Targeting of p0071 to desmosomes and adherens junctions is mediated by different protein domains

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Summary

p0071, a member of the armadillo protein family, is most closely related to p120^{ctn} and the plakophilins 1-3. Whereas plakophilins are desmosomal plaque proteins, p120^{ctn} localizes to adherens junctions and interacts with classical cadherins. In contrast, p0071 has been described as a protein with dual localization in adherens junctions and desmosomes depending on the cell type examined. Here we have analyzed the localization of p0071 and its domains in detail. Although by sequence analysis, p0071 is more closely related to the adherens junction proteins p120^{ctn}, ARVCF and δ-catenin, endogenous p0071 associated preferentially with desmosomes in MCF-7 epithelial cells. Overexpressed p0071 localized along cell borders and overlapped only partially with desmosomal markers but colocalized with non-desmosomal cadherins and recruited cadherins to the membrane. The head domain of p0071 was sufficient for desmosomal targeting, whereas the arm repeat domain associated with adherens junctions and enhanced membrane association of classical cadherins. The tail domain localized preferentially to the nucleus and associated with desmosomes. To examine the mechanism underlying this dual localization more closely we determined binding partners of p0071 by using yeast-two-hybrid and mom-targeting assays. These approaches show that the head domain interacted with desmosomal proteins desmocollin 3a and desmoplakin, whereas the armadillo repeat domain binds to non-desmosomal cadherins. Head and armadillo repeat domains both interacted with plakoglobin by binding to different sites. Our data suggest that, in addition to plakoglobin, p0071 is the second armadillo protein present in both types of adhesive junctions and may play a role in regulating crosstalk between adherens junctions and desmosomes.

Key words: p0071, Plakophilin, Plakoglobin, Cadherin, Desmosome

Introduction

Adhesive interactions between epithelial cells are mediated through adherens junctions and desmosomes that anchor actin filaments and intermediate filaments to sites of cell cell contact (Green and Gaudry, 2000; Nagafuchi, 2001; Vasioukhin and Fuchs, 2001). Both types of junctions are composed of distinct but related sets of proteins. Adhesion is mediated by transmembrane proteins of the cadherin family. Their extracellular domains interact with cadherins from opposing cells. Since individual interactions are weak, the maintenance of cadherin-mediated adhesion depends upon clustering of cadherins through junctional plaque proteins and their association with the cytoskeleton (Adams et al., 1996; Vasioukhin and Fuchs, 2001; Yap et al., 1998).

In adherens junctions, β-catenin or γ-catenin (plakoglobin), the vertebrate homologues of armadillo, link α-catenin to the intracellular domain of cadherins (Aberle et al., 1996; Gumbiner and McCrea, 1993; Kemler, 1993; Obama and Ozawa, 1997). P120^{ctn}, another armadillo repeat protein located in cell contacts, binds to E-cadherin simultaneously with β-catenin or γ-catenin (Aghib and McCrea, 1995; Daniel and Reynolds, 1995; Ohkubo and Ozawa, 1999; Reynolds et al., 1996; Shibamoto et al., 1995; Staddon et al., 1995). The β-catenin-binding site maps to the C-terminal region of cadherins, whereas p120^{ctn} binds the juxtamembrane region, which plays an important role in cadherin clustering and regulating adhesion. ARVCF and NPRAP/δ-catenin, two p120^{ctn}-related armadillo repeat proteins, can bind to the same cadherin domain (Kaufmann et al., 2000; Lu et al., 1999; Mariner et al., 2000; Paulson et al., 2000; Waibler et al., 2001). These proteins are much less abundant than p120^{ctn} and their role in regulating adherens junction function is not well understood.

Desmosomes contain two types of cadherins, the desmogleins (dsgs) and desmocollins (dscs). The three different desmoglein and desmocollin genes (*dsg 1-3* and *dsc 1-3*) are differentially expressed (Koch and Franke, 1994; Schmidt et al., 1994). The intracellular domains of the desmosomal cadherins associate with plakoglobin, which also binds desmoplakin. Direct interactions between desmosomal cadherins and desmoplakin have been reported in vitro (Smith and Fuchs, 1998) but it appears that plakoglobin is necessary to link these proteins in vivo (Kowalczyk et al., 1996; Kowalczyk et al., 1997). Desmoplakin binds to plakoglobin through its N-terminal domain and to intermediate filaments through its C-terminal domain and connects desmosomes to the cytoskeleton (Stappenbeck et al., 1993; Stappenbeck et al., 1994). The importance of plakoglobin and desmoplakin in

desmosome function became evident in vivo. In mice, lack of plakoglobin results in an embryonic lethal phenotype due to reduced intercellular adhesion between cardiomyocytes (Bierkamp et al., 1996; Ruiz et al., 1996). A C-terminal truncation of plakoglobin has been described in arrhythmogenic right ventricular cardiomyopathy with palmoplantar keratoderma and woolly hair (Naxos disease), which suggests that plakoglobin and perhaps other proteins involved in cell-cell adhesion play an important part in maintaining myocyte integrity (McKoy et al., 2000). Mice lacking desmoplakin expression in their skin revealed reduced intercellular adhesion upon mechanical stress and showed defects in epithelial sheet formation (Vasioukhin et al., 2001). A mutation in the desmoplakin gene, resulting in a null allele and haploinsufficiency, was observed in patients with striate palmoplantar keratoderma. Affected skin demonstrated loosening of intercellular connections, disruption of desmosome-keratin interactions and rudimentary desmosomal structures (Armstrong et al., 1999). A generalized striate keratoderma particularly affecting the palmoplantar epidermis, woolly hair, and a dilated left ventricular cardiomyopathy was described in patients with a recessive mutation in the desmoplakin gene. Histology of the skin revealed large intercellular spaces with a perinuclear localization of keratin filaments in suprabasal keratinocytes, suggesting a collapsed intermediate filament network (Norgett et al., 2000).

Additional components of the desmosomal plaque are plakophilins 1-3 (Bonne et al., 1999; Hatzfeld, 1997; Hatzfeld et al., 1994; Heid et al., 1994; Mertens et al., 1996; Schmidt et al., 1999; Schmidt et al., 1997). Plakophilin 3 is widely expressed in all desmosomes, whereas plakophilin 1 and 2 show more restricted expression patterns and are found mainly in stratified or simple epithelia, respectively. Plakophilin 1 interacts with the suprabasal desmoglein isoform dsg1 and the desmoplakin N-terminal domain (Hatzfeld et al., 2000), and plakophilin 2 binds to dsg1 and 2, as well as plakoglobin and desmoplakin (Chen et al., 2002). An essential function of plakophilin 1 in stabilizing intercellular adhesion has been suggested by the phenotype caused by lack of plakophilin 1 (McGrath, 1999; McGrath et al., 1997). Null-mutations in the plakophilin 1 gene cause a genetic skin disease in humans called skin fragility syndrome. Desmosomes in the skin from patients are small and poorly formed with widening of keratinocyte intercellular spaces and perturbed desmosome-keratin filament interactions. Moreover, desmoplakin was found predominantly cytoplasmic suggesting a role for plakophilin 1 in organizing desmosomal plaque proteins. Ectopic expression of plakophilin 1 induced recruitment of desmosomal proteins to the plasma membrane through its interaction with several desmosomal proteins and suggests a function of plakophilin 1 in determining desmosome size and organization (Hatzfeld et al., 2000; Kowalczyk et al., 1999).

The ability of cells to organize desmosomal proteins into a functional structure critically depends on the presence of adherens junctions. Plakoglobin, so far the only protein common to both types of cell contacts, has been implicated in crosstalk between adherens junctions and desmosomes (Lewis et al., 1997).

p0071 is another armadillo repeat protein located in intercellular junctions (Hatzfeld and Nachtsheim, 1996). It is most closely related to NPRAP/δ-catenin and p120^{ctn}, both of which associate with classical cadherins (Hatzfeld, 1999). In contrast, a dual localization pattern in adherens junctions and desmosomes depending on the cell type has been reported for p0071 (Hatzfeld and Nachtsheim, 1996). In order to characterize the function of this protein in intercellular adhesion we have characterized its intracellular localization and show that the N-terminal head domain associates with desmosomes, whereas the armadillo repeat domain colocalizes with classical cadherins and has the capacity to recruit cadherins to the plasma membrane. A direct interaction between the head domain and desmocollin 3a, desmoplakin and plakoglobin was consistent with targeting of this domain to desmosomes. In contrast, the armadillo repeats differentially interact with several non-desmosomal cadherins. These findings indicate that p0071, like plakoglobin, can associate with both types of intercellular junctions and may have a function in crosstalk between these adhesive structures.

Materials and Methods

Cell culture and transfection

MCF-7 and PtK2 cells were cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 10% FCS and penicillin/streptomycin. Cells were plated 12-16 hours before transfection, transfected by the calcium phosphate precipitation method and processed for immunofluorescence analysis after ~30 hours as described (Hatzfeld et al., 2000).

Plasmids and cDNA constructs

RNA was prepared according to the LiCl/urea extraction method (Auffray and Rougeon, 1980) and cDNA synthesized by rtPCR using expand reverse transcriptase and high fidelity or long template polymerase (Roche Diagnostics, Mannheim, FRG). Suitable restriction sites for cloning were included in the primer sequences. PCR products were ligated into the PCR2.1 vector using TOPO TA cloning (Invitrogen, Karlsruhe, FRG). All PCR products were sequenced completely.

The following p0071 constructs were generated with a 5′ *Mun*I or *Eco*RI restriction site and a 3′ *Sal*I or *Xho*I restriction site and cloned into the pEGFP c2 vector (BD Clontech, Heidelberg, Germany): p0071 wt (aa 1-1193, short splice variant of the tail domain lacking aa 1043-1085); p0071 N-terminal head (aa 1-509); head aa 149-509; p0071 headless (aa 510-1193, short splice variant); p0071 tail (aa 989-1193, short splice variant); p0071 arm repeats (aa 510-988); arm repeats 1-5 (aa 510-745); arm repeats 2-10 (aa 552-988); and arm repeats 4-10 (aa 639-988). The same constructs including an additional Kozak sequence between the 5′-*Mun*I restriction site and the start codon and lacking a stop codon were cloned into the *Eco*RI and *Xho*I sites of pcDNA4/TO/myc-His (Invitrogen) and into the *Eco*RI and *Sal*I sites of pDsRed1-N1 (BD Clontech), which had been mutagenized to obtain the correct reading frame (Quick change mutagenesis kit, Stratagene, Amsterdam, The Netherlands).

The p0071 head and arm repeat domain constructs were also cloned into the yeast two-hybrid vectors pGBKT7 and pAS-2-1 (BD Clontech). Head domain clones showed autoactivation of the His and LacZ reporter genes except for one clone in pAS2-1. Sequence analysis revealed that this clone contained an internal deletion leaving an N-terminal head domain fragment of aa 1-198. The largest N-terminally deleted head domain construct without autoactivation (aa 209-509) and the p0071 arm repeats (repeats 1-10, 1-5, 2-10 and 4-10) were cloned into pGBKT7.

Vectors encoding cytoplasmic domains of human desmogleins (dsg) 1-3 and desmocollins (dsc) 1a-3b have been described (Hatzfeld et al., 2000). To exclude false-negative results due to low expression

levels in the pGAD424 vector used earlier, all fragments were subcloned into the pGADT7 vector, which allows high protein expression (BD Clontech). The human E- (aa 734-884), N- (aa 746-906) and OB-cadherin/cadherin 11 (aa 638-797) cytoplasmic tails were cloned into pGADT7. All dsgs, dscs and cadherin cytoplasmic domains were also inserted in the pcDNAmom-flag vector as described (Kaufmann et al., 2000). The E-cadherin cytoplasmic domain was also fused to the 4A6-birch profilin antibody epitope (4A6-tag) (Kaufmann et al., 2000).

An N-terminal deleted plakoglobin clone in pACT lacking the head portion and repeats 1-2 (pg-3-13+C, aa 230-745, GenBank accession number Z68228) was isolated in a two-hybrid screen for p0071-repeat-domain-interacting proteins. Candidate clones were isolated from a Hela cell cDNA library (BD Clontech). Plakoglobin constructs in pACT containing the N-terminus through arm repeat 6 (residues 1-375) with a C-terminal myc tag (pg-N+1-6) and arm repeats 4-9 (residues 249-490, pg-4-9) were generated by PCR and cloned into pACTII for expression in yeast. Full length myc-tagged plakoglobin (pg-myc) and the N- and C-terminally deleted plakoglobin (pg-ΔNΔC) in a eukaryotic expression vector under the control of a β-actin promotor were generated as previously described (Kowalczyk et al., 1994; Palka and Green, 1997). A plakoglobin construct lacking the majority of the central arm repeat region (pg-ΔSac) was constructed by partial digestion of pg-wt with *Sac*I and ligation in frame to yield a plasmid containing residues 1-146 fused to residues 668-745.

The p120^{ctn} (isoform A) and NPRAP/δ-catenin tail domains were amplified by PCR from Hela cDNA or a human brain cDNA library (Stratagene), respectively. The fragments were cloned into the pRSET vector containing an N-terminal His-tag sequence (Invitrogen) and expressed in BL21DE3 bacteria. The PKP1 repeat construct (including the short tail) has been described before (Hatzfeld et al., 2000).

Antibodies and immunofluorescence

Rabbit polyclonal anti-p0071 arm repeat (serum 678) and rabbit polyclonal anti-plakophilin 1 antibodies have been described (Hatzfeld et al., 2000; Hatzfeld and Nachtsheim, 1996). The monoclonal antibody 6D1-10 was produced by immunizing mice with the recombinant p0071 tail domain (aa 989-1193, short splice variant, Davids Biotechnologie, Regensburg, Germany) expressed from the pRSET vector. Protein expression was performed in BL21DE3 bacteria using standard methods and His-tagged recombinant protein purified using Ni-NTA resin (Qiagen, Hilden, FRG). Rabbit antiserum 616 against desmoplakin was prepared by immunizing a rabbit with gel-purified bovine desmoplakin. Desmosomes were prepared from bovine snout epithelia. Desmosomal proteins were separated by SDS-PAGE, protein bands cut out of the gel and the protein eluted by diffusion in 0.01% SDS (Hatzfeld et al., 1994). Antibody 4A6 against the birch profilin tag was kindly provided by B. M. Jockusch.

The following commercial antibodies were used: mouse monoclonal anti-desmoplakin 1+2; anti-desmoglein 1+2; anti-plakophilin 2; anti-E-cadherin (Progen, Heidelberg, Germany); rabbit polyclonal anti-Pan-cadherin; mouse monoclonal anti-plakoglobin; anti-c-myc clone 9E10; anti-flag M2 (Sigma, Taufkirchen, FRG); monoclonal anti-p120 (Transduction, BD Pharmingen, Heidelberg, FRG); and mouse monoclonal anti-His-tag (Qiagen). The monoclonal antibody against plakophilin 3 (clone 23E3) was kindly provided by S. Bonné and F. van Roy (Ghent, Belgium). Secondary antibodies used in this study were: Cy3 donkey anti-rabbit and anti-mouse IgG; Cy3 goat anti-mouse IgG+IgM (Dianova, Hamburg, FRG); Alexa 488 goat anti-mouse and goat anti-rabbit IgG; Alexa 350 goat anti-rabbit IgG (Molecular probes, MoBiTec Göttingen, FRG); alkaline-phosphatase (AP)-coupled goat anti-mouse and monoclonal mouse anti-rabbit IgG (Sigma); and AP anti-mouse IgM (Jackson/Dianova, Hamburg).

For immunofluorescence analysis cells were either fixed in 3.7% paraformaldehyde for 15 minutes at room temperature or in methanol at –20°C for 10 minutes, followed by permeabilization in 0.5% Triton in PBS for 20 minutes. For immunolabeling of endogenous p0071, cells were washed in PBS and extracted in 0.5% Triton in PBS for 20 minutes at 4°C. Cells were fixed in 3.7% paraformaldehyde for 20 minutes at room temperature and washed in PBS again. After fixation and permeabilization, cells were incubated in PBS containing 1% nonfat milk for 30 minutes, incubated with the primary antibody either for 2 hours at room temperature or overnight at 4°C, washed three times with PBS and treated with secondary antibodies for 1 hour at room temperature. Coverslips were mounted in Mowiol and analyzed with a Nikon Eclipse E600 microscope equipped with a Nikon FDX-35 camera.

Gel electrophoresis and western blotting

Total extracts from *E. coli* BL21DE3 cells were prepared by boiling cell pellets in SDS-sample buffer. Rat kidney was homogenized in Tris buffer (10 mM Tris pH 7.5, 5 mM EDTA, 2 mM EGTA, 150 mM NaCl). SDS and β-mercaptoethanol were added to a final concentration of 5% and 10%, respectively and the sample was centrifuged at 12,000 ***g*** for 15 minutes. Samples were separated on 15% or 7% SDS-gels. Electrophoresis and blotting was performed following standard protocols.

Yeast two-hybrid assay

The yeast strain YRG2 (Stratagene) was transformed by electroporation. Double transformants were grown on plates lacking leucine and tryptophane. Expression of the His-reporter gene was analyzed on plates lacking histidine in addition to leucine and tryptophane. LacZ reporter gene expression was analyzed with the colony-lift filter assay and quantitated using the ONPG (o-nitrophenyl-β-D-galactopyranoside) substrate as described in the yeast protocols handbook (BD Clontech).

Results

Endogenous p0071 associates with desmosomes in MCF-7 cells

In order to determine the intracellular localization of p0071 we generated a new monoclonal antibody (6D1-10) directed against the p0071 tail domain. Analysis of recombinant protein domains, including the closest relatives NPRAP/δ-catenin and p120^{ctn} tail domains, showed crossreactivity with NPRAP/δ-catenin but not with p120^{ctn} or plakophilin 1 (Fig. 1A). Since NPRAP expression is restricted to neuronal cells (Paffenholz and Franke, 1997), we conclude that the 6D1-10 monoclonal antibody specifically detects p0071 in epithelial cells and tissues. Western blot analysis of a total extract from rat kidney revealed a single band of 135 kDa indicating the specificity of the antibody (Fig. 1B). The p120^{ctn} and plakophilin antibodies recognized distinct fragments supporting the data with recombinant protein fragments. In total cell extracts from cultured cells the signal was very weak, which suggests that p0071 may be less abundant than p120^{ctn}. In immunofluorescence experiments the 6D1-10 antibody labeled desmosomes in MCF-7 cells, as shown by double immunofluorescence with antibodies against desmoplakin (Fig. 1C). Double labeling with cadherin antibodies showed that p0071 and cadherins were concentrated in distinct regions of the plasma membrane and overlapped only partially (Fig. 1C), suggesting that p0071 associates preferentially with desmosomes in MCF-7 cells. In addition to its membrane

association, p0071 shows a punctate cytoplasmic staining pattern as reported earlier using different reagents (Hatzfeld and Nachtsheim, 1996).

Transfected wt p0071 associates with cell contacts and recruits non-desmosomal cadherins to the plasma membrane of MCF-7 cells

Overexpressed wild-type (wt) p0071 with a DsRed tag at its C-terminus exhibited a prominent membrane association in MCF-7 cells. In contrast to the endogenous protein, exogenous p0071 distributed in a linear pattern along the plasma membrane and overlapped only partially with desmoplakin (Fig. 2). Moreover, desmoplakin staining was excluded from membrane regions highly enriched in p0071 (Fig. 2). This could be due to either epitope masking resulting from the high density of cell contact proteins recruited to the membrane or a displacement of desmosomal proteins from regions highly enriched in p0071. Since desmoplakin labeling was not recovered after extraction with detergent buffers prior to fixation, and immunodetection of dsg and dsc was also lost, we conclude that the effect is probably not caused by epitope masking. Double labeling of p0071wt DsRed-expressing cells with cadherin antibodies revealed colocalization of these proteins along the plasma membrane. In addition, regions rich in p0071wt protein were also enriched in E-cadherin and plakoglobin (not shown) implying that p0071 either recruits cadherins to the plasma membrane or stabilizes membrane-associated cadherins by clustering (Fig. 2).

Since localization of exogenous p0071 differs from that of endogenous p0071, we used constructs with different tags in order to exclude the possibility that the tag influenced localization of p0071. P0071 wt containing either a C-terminal myc tag (not shown) or an N-terminal EGFP tag (Fig. 2) showed the same intracellular distribution as DsRed-tagged p0071 wt. As before, p0071 was preferentially found along the plasma membrane and colocalized with non-desmosomal cadherins, which suggests that p0071 intracellular distribution is not determined by the tag sequence and that high expression levels favor association with adherens junctions.

The p0071 head and tail domains are responsible for desmosomal targeting

In order to gain insight into the regulation of intracellular localization and membrane association of p0071, we generated constructs comprising the N-terminal head domain and several fragments thereof, a headless mutant, the armadillo repeat domain and the C-terminal tail domain. Fig. 3A gives an overview of the p0071 constructs used in this study.

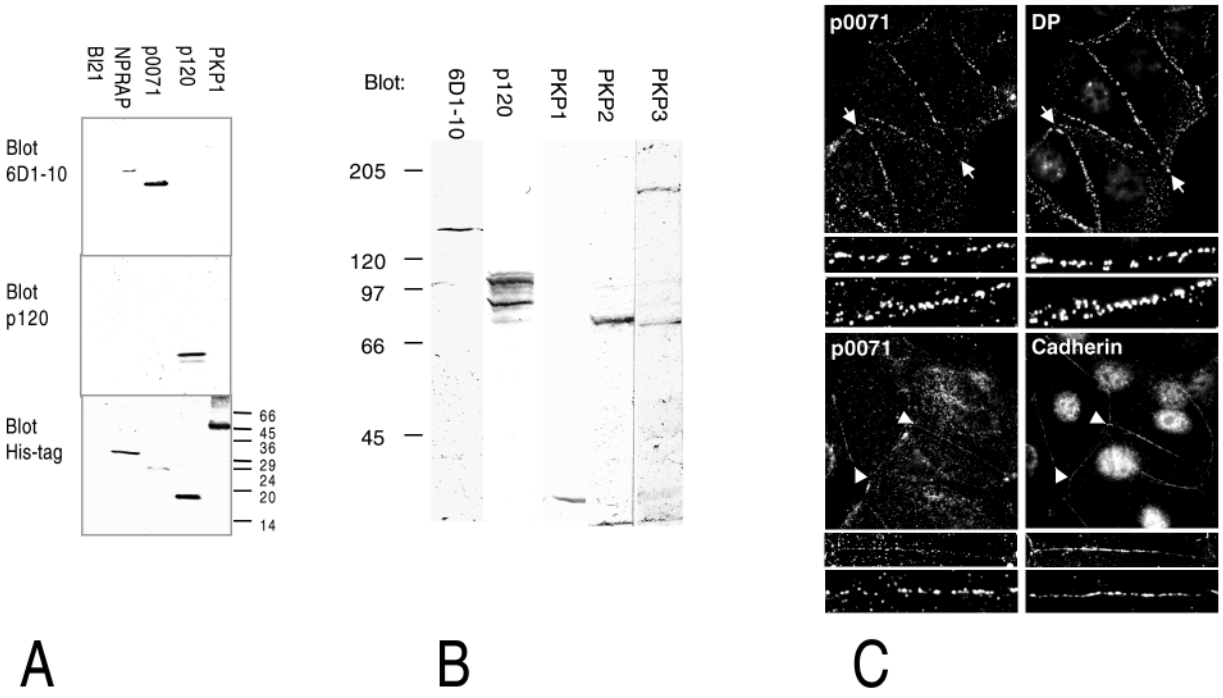


Fig. 1. (A) Western blot analysis with recombinant protein fragments expressed in *E. coli* BL21DE3 cells. BL21DE3 cells not expressing exogenous protein (BL21) or expressing the p0071 tail (p0071), the NPRAP/δ-catenin tail (NPRAP), the p120^{ctn} tail (p120^{ctn}) or the plakophilin 1 repeat + tail domains (PKP1) were lysed in SDS sample buffer, separated on 15% gels and transferred to membranes. Membranes were probed with 6D1-10-, p120^{ctn}- and His-tag monoclonal antibodies as indicated. 6D1-10 antibody recognized NPRAP and p0071, but not p120^{ctn} or plakophilin 1, the p120^{ctn} antibody was specific for the p120^{ctn} tail and the His-tag antibody detected all recombinant proteins. (B) Total cell extracts from kidney were prepared in SDS sample buffer, separated on 7% gels and transferred to membranes. Individual lanes were probed with antibodies to p0071 (6D1-10), p120^{ctn} and plakophilin 1, 2 and 3, as indicated. 6D1-10 detected a single band of 135 kDa that differs from protein bands detected by p120^{ctn}- and plakophilin antibodies. (C) Intracellular localization of endogenous p0071 in MCF-7 cells as detected by antibody 6D1-10. MCF-7 cells were extracted with Triton-X 100 prior to fixation and double labeled with the 6D1-10 monoclonal antibody (p0071) and rabbit anti-desmoplakin (serum 616) and rabbit polyclonal anti-Pan-cadherin (cadherin). Plasma membrane regions denoted by arrows are shown in detail at higher magnification. Bars, 3 μm.

Ectopic expression of the p0071 head domain resulted in a punctate staining pattern along the plasma membrane and some punctate cytoplasmic staining (Fig. 4A) resembling the localization pattern of the endogenous protein (see Fig. 1C). Double labeling with desmoplakin antibodies showed that these proteins colocalized, suggesting that protein interactions of the p0071 head domain are capable of targeting the protein to desmosomes. Colocalization with desmosomes was also observed with the p0071 head domain mutant comprising aa 149-509. This construct lacks the N-terminal conserved coiled-coil motif characteristic of p120^{ctn}-related proteins (Anastasiadis and Reynolds, 2000), indicating that this motif is not essential for plasma membrane targeting (Fig. 4B). The p0071 tail domain localized in the nucleus of all transfected cells. In addition, it was found to varying extents in a diffuse-cytoplasmic- and a punctate-membrane-associated pattern. Double labeling with E-cadherin and desmoplakin antibodies showed that the p0071 tail, like the head domain, colocalized with desmosomes (Fig. 4C,D).

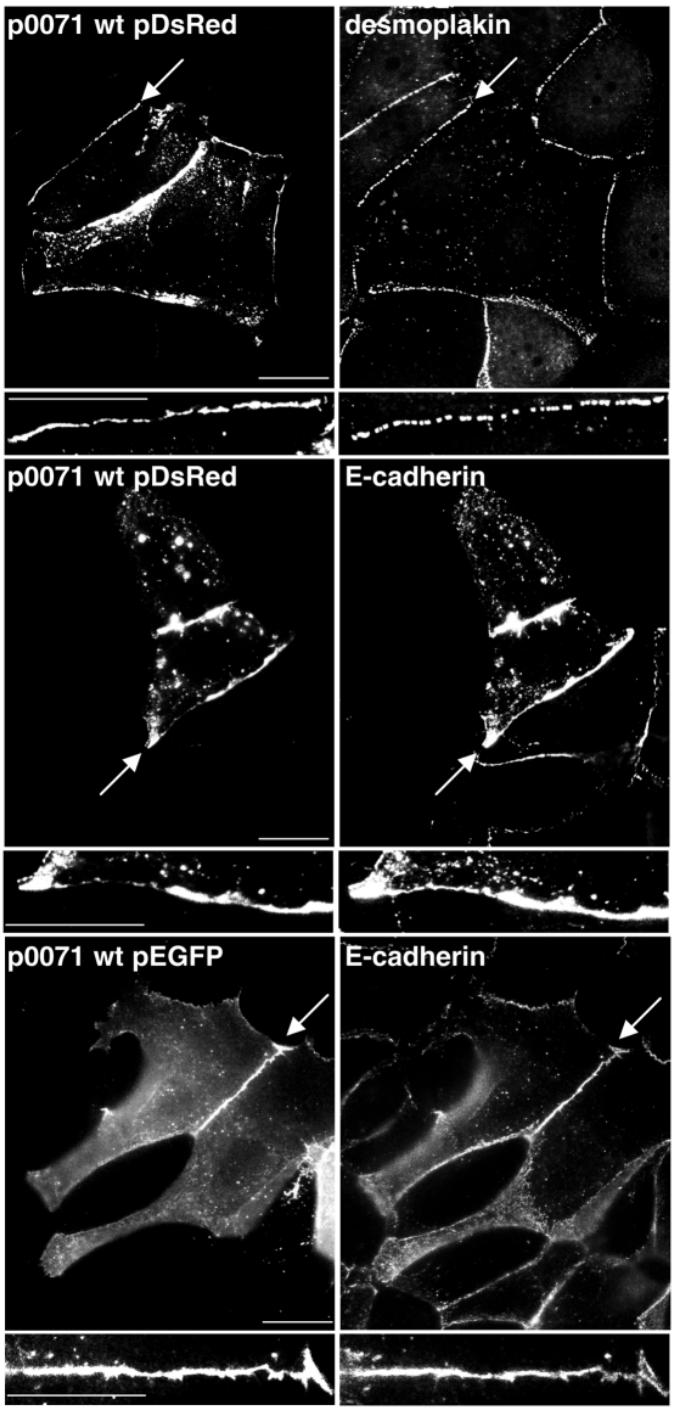


Fig. 2. Overexpression of p0071 wt in MCF-7 cells. P0071 wt with a C-terminal DsRed tag or an N-terminal EGFP tag was expressed in MCF-7 cells. Cells were fixed in methanol and labeled with desmoplakin (serum 616) or E-cadherin antibodies. Plasma membrane regions denoted by arrows are shown in detail at higher magnification. Note that desmoplakin staining disappears from the membrane regions enriched in p0071, whereas E-cadherin staining is increased. Bars, 3 μm.

Headless p0071 and the armadillo repeat domain colocalize with non-desmosomal cadherins

Ectopic expression of p0071 headless revealed a linear staining pattern along the plasma membrane that colocalized exactly with E-cadherin (Fig. 4E,F). Similarly to p0071 wt, the headless fragment recruited non-desmosomal cadherins to sites of cell cell contact (Fig. 4F). Double labeling with desmoplakin antibodies showed that p0071 headless was targeted to structures different from desmosomes. Membrane regions highly enriched in p0071 headless showed reduced staining for desmosomal markers (Fig. 4G).

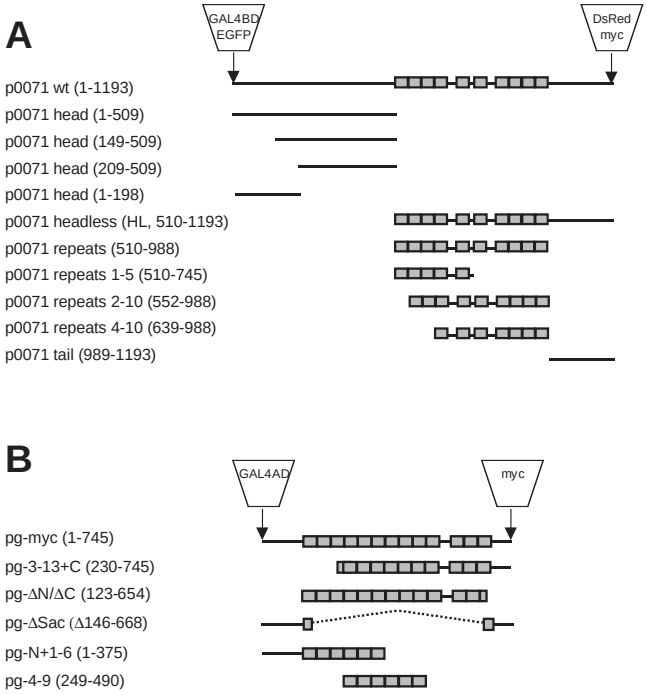


Fig. 3. (A) p0071 constructs used in this study. All constructs contain the short splice variant of the tail domain. (B) Plakoglobin (pg) constructs used in this study. Localization of tag sequences is indicated on top. Boxes represent the armadillo repeat units. For details see Materials and Methods.

The armadillo repeat domain showed localization along the membrane similar to that of the headless construct, but in most cells a large portion of the protein was cytoplasmic, which suggests that the tail domain contributes to membrane targeting (Fig. 4H). Membrane-associated p0071 repeats colocalized with E-cadherin but not with desmoplakin (Fig. 4H,I,J). Again, cadherin was recruited to the plasma membrane of transfected cells with high expression levels (Fig. 4I). Recruitment of cadherin was observed with all constructs containing arm repeats, and strong recruitment was always accompanied by the exclusion of desmosomal markers from the respective regions.

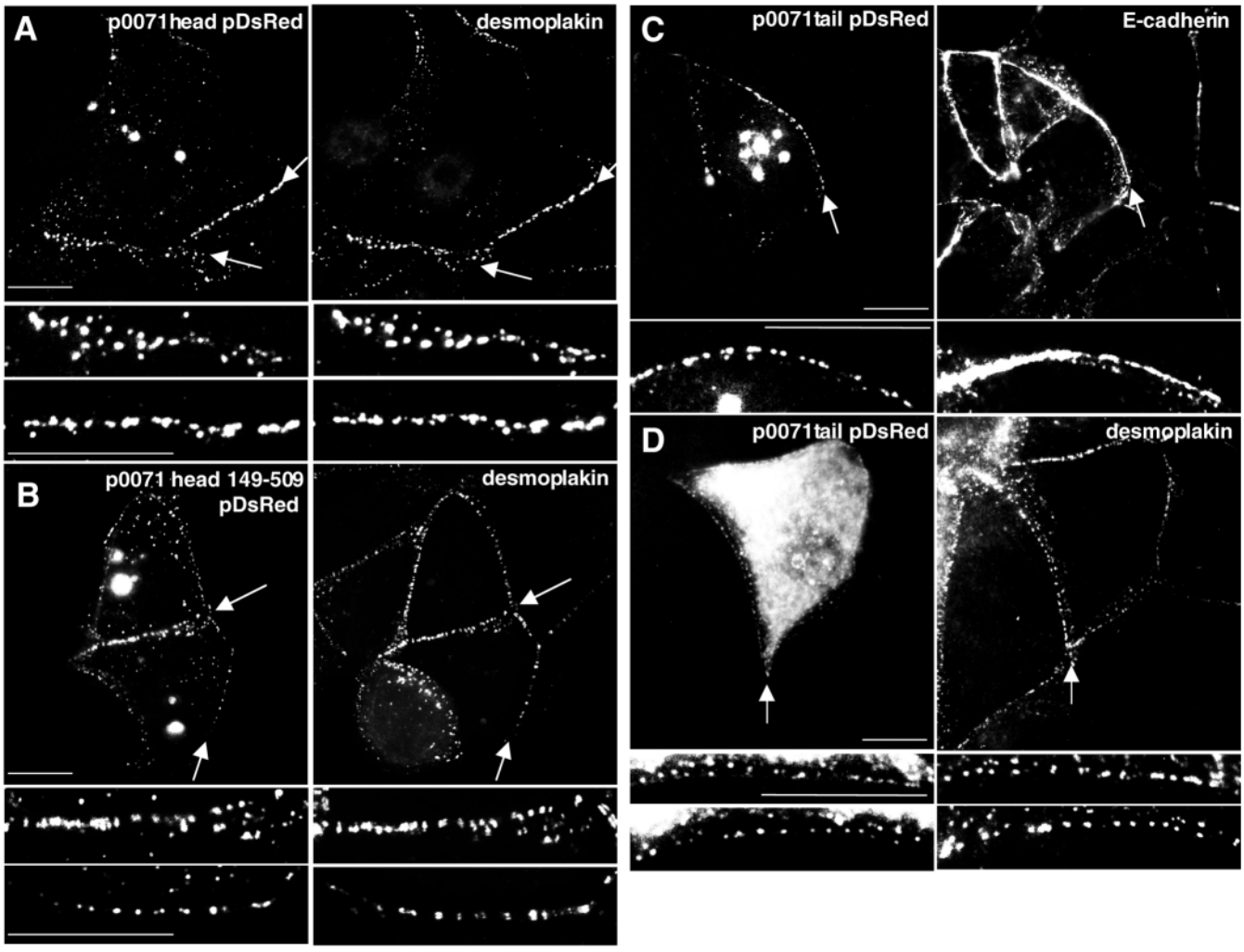
The p0071 head domain binds directly to dsc3a, plakoglobin and the DP-N-terminus whereas the repeat domain interacts with non-desmosomal cadherins

Direct interactions between p0071 domains and putative binding partners in cell cell contacts were examined by yeast two-hybrid analysis. Since the head domain showed autoactivation in this system we used two fragments coding for aa 1-198 and 209-509 of the head, respectively.

p0071 head 1-198 interacted with the dsc3a intracellular domain and with a plakoglobin fragment lacking the N-terminus including repeats 1-2 (pg-3-13+C). It did not interact with plakoglobin head + repeats 1-6 (pg-N+1-6) or repeats 4-9 (pg-4-9), suggesting that the p0071 N-terminal fragment binds to the plakoglobin tail (for an overview of pg-constructs see Fig. 3B). An interaction with desmoplakin was detected when analyzing activation of the His-reporter gene, but could not be confirmed by analyzing lacZ reporter gene activation (Fig. 5A).

p0071 head (aa 209-509) interacted with the desmoplakin N-terminal domain but again activation of the lacZ reporter gene was weak. In addition a strong interaction with pg-3-13+C but not with pg-N+1-6 or pg-4-9 was observed (Fig. 5A). No interaction was detected between p0071 head domain fragments and non-desmosomal cadherins, desmogleins or desmocollin isoforms other than dsc3a.

The repeat domain of p0071 interacted with all non-desmosomal cadherins analyzed (E-, N-, OB-cadherin/cadherin 11, Fig. 5A). Deletion of p0071 arm repeats 1 or 1-3 resulted in a loss of E-cadherin interaction, whereas repeats 1-5 were sufficient to mediate the p0071-E-cadherin interaction, which suggests that repeat 1 is important for cadherin binding. Moreover, p0071 repeats also interacted with plakophilin 2 (Fig. 5A), pg-3-13+C and with pg-N+1-6 but not with pg-4-9, suggesting that the binding site of the p0071 head and repeat domains in plakoglobin differ from each other and that p0071 repeat binding depends on arm repeat 3 of plakoglobin, a region that is involved in plakoglobin-desmoglein interactions.



The results of p0071 two-hybrid interactions are summarized in Table 1.

E-Cadherin is the typical classical cadherin of all normal epithelial cells and tissues. However, several carcinoma cells express more than one cadherin and N-cadherin has been detected in several tumor cell lines. To investigate whether

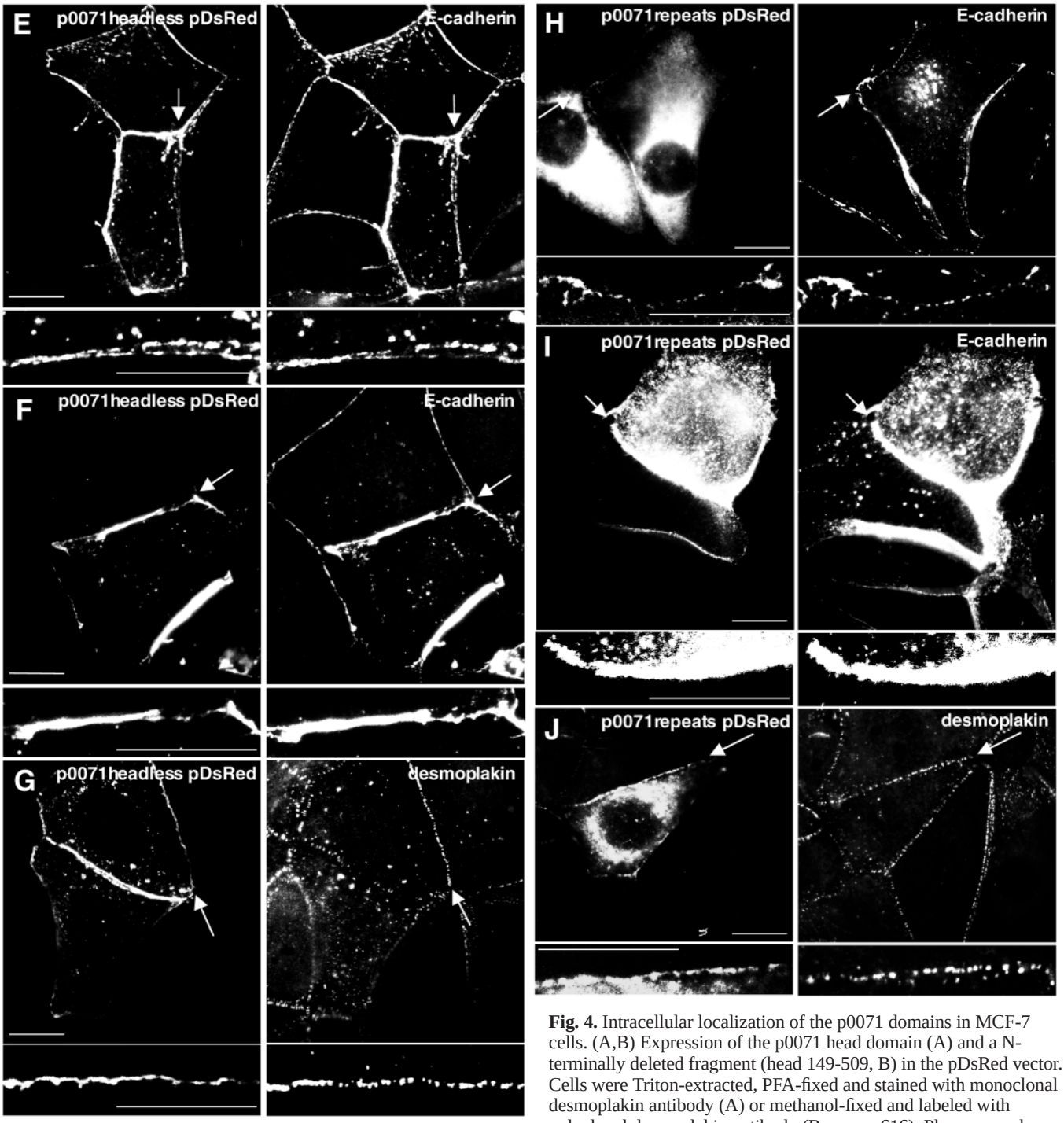
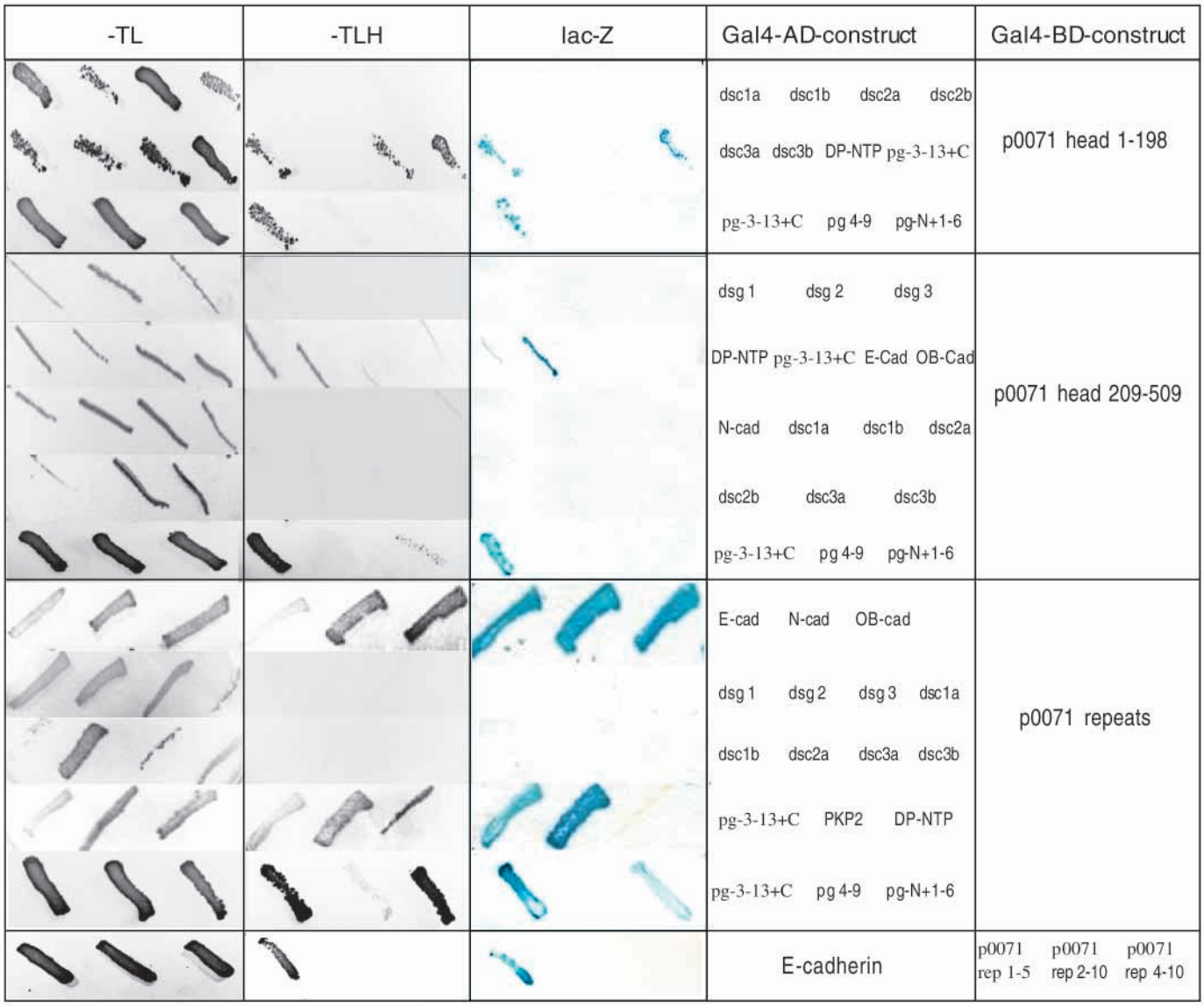


Fig. 4. Intracellular localization of the p0071 domains in MCF-7 cells. (A,B) Expression of the p0071 head domain (A) and a N-terminally deleted fragment (head 149-509, B) in the pDsRed vector. Cells were Triton-extracted, PFA-fixed and stained with monoclonal desmoplakin antibody (A) or methanol-fixed and labeled with polyclonal desmoplakin antibody (B, serum 616). Plasma membrane regions denoted by arrows are shown in detail at higher magnification. (C,D) Intracellular targeting of the p0071 tail domain. Cells transfected with the p0071 tail pDsRed were fixed in methanol and stained with anti-E-cadherin (C) or desmoplakin antibodies (D). Membrane regions denoted by arrows are shown at higher magnification. (E,F,G) Intracellular localization of p0071 headless pDsRed. Cells were fixed in methanol and labeled with E-cadherin (E,F) or desmoplakin (G, serum 616) antibodies. Enlargement of membrane regions denoted by arrows shows colocalization with E-cadherin (E,F), whereas desmoplakin staining is distinct from p0071 headless localization (G). Note that desmoplakin staining is sparse along the membrane enriched in p0071 headless (G). (H,I,J) Localization of the p0071 repeats pDsRed. Cells were fixed in methanol and labeled with E-cadherin (H,I) or desmoplakin (J, serum 616) antibodies. Enlargement of membrane regions denoted by arrows shows colocalization with E-cadherin (H,I), whereas desmoplakin staining is distinct from p0071 repeat domain localization (J). Bars, 3 μm.

p0071 preferentially interacts with one of the cadherins we quantitated p0071-cadherin interactions. Whereas the p0071-E-cadherin and OB-cadherin/cadherin 11 interactions were very similar, p0071-N-cadherin was more than three times as efficient in activating the lacZ reporter gene (Fig. 5B). Whether this preference for N-cadherin observed in the two-hybrid system is of physiological significance remains to be determined in other experimental systems.



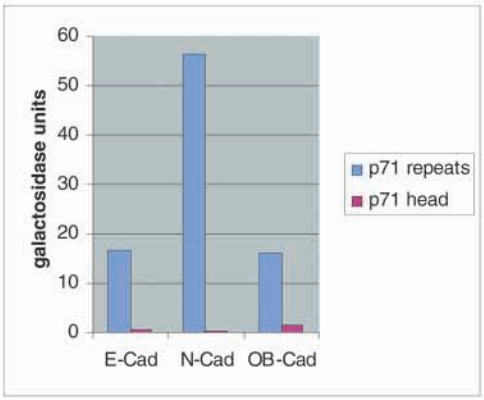


Fig. 5. Yeast two-hybrid interaction analysis using p0071 head 1-198, p0071 head 209-509 and p0071 repeats as baits. (A) Cotransformations of p0071 constructs in pGBKT7 with non-desmosomal cadherin-, dsg and dsc cytoplasmic domains, pg domains, PKP2 and the desmoplakin N-terminus (DP-NTP) were streaked in parallel on plates lacking tryptophane and leucine (–TL) and tryptophane, leucine and histidine (–TLH). Colonies from –TLH plates were blotted on filters and analyzed for lacZ reporter gene activation (lac-Z). The p0071 head 1-198 interacted with dsc3a and pg-3-13+C, but not pg-4-9 or pg-N+1-6. Cotransformants expressing DP-NTP activate the his reporter gene, but not the lac-Z reporter gene. The p0071 head 209-509 interacted with pg-3-13+C and DP-NTP although lac-Z reporter gene activation was weak for the latter interaction. p0071 arm repeats interacted with E-, N-, OB-cadherin/cadherin 11, pg-3-13+C, pg-N+1-6 and PKP2. Again, cotransformants with p0071 repeats and DP-NTP activated the his reporter gene but not the lac-Z reporter gene. E-cadherin interacted with p0071 rep1-5, but not p0071 rep2-10 or p0071 rep4-10, indicating that repeat1 is important for the interaction. (B) Quantitation of lac-Z reporter gene activation by p0071 arm-repeat–cadherin interactions and p0071-head (aa 1-198)–cadherin interactions using ONPG. Lac-Z activity was measured for >5 independent transformants. Lac-Z activation of the N-cadherin-p0071 cotransformants is ~3.5-fold higher compared with that of E- and OB-cadherin-p0071 cotransformants.

Table 1. Two-hybrid interaction data

	E-Cad	N-Cad	OB-Cad	Dsg 1	Dsg 2	Dsg 3	Dsc 1a	Dsc 1b	Dsc 2a	Dsc 2b	Dsc 3a	Dsc 3b	DP NTP	pg 3-13+C	pg-N+1-6	pg-4-9
p0071 head 1-198	–	–	–	–	–	–	–	–	–	–	+	–	+/–	+	–	–
p0071 head 209-509	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–
p0071 repeats 510-988	+	+	+	–	–	–	–	–	–	–	–	–	+/–	+	+	–

+, activation of both reporter genes; +/–, His reporter but not LacZ reporter gene activation. Cadherin, dsg and dsc clones represent the cytoplasmic domains; DP-NTP represents the N-terminal domain of desmoplakin; pg-3-13+C comprises plakoglobin arm repeats 3-13 and the C-terminal tail domain; pg-N+1-6 comprises the plakoglobin N-terminus including repeats 1-6; and pg-4-9 represents plakoglobin armadillo repeats 4-9.

Table 2. Mom-targeting assay with cytoplasmic domains of transmembrane cell adhesion molecules anchored on mitochondria

	E-Cad mom	Dsg 1 mom	Dsg 2 mom	Dsg 3 mom	Dsc 1a mom	Dsc 1b mom	Dsc 2a mom	Dsc 2b mom	Dsc 3a mom	Dsc 3b mom
p0071 head 1-509	–	–	–	–	–	–	–	–	+	–
p0071 repeats 510-988	+	–	–	–	–	–	–	–	–	–
p0071 tail 989-1193	–	–	–	–	–	–	–	–	–	–

Recruitment of p0071 domains to mitochondria was analyzed.

The finding that the p0071 arm repeat domain interacts with non-desmosomal cadherins is consistent with its colocalization with E-cadherin in transfected cells and with its capacity to recruit non-desmosomal cadherins to sites of cell cell contact.

In vivo interactions of p0071 head and p0071 armadillo repeat domains

In order to verify the two-hybrid interaction data we used an intracellular targeting assay to investigate protein-protein interactions in mammalian cells. The cytoplasmic domains of dsg1-3, dsc1a-3b and E-cadherin were fused to the TOM70 mitochondrial membrane anchor, thus replacing the transmembrane domains of the full length proteins (Kaufmann et al., 2000). The constructs also contained a flag tag to facilitate detection. Owing to the TOM70 membrane anchor, these proteins are expressed on the cytoplasmic surface of mitochondria. Double transfection experiments with the mitochondria-anchored cadherin cytoplasmic domains and the p0071 head domain showed no colocalization with E-cadherin, (Fig. 6; Table 2), the desmogleins 1-3, desmocollins 1b, 2b and 3b (not shown) and desmocollins 1a and 2a (Fig. 6). In contrast, the desmocollin 3a domain was able to recruit the p0071 head domain to mitochondria and both proteins colocalized (Fig. 6). This finding is in agreement with the two-hybrid interaction data, where an interaction between the p0071 head domain and desmocollin 3a but not with other desmocollin or desmoglein isoforms or non-desmosomal cadherins was observed.

We also examined interactions between the p0071 repeat and tail domains with desmoglein and desmocollin isoforms and E-cadherin. In contrast to the head and tail domains p0071 repeats were recruited to E-cadherin coated mitochondria (Fig. 6), indicating that the E-cadherin interaction is exclusively mediated by the arm repeat domain in vivo. Again, this finding is consistent with the two-hybrid interaction data. No recruitment was detected for the desmoglein and desmocollin isoforms nor the arm repeat or the tail domain of p0071 (data not shown). Interactions between p0071 and plakoglobin or the DP-N-terminus could not be analyzed by this assay since these proteins and protein fragments contained intracellular targeting signals that were dominant over the TOM70 membrane anchor.

Interactions between p0071 and plakoglobin

All p0071 domains interacted with plakoglobin in the yeast two-hybrid assay and use of plakoglobin fragments suggested an interaction of the p0071 head with the plakoglobin tail. In contrast, the p0071 repeat interaction with plakoglobin did not depend on the end domains. To verify the p0071-plakoglobin interactions in mammalian cells we cotransfected p0071 domains with plakoglobin fragments and analyzed the extent of colocalization. Transfection of p0071wt together with plakoglobin (pg-myc) resulted in colocalization of both proteins along the plasma membrane. The overlap observed was not identical with desmosomal labeling (Fig. 7). P0071 headless colocalized with N- and C-terminally deleted plakoglobin (pg-ΔNΔC), whereas a plakoglobin fragment lacking almost the entire repeat region (pg-ΔSac) distributed in a diffuse pattern in the cytoplasm and the nucleus and was not recruited to the membrane. This supports our conclusion that the p0071 repeat-plakoglobin interaction relies on the presence of the plakoglobin armadillo domain. In contrast, cotransfection of the p0071 head domain with pg-ΔNΔC revealed no, or very restricted, colocalization of these two polypeptides. This plakoglobin fragment showed predominantly a punctate distribution in the cytoplasm with poor membrane association in MCF-7 cells. The plakoglobin fragment lacking most of its repeat region (pg-ΔSac) showed partial membrane association when cotransfected with the p0071 head domain, consistent with the finding in the yeast two-hybrid assay.

Discussion

p0071 plays a unique role among p120^{ctn} family members because of its dual localization in adherens junctions and desmosomes (Hatzfeld and Nachtsheim, 1996). In the present study we show that targeting to adherens junctions and desmosomes is mediated by different domains of p0071 that

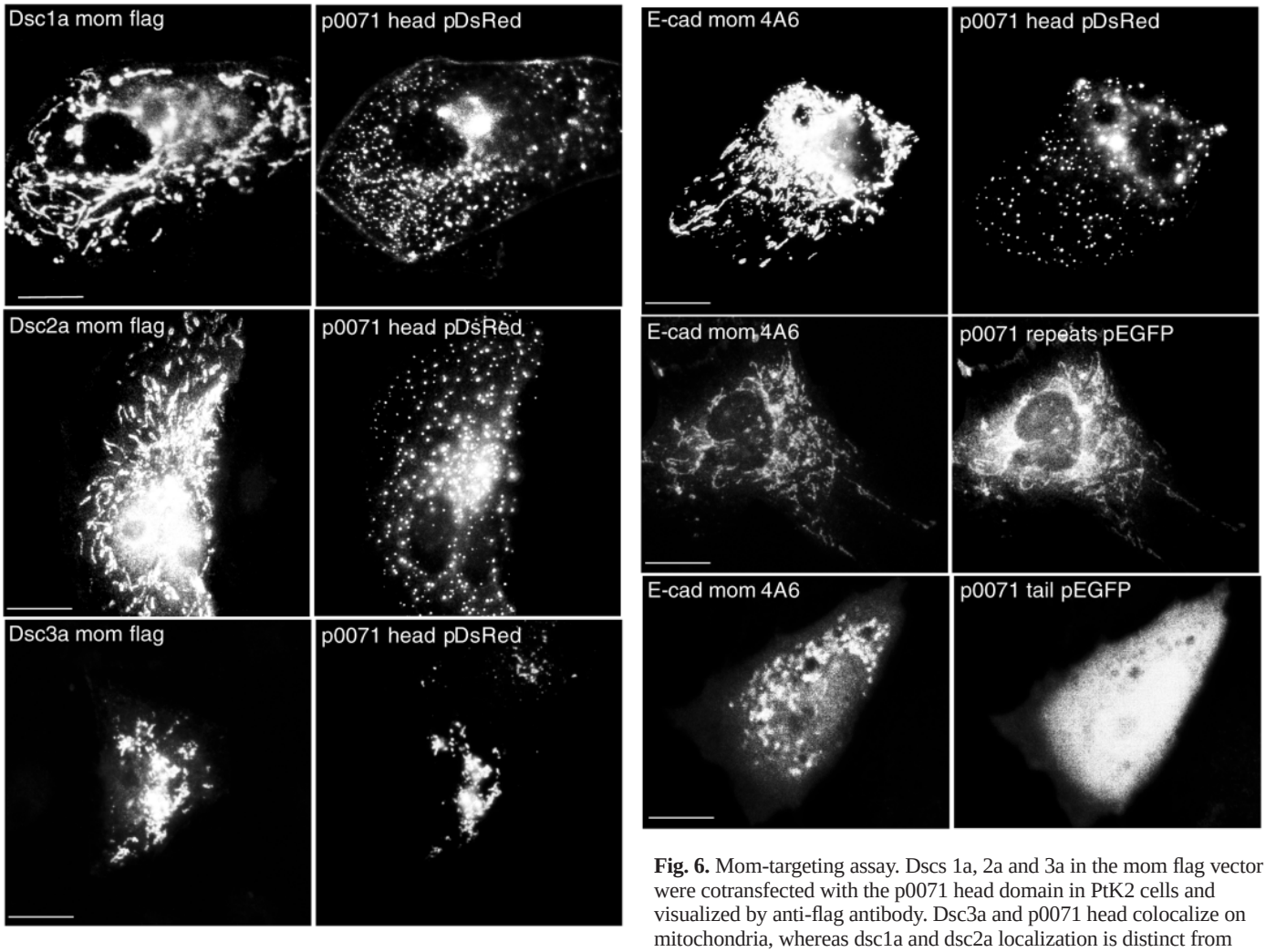


Fig. 6. Mom-targeting assay. Dscs 1a, 2a and 3a in the mom flag vector were cotransfected with the p0071 head domain in PtK2 cells and visualized by anti-flag antibody. Dsc3a and p0071 head colocalize on mitochondria, whereas dsc1a and dsc2a localization is distinct from p0071 head distribution. E-cadherin in the mom-vector with 4A6 tag was cotransfected with the p0071 head, repeat and tail domains. Whereas the p0071 repeat domain was recruited to E-cadherin-coated mitochondria, head and tail domains of p0071 were not recruited. Bars, 3 μm.

interact with either desmosomal or adherens junction proteins.

P0071 is both a desmosomal protein and an adherens-junction-associated protein

Although p0071 is by its sequence more closely related to NPRAP/δ-catenin, p120^{ctn} and ARVCF, its intracellular localization resembles more that of plakophilins 1-3, since in most cells analyzed p0071 colocalized with desmosomal markers (Hatzfeld and Nachtsheim, 1996). In order to exclude that this observation was based on antibody crossreactivity with plakophilins we have generated a new monoclonal antibody directed against the p0071 tail domain that shows no sequence homology to plakophilins. The 6D1-10 monoclonal antibody showed no crossreactivity with either the desmosomal plakophilins 1-3 or p120^{ctn} but it also reacted with the closest relative of p0071, NPRAP/δ-catenin. Since this protein is expressed only in neuronal cells and was not detected at the RNA or protein level in MCF-7 cells (M.H., unpublished), we conclude that in epithelial cells the signal obtained is specific for p0071. By using this new monoclonal antibody we confirmed our earlier results and showed colocalization of endogenous p0071 with desmosomes in MCF-7 epithelial cells.

In contrast to endogenous p0071, overexpressed p0071wt colocalized with non-desmosomal cadherins and was able to recruit cadherins to the plasma membrane. The same phenotype has been described for NPRAP/δ-catenin (Lu et al., 1999), the closest relative of p0071. Thus, at high expression levels p0071 behaves like NPRAP/δ-catenin, ARVCF and p120^{ctn} (Anastasiadis and Reynolds, 2000). Desmosomal markers were displaced from plasma membrane sites highly enriched in p0071, which suggests that unregulated p0071 expression interferes with the normal balance between desmosomes and adherens junctions. Therefore, it is conceivable that the level of p0071 expression not only determines its intracellular localization but also influences number and size of both adhesive structures.

P0071 interactions with desmosomal and adherens junction proteins are mediated by different domains

Binding partners of plakophilins, p120^{ctn}, ARVCF and NPRAP/δ-catenin are either desmosomal proteins [for

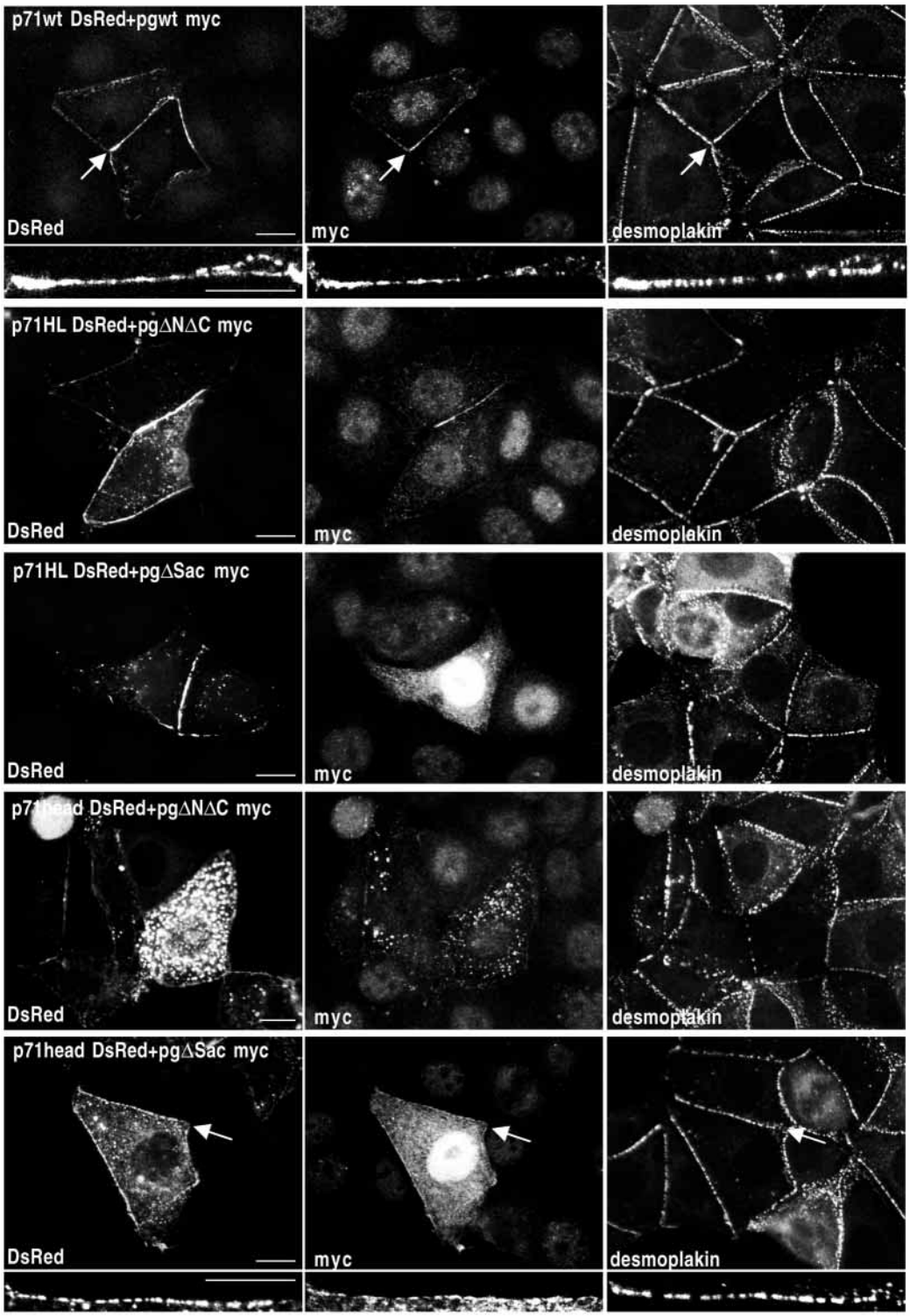


Fig. 7. Cotransfection studies of p0071 domains and plakoglobin domains. Cells were fixed in paraformaldehyde and labeled with the myc antibody recognizing plakoglobin constructs and desmoplakin antibody (serum 616) to visualize desmosomes. P0071wt DsRed and plakoglobin-myc colocalize along the plasma membrane. This localization partially overlaps with desmoplakin staining as shown in detail at higher magnification. p0071 headless DsRed colocalizes with pg-ΔCΔN along the membrane, whereas the same plakoglobin construct localizes preferentially in the cytoplasm when cotransfected with the p0071 head domain. In contrast, cotransfection of p0071 headless and pg-ΔSac shows membrane association of p0071 headless as noted before and cytoplasmic and nuclear localization of pg-ΔSac. Double transfection of pg-ΔSac with the p0071 head domain showes only partial recruitment of plakoglobin end domains to the membrane. Bars, 3 μm.

plakophilins 1-3 (Chen et al., 2002; Hatzfeld et al., 2000) S. Bonné, B. Gilbert, M.H. et al., unpublished] or the non-desmosomal cadherins [for $p120^{ctn}$, δ-catenin and ARVCF (Kaufmann et al., 2000; Lu et al., 1999; Mariner et al., 2000; Reynolds et al., 1996; Shibamoto et al., 1995; Staddon et al., 1995; Thoreson et al., 2000; Waibler et al., 2001)]. In contrast, p0071 colocalizes with both types of junctions, which suggests interactions with different types of junctional proteins. To elucidate this seemingly contradictory behaviour of endogenous and exogenous p0071 we have analyzed targeting of individual domains of p0071 and identified direct binding partners in the yeast two-hybrid system.

Both N- and C-terminal domains were able to associate with desmosomes and the N-terminal domain interacted with the desmosomal proteins dsc3a, desmoplakin and plakoglobin but did not interact with adherens junction proteins. Fig. 8A shows

the direct binding partners of p0071 domains, Fig. 8B summarizes p0071 interactions in the desmosome. As anticipated, the N-terminal coiled-coil motif conserved between p120^{ctn}, NPRAP/δ-catenin, ARVCF and p0071 but not the plakophilins 1-3 (Anastasiadis and Reynolds, 2000) was not essential for desmosome targeting, which seems to be a unique feature of p0071. In addition, head and tail domains of p0071 were detected in the nucleus. In desmosome-bearing cells, the head domain associated preferentially with desmosomes, whereas in cells lacking desmosomes the head was not able to associate with the membrane and localized predominantly in the nucleus (M.H., unpublished). As described for ARVCF (Mariner et al., 2000) and plakophilin 1 (Hatzfeld et al., 2000) N-terminal sequences seem to be responsible for nuclear targeting despite the presence of a putative nuclear localization signal in the arm repeat region of all p120^{ctn} family members. Although a signaling function of p120^{ctn} family members is generally accepted, the extent of nuclear localization varies considerably between the members of the family and nuclear binding partners have not yet been identified with the exception of Kaiso, a transcription factor interacting with p120^{ctn} (Daniel and Reynolds, 1999).

The tail domain localized preferentially in the nucleus in all cell types analyzed and only minor amounts were cytoplasmic and desmosome-associated. Since there is no classical nuclear localization signal (NLS) in the tail domain the protein may be imported into the nucleus only after associating with a bona fide nuclear protein. In addition, an interaction of the p0071 tail with the PDZ-domain proteins papin and erbin has recently been reported (Deguchi et al., 2000; Izawa et al., 2002; Jaulin-Bastard et al., 2002; Laura et al., 2002). Erbin seems to be able to associate with different types of junctions, adherens junctions, desmosomes and hemidesmosomes (Favre et al., 2001) and several lines of evidence support a role in appropriate organization of cytoskeletal elements and epithelial cell polarity in a rho-dependent manner. Recent data suggest that membrane association of papin and erbin does not depend on p0071 but that these proteins come to the regions of cell contact independently and interact with each other on the lateral membrane (Ohno et al., 2002).

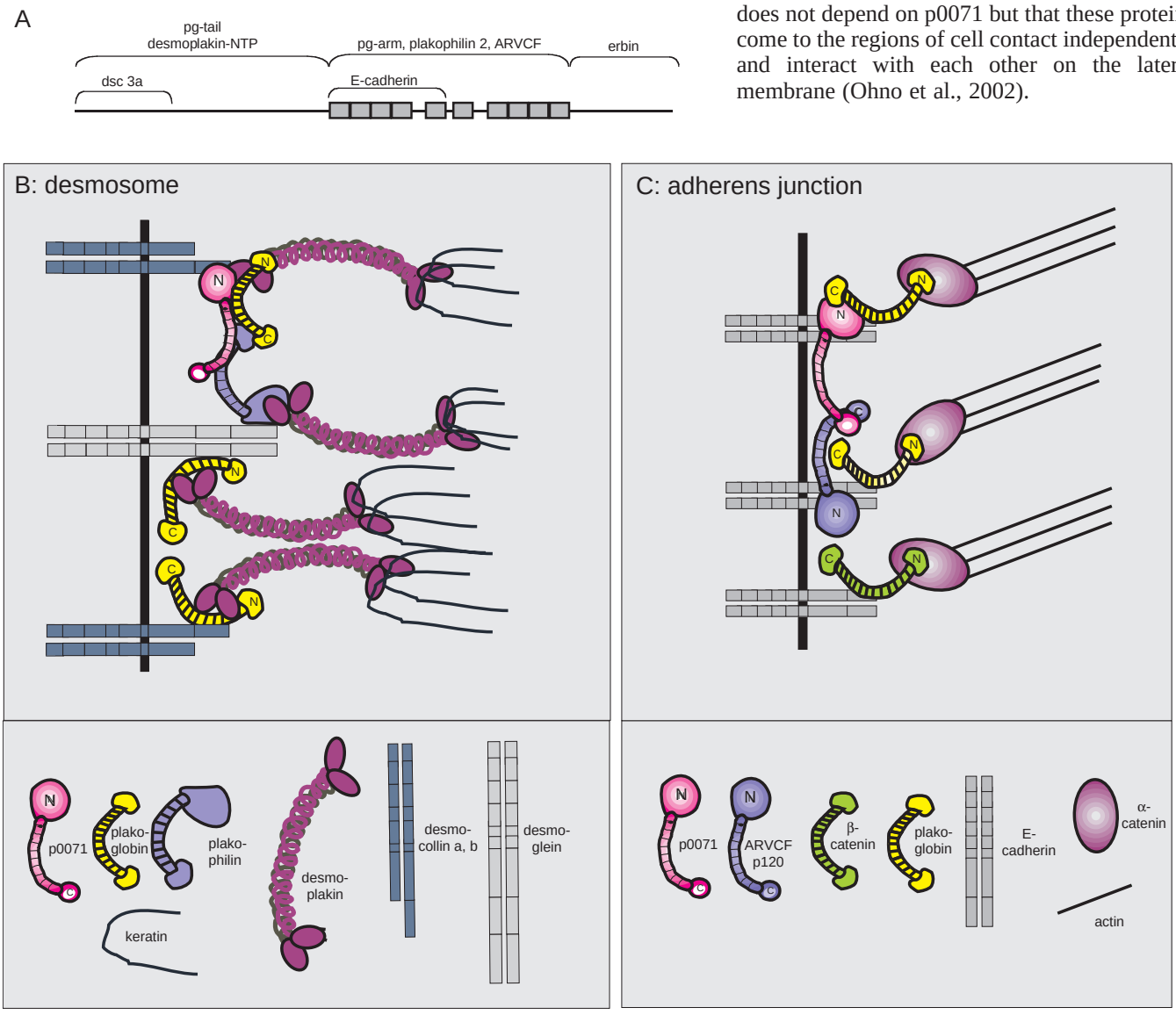


Fig. 8. (A) Binding sites of cell contact proteins in the p0071 molecule. (B,C) Model depicting p0071 interactions in desmosomes (B) and adherens junctions (C).

In contrast to the end domains, the central arm repeat domain of p0071 behaves similarly to p120^{ctn}, NPRAP/δ-catenin and ARVCF and associates directly with various non-desmosomal cadherins of adherens junctions (summarized in Fig. 8C). Deletion of p0071 repeat 1 abolished this interaction, which suggests that this region is important for p0071–E-cadherin binding. Interestingly, a splice variant in repeat 1 of mouse p0071 has been described before (Hatzfeld and Nachtsheim, 1996), and on the basis of preliminary observations a putative correlation between expression of the longer splice variant and cadherin association of p0071 had been suggested. The finding that repeat 1 is important for the interaction is in agreement with the hypothesis of a differential interaction of both splice variants with non-desmosomal cadherins. However, we did not detect the longer rare splice variant in human tumor cell lines derived from tissues corresponding to the mouse tissues expressing this splice variant. Further investigation of the two splice variants and their association with cadherins will be necessary to clarify whether these alternative splice variants differ in their affinity for cadherins and if this mechanism may contribute to the regulation of the intracellular localization of p0071.

Although p120^{ctn} and p120^{ctn}-like proteins may share redundant functions, it is clear that their roles in cell adhesion and motility are not identical: whereas p120^{ctn} and NPRAP/δ-catenin overexpression produced a strong branching phenotype (Lu et al., 1999; Reynolds et al., 1996), this effect is much weaker for p0071 and ARVCF (Mariner et al., 2000). The strength of the phenotype also depended on the cell type analyzed and one aspect possibly involved in mediating these differences is the composition of junctional structures. In contrast to E-cadherin, which mediates strong adhesion (Behrens et al., 1989; Behrens et al., 1993; Hermiston et al., 1996; Nabeshima et al., 1997), N-cadherin has been implicated in elevated cell motility and metastasis (Cavallaro et al., 2002; Hazan et al., 2000). It is not known how adhesion is regulated by the interaction of p120^{ctn} family members with the juxtamembrane domain of cadherins. Conflicting results have been described, since p120^{ctn} either promoted or reduced adhesion (Anastasiadis and Reynolds, 2000; Aono et al., 1999; Chen et al., 1997; Lu et al., 1999; Mariner et al., 2000; Paulson et al., 2000; Thoreson et al., 2000; Yap et al., 1998; Horikawa and Takeichi, 2001). Therefore, the mechanisms by which differential interactions of p0071 with cadherin family members might modulate the balance between adhesion and motility remain poorly understood.

P0071-plakoglobin interactions may be involved in regulating the balance between adherens junctions and desmosomes

The head and arm repeat domains of p0071 interacted with plakoglobin, so far the only known protein present in both adherens junctions and desmosomes (Cowin et al., 1986). Using plakoglobin deletion clones we present evidence that the p0071 head and arm repeats may bind to different sites in plakoglobin. Since we could not confirm these direct interactions in in vitro binding assays it is formally possible that they are artefacts of the two-hybrid system. However, it is also possible that interactions are stabilized in multiprotein complexes in vivo by cooperative binding mechanisms that make it difficult to analyze the individual interactions in vitro. Moreover, p0071 is not easily solubilized from desmosome-containing cells complicating co-immunoprecipitation analyses of such stable protein complexes. This explanation is supported by the results of the transfection experiments with p0071 and plakoglobin fragments, which are consistent with the two-hybrid data.

p0071 is possibly the first binding partner of the plakoglobin tail domain that has been implicated in regulating its subcellular distribution and desmosome size (Palka and Green, 1997). A role for the plakoglobin C-terminus in determining desmosome size is based on the observation that expression of C-terminal truncated plakoglobin in COS cells leads to striking alterations in desmosome morphology with formation of extremely long junctions or groups of tandemly linked desmosomes. Together with the data presented here, it seems plausible that protein-protein interactions between the plakoglobin tail and p0071 may contribute to this size-limiting function of the plakoglobin tail. Binding of the p0071 head domain to plakoglobin leaves the plakoglobin-dsg binding site accessible, which is consistent with the finding that the head domain targets p0071 to desmosomes (see Fig. 8B). In contrast, the p0071 arm-repeat binding site in plakoglobin overlaps with its dsg binding site so that binding of p0071 repeats could prevent plakoglobin from interacting with desmosomal cadherins, which is consistent with its role in targeting p0071 to adherens junctions (see Fig. 8C). It is conceivable that the interaction between plakoglobin and p0071 plays a role in targeting these two proteins either to desmosmes or to adherens junctions. The differential interaction of p0071 with plakoglobin described here might be one mechanism required for the formation and regulation of the two types of intercellular junctions.

A role for plakoglobin in regulating desmosome formation has been suggested in an in vitro model using adhesion-defective cells (Lewis et al., 1997). In this system, plakoglobin had to be linked to E-cadherin before cells began to assemble desmosomes. In vivo, the situation is more complex because β-catenin can partially compensate for the loss of plakoglobin (Ruiz et al., 1996; Bierkamp et al., 1996). In general, formation of adherens junctions seems to preceed desmosome formation and adherens junctions and desmosomes alternate in a regular pattern along the plasma membrane of epithelial cells. A role of p0071 in regulating the balance between desmosomal and cadherin-mediated adhesion is suggested by the observation that plasma membrane sites highly enriched in p0071 not only recruited non-desmosomal cadherins but also revealed displacement of desmosomal markers. This suggests that unregulated p0071 expression interferes with the normal balance between these structures. Elucidating the mechanisms that direct p0071 to either desmosomes or adherens junctions will help to further clarify its role in regulating cell cell adhesion.

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