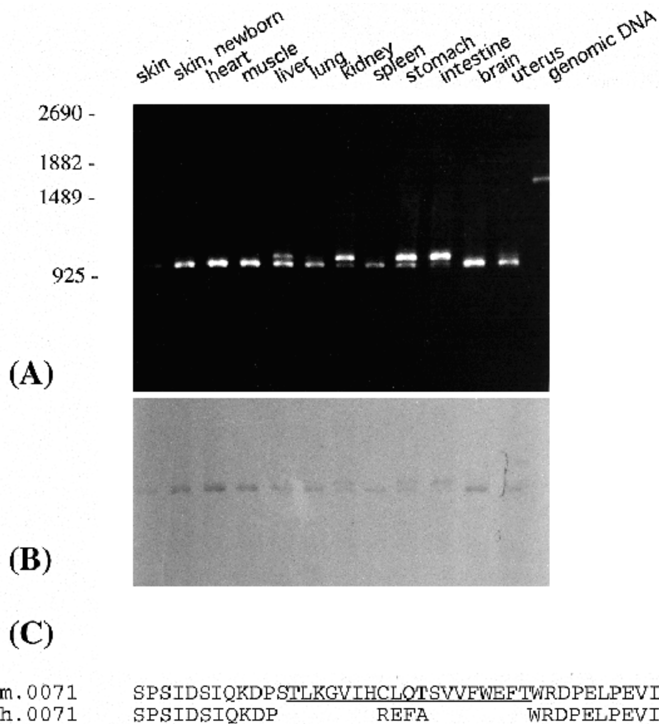

Fig. 7. rt-PCR analysis of p0071 RNA from various mouse tissues. Tissues are given on top of each lane. A 1.5 μg sample of total RNA was used in each experiment to amplify a 959 bp fragment; 2% of the PCR reaction mixture was loaded onto the gel. (A) Ethidium bromide stained gel, (B) Southern blot analysis of the same samples after transfer to a nylon membrane. Two fragments that differ slightly in size were amplified, both reacted with the p0071 specific probe. (C) Amino acid sequence of the 20 amino acid insertion at amino acid 284 of the partial mouse cDNA clone.

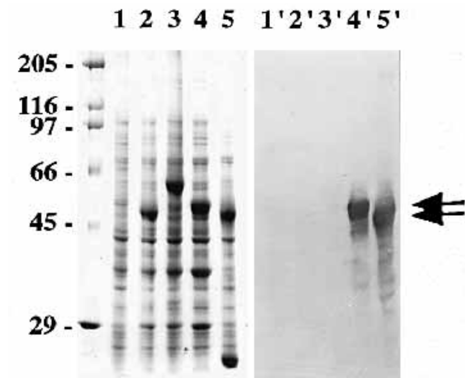

Fig. 8. Specificity of the p0071 antibody. Total extracts of bacteria (lane 1), bacteria expressing the B6P armadillo repeat region (lane 2), the plakoglobin repeat region (lane 3), the p0071 repeat region in pRSET (lane 4) and the p0071 repeat region in pINDU (lane 5) were separated on 10% SDS-gels and stained with Coomassie brillant blue. Molecular masses of the reference proteins are given at the left in kDa. Samples run in parallel were transferred to nitrocellulose and tested with the affinity purified antibody (lanes 1′ to 5′). The antibody reacts with the p0071 repeat domain in pRSET (lane 4′) and in pINDU (lane 5′, arrows) but does not cross react with any of the related protein fragments.

(Weis et al., 1995), Importin1 or pendulin (Küssel and Frasch, 1995), a microtubule associated protein in flagella, PF16 (Smith and Lefebvre, 1996) an exchange factor for ras related small G proteins (smg GDS) (Peifer et al., 1994a) and others. The central armadillo repeat domain of all proteins is flanked by amino- and carboxy-terminal domains that are completely variable in length and in sequence. In addition the number and organisation of the individual motifs varies between members of the gene family. Sequence comparisons reveal that there are several groups of closely related proteins among the members of the *armadillo* gene family. The proteins of one subfamily share not only a higher degree of sequence conservation but also an identical number and organisation of armadillo related motifs. These subfamilies include the *armadillo*, plakoglobin and β-catenin group, the SRP1, pendulin and α-importin group and the p120, B6P/plakophilin and p0071 group. Given the much higher degree of sequence conservation between p0071, p120 and B6P/plakophilin compared to other members of the gene family and their identical subunit organisation we conclude that these proteins constitute a family of closely related proteins. Based on the findings that all three proteins are components of the junctional plaque we suggest that similarities go beyond mere structural features and include some functional characteristics.

Association of p0071 with intercellular junctions

Since p120 and B6P/plakophilin as well as the more distantly related armadillo family members plakoglobin and β-catenin localize to the junctional plaques of desmosomes and adherens junctions we decided to explore the possible relationship between p0071 and cell-cell contacts. p0071 was predominantly detected in the epithelial compartment of those tissues we examined and the antibody localized predominantly to regions of cell-cell contact consistent with a possible association with intercellular junctions. However, the high number of cell junctions in these tissues prevented a distinction between an exclusively junctional localisation or a more general association with the plasma membrane. We therefore used cultured epithelial cells to assess the intracellular localization of p0071. In HeLa, A431 and HaCaT cells p0071 colocalizes with desmoplakin in the junctional plaque of desmosomes indicative of a junctional association in these cells. In contrast in MDBK cells immunostaining was observed along the entire plasma membrane and not restricted to desmosomes. The antibody staining in these cells resembles that of the classical cadherins which are found along the entire lateral surface of cultured cells including the regions of cell-cell junctions. In addition to the staining of cell borders we observed a cytoplasmic staining in all cells that was not diffuse as expected for a soluble protein but showed a punctate pattern associated with the cytoskeleton.

The difference in antibody labelling between MDBK and HeLa, A431 and HaCaT cells may be explained either by different localisation of the antigen in MDBK cells compared to HeLa, A431 and HaCaT cells or by the presence of an additional cell type specific protein in MDBK cells that is closely related to p0071 and cross reacts with the antibody. Although we could show that the antibody does not cross react with several other members of the *armadillo* gene family (see Fig.

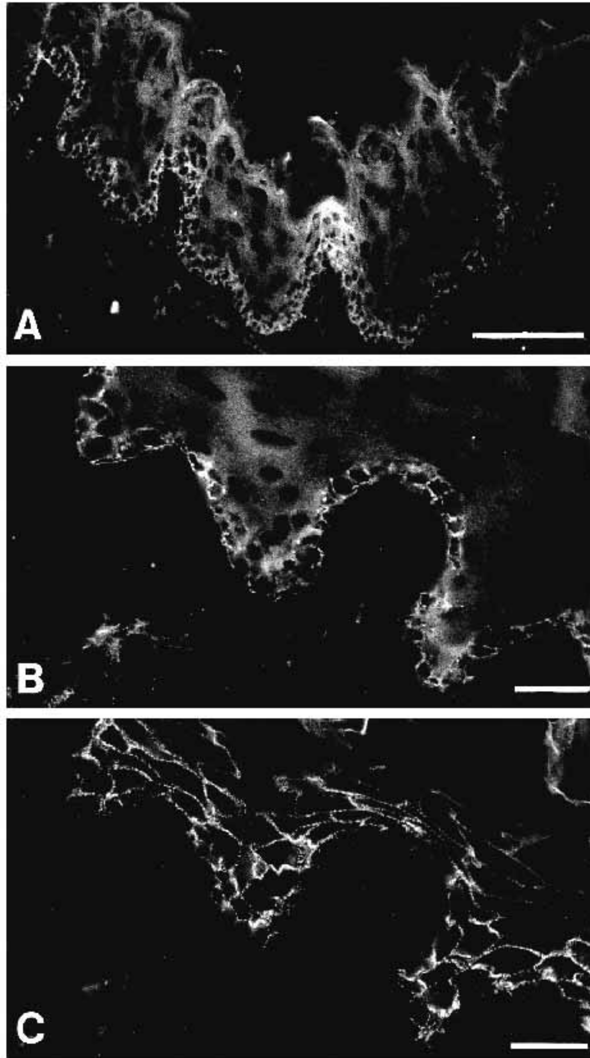

Fig. 9. p0071 antibodies localize to the intercellular borders of epithelial cells in stratified tissues. Indirect immunofluorescence analysis with affinity purified p0071 (A and B) and desmoplakin antibody (C) on rat tongue. The p0071 antibody localizes to the intercellular borders predominantly in the basal cell layers. Bars: in A, 100 μm; in B and C, 25 μm.

8) we cannot exclude the possibility that additional and as yet unidentified members exist that are more closely related to p0071 than the proteins tested and might therefore cross react with the antibody. On the other hand we have shown that several splice variants exist that reveal a tissue specific expression pattern suggesting that these variants might have specific binding partners and as a consequence might also differ in their intracellular localisation. In mouse kidney, stomach and intestine a splice variant containing a 20 amino acid insert was detected in rt-PCR analysis which was not detected in stratified epithelia such as skin. This splice variant is probably also expressed in the kidney derived cell line MDBK but not in HaCaT, A431 or HeLa cells suggesting that the insert might change the binding characteristics of p0071 and as a consequence the localization of p0071.

Our findings on the intracellular localisation of p0071 place this protein among the plaque associated armadillo family members suggesting that p0071 binds to cadherin family members. Interactions between armadillo family members and cadherin family members seem to be mediated through parts of the armadillo repeat regions as demonstrated for plakoglobin (Cowin and Burke, 1996; Sacco et al., 1995) β-catenin (Hülsken et al., 1994b), p120 (Daniel and Reynolds, 1995) and B6P/plakophilin1 (C. Nachtsheim and M. Hatzfeld, unpublished results) suggesting that p0071 also interacts directly with cadherin family members through its armadillo repeat domain.

In spite of its desmosomal localisation p0071 could not be detected in immunoblots of desmosomal fractions isolated from bovine snout epidermis suggesting that this protein is a minor component of desmosomes. Similarly p120 is only a minor component of adherens junctions and the p120/E-cadherin complex represents only part of the entire pool of each molecule (Aghib and McCrea, 1995; Reynolds et al., 1994; Shibamoto et al., 1995; Staddon et al., 1995). Clearly p0071 and p120 interact more tenuously with the cadherin complexes than do the related molecules plakoglobin, β-catenin and B6P/plakophilin1. Given the extremely harsh isolation procedure for desmosomes it seems not surprising that some associated proteins including p0071 may be lost during this procedure.

What are the functions of p0071, p120 and B6P/plakophilin1?

p0071, p120, B6P/plakophilin1 share with each other and armadillo, plakoglobin and β-catenin not only sequence characteristics but also a localisation pattern strongly suggestive of a role in regulating cell adhesion.

Interaction with the catenin complex is essential for cadherin function (Kintner 1992). β-Catenin seems to be involved in regulating adhesive interactions and its function is controlled by tyrosine phosphorylation through epidermal growth factor receptor (Hoschuetzky et al., 1994) or src kinase (Behrens et al., 1993).

The precise function of p120 is not yet known but, interestingly, the protein is tyrosine and serine/threonine phosphorylated upon stimulation of cells with certain growth factors that activate receptor tyrosine kinases such as EGF and PDGF (Kanner et al., 1991; Reynolds et al., 1992). In addition p120 binds to a cytoplasmic tyrosine kinase, FER (Kim and Wong, 1995) and is tyrosine phosphorylated in cells transformed with v-src where its tyrosine phosphorylation correlates with a transformed phenotype. Given its localization in cell cell contacts where c-src and EGF-receptor have been detected (Hoschuetzky et al., 1994; Tsukita et al., 1991) p120 may be a biologically significant target of kinase signalling pathways aimed at regulating cell adhesive properties and it has been speculated that p120 is involved in regulating contact inhibition. Based on the similarity between p0071, and p120 we suppose that p0071 might also be involved in regulating adhesive interactions and cytoskeletal organisation at the junctional plaque. This is consistent with the finding that p0071 is not a major structural component of the desmosomal plaque but rather a minor more tenuously associated protein.

Besides their role in cell contacts armadillo, plakoglobin and β-catenin are also involved in signalling cascades that function in pattern determination. Recent experiments suggest that this

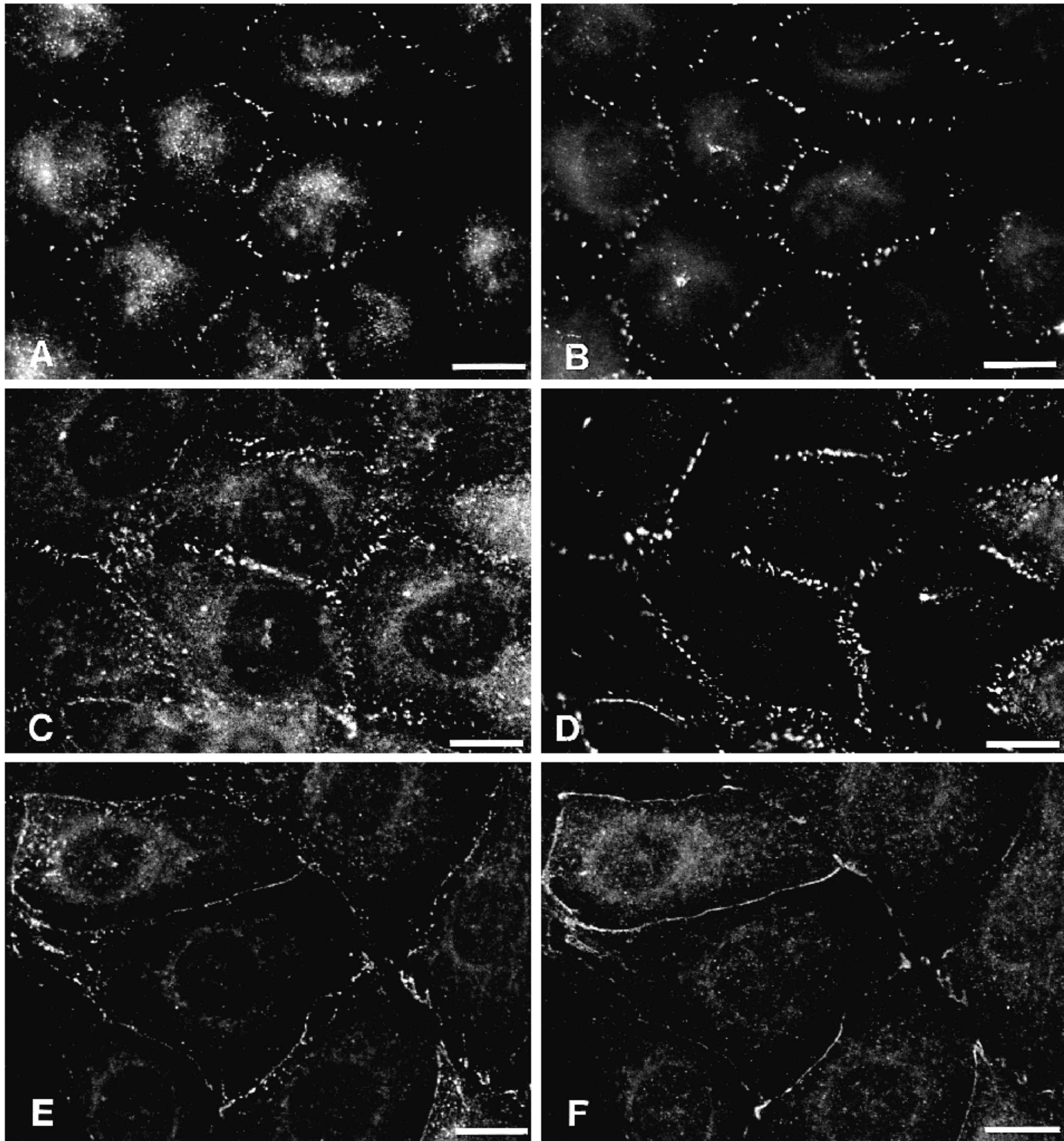

Fig. 10. p0071 antibodies localize to the intercellular borders of cultured epithelial cells. Double immunofluorescence staining using affinity purified p0071 antibodies (A,C,E) and desmoplakin antibodies (B,D,F) shows overlapping localization of both proteins in HeLa (A and B) and in HaCaT (C and D) cells. In MDBK cells p0071 antibodies localize along the entire plasma membrane (E) Double immunofluorescence staining with E-cadherin antibodies (F) shows colocalization of these two proteins in MDBK cells. Bars, 20 μm.

signalling function is regulated by cell adhesion and cadherins (Fagotto et al., 1996; Funayama et al., 1995; Karnovsky and Klymkowsky, 1995; McCrea et al., 1993). In addition a nuclear localization was observed for these proteins at elevated expression levels. This has prompted the speculation that the proteins might be translocated into the nucleus in the course of their signalling activity where they interact with nuclear target proteins. Such speculations immediately raise the question of how the proteins might be translocated into the cell nucleus, since neither β-catenin nor plakoglobin contain any obvious nuclear localisation signals. In this context it is interesting to note that p0071 contains a stretch of 8 basic amino acids at the end of repeat 6 that might function as a nuclear localization signal. Further experiments have to clarify whether or not armadillo related proteins are specifically translocated into the nucleus and how this transport is regulated.

Other factors seem to be involved in regulating the intracellular localisation of armadillo related proteins. For example the tumor suppressor gene product APC which itself is a member of the armadillo gene family competes with cadherins for

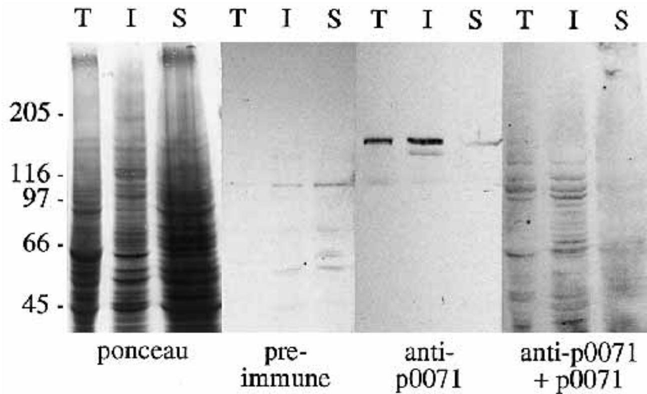

Fig. 11. Immunoblot analysis of total cellular extracts (T) and detergent insoluble (I) and soluble (S) fractions of HeLa cells. Molecular masses of the reference proteins are given at the left. Fractions were stained with Ponceau S and subjected to immunoblot analysis with p0071 preimmune serum, affinity purified p0071 antibody and affinity purified antibody in the presence of recombinant p0071 repeat domain as indicated. A 150 kDa band not detected by the preimmune serum was specifically labelled by the p0071 antibody. Addition of soluble p0071 removed this reactivity.

binding to plakoglobin and β-catenin (Hülsken et al., 1994a) and seems to downregulate their expression (Cowin and Burke, 1996). Since APC blocks cell cycle progression from G_0/G_1 to S phase (Baeg et al., 1995) it may represent a link between growth control and cell adhesion.

Taken together these data suggest that armadillo related proteins play a general role in controlling cell adhesion and in linking the cytoskeleton to various adhesion receptors. Based on the modular composition of the proteins it has been suggested that they mediate protein protein interactions within signalling cascades and that the motifs represent specific binding sites. It will therefore be important to assess binding partners of the armadillo related proteins including their upstream and downstream effectors in cell signalling to elucidate their role in growth control.

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