Protein p0071 – an armadillo plaque protein that characterizes a specific subtype of adherens junctions

Ilse Hofmann[1,2,*], Tanja Schlechter[1,2,4], Cäcilia Kuhn[3], Michaela Hergt[3] and Werner W. Franke[3]

[1]Joint Research Division Vascular Biology, Medical Faculty Mannheim, University of Heidelberg, Ludolf-Krehl-Street 13-17, 68167 Mannheim, Germany
[2]The German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, 69120 Heidelberg, Germany
[3]Helmholtz Group for Cell Biology, German Cancer Research Center, Im Neuenheimer Feld 581, 69120 Heidelberg, Germany
[4]Progen Biotechnik, Maass-Street 30, 69123 Heidelberg, Germany
*Author for correspondence (e-mail: i.hofmann@dkfz.de)

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In vertebrates, cell-cell-adhering proteins of both the punctum adhaerens and the desmosome type are characterized by transmembrane glycoproteins of the cadherin superfamily, and by cytoplasmic plaques, which comprise the common plaque protein plakoglobin (Cowin et al., 1986; Franke et al., 1989), as well as proteins that are specific for either the desmosome, such as desmoplakin (Franke et al., 1982; Virata et al., 1992) and one or two members of the plakophilin 1 to plakophilin 3 group of proteins, or the adherens junctions such as α- and β-catenin (Ozawa et al., 1989; Nagafuchi and Takeichi, 1989), protein p120 (Reynolds et al., 1994) and protein ARVCF (Mariner et al., 2000; Walter et al., 2008). In addition, another plaque protein with the molecular mass of 135 kDa that also belongs to the family of armadillo-type plaque proteins (Peifer and Wieschhaus, 1990; Peifer et al., 1994) has been discovered by Hatzfeld and Nachtsheim (Hatzfeld and Nachtsheim, 1996) and reported to also be a 'dual-junction' molecule occurring in the plaques of both desmosomes and adherens junctions (Calkins et al., 2003; Hatzfeld et al., 2003; Setzer et al., 2004) (for a review, see Hatzfeld, 2005). Molecular binding partners of this protein – named p0071 – that are involved in the recruiting of the protein to plaques and its integration into plaques have also been described (e.g. Deguchi et al., 2000; Hatzfeld et al., 2003; Izawa et al., 2002; Jaulin-Bastard et al., 2002; Ohno et al., 2002).

To elucidate the distribution and functional contribution(s) of such plaque proteins in various developmental situations or in malignant growth and spread, one needs to identify the proteins at question sensitively and unequivocally in biochemical and cell biological experiments. Unfortunately, specific, well-defined and reliable antibodies against p0071 are not generally available, and the cell-type- and cell-structure-specificity as well as the nature of its molecular partners in functional complexes have not yet been adequately determined. One consequence of this lack of defined antibodies is the fact that the application of p0071 as a cell-type marker has so far not found its way into diagnostic pathology. Therefore, we have generated a number of cross-species-reactive antibodies that specifically recognize certain epitopes of protein p0071 and are useful in biochemical and cell biological studies. Using immunofluorescence and immunoelectron microscopy, as well as immunoprecipitation experiments, these antibodies have allowed the exclusion of a desmosomal location and the definition of a specific subtype of adherens junctions.

We have systematically prepared antibodies against various domains of p0071 (cf. Hatzfeld and Nachtsheim, 1996; Deguchi et al., 2000). The epitopes of those antibodies, which we have found most useful, are systematically presented in Fig. 1A, including murine monoclonal antibodies [designated clones p0071-1 to p0071-3 against peptide 1-1, clone p0071-4 against peptide 2-2, clones p0071-5 and p0071-6 against peptide 2-4, as well as guinea pig polyclonal antibodies against the cocktail of peptides 1-1, 1-2, 1-3 and 1-4 of the human (accession number NP_003619) and the murine (accession number AAH79848) sequence of p0071]. The antibodies were characterized and compared with antibodies against other junction proteins by using immunoblotting, coimmunoprecipitation, as well as light and electron microscopic immunolocalization techniques [for methods see Borrmann et al. (Borrmann et al., 2006)]. All murine monoclonal as well as guinea pig polyclonal antibodies mentioned here react with the p0071 polypeptide and localize to the same structures. The antibodies will soon be commercially available as single antibodies and as antibody cocktails covering several p0071 epitopes (for more information contact Progen Biotechnik, Heidelberg, Germany).

Specificity for polypetides with the molecular mass of 135 kDa – i.e. p0071 – in a diversity of human, rat and mouse cells and tissues is evident from Fig. 1B,C. Analysis of our immunoprecipitation experiments showed that constitutive proteins of adherens junctions co-immunoprecipitated (Fig. 1D shows examples for E-cadherin and β-catenin), whereas desmosomal proteins did not appear in such precipitates (Fig. 1D) [for immunoprecipitation methods see, for example, Hofmann et al. or Borrmann et al. (Hofmann et al., 2000; Borrmann et al., 2006)]. In addition, we noticed in carefully conducted immunoelectron microscopy experiments using various epithelial and non-epithelial tissues as well as cultured cells that all of our antibodies against p0071 reacted intensively and specifically with the plaques of adherens junctions, including even tiny puncta adhaerentia, but not at all with those of desmosomes (Fig. 1E). This exclusive specificity for adherens junctions and the exclusion of desmosomes was also demonstrated by high-resolution double-label immuno-fluorescence microscopy, in particular using monolayer cultures of epithelial cells. Fig. 2A presents an example of a human breast carcinoma cell (MCF-7 cells) monolayer culture, which shows mutually exclusive labeling of desmosomes and puncta adhaerentia.

We obtained corresponding immuno-localization results on cryostat sections of frozen tissues and – using some of the antibodies and the antigen-retrieval technique – also on sections of fixed and paraffin-embedded tissue samples. As an example, Fig. 2B-B″ presents lactating alveolar epithelial cells of a bovine mammary gland, showing colocalization of p0071 with β-catenin on epithelial plasma membranes. Similar results were obtained when analyzing a wide selection of single- and multi-layered epithelia (data not shown) or blood vessel endothelial cells (Fig. 2B-B″, arrows).

It has been reported previously that the junctional plaque protein p0071 also occurs specifically in sarcomeric I-bands of cross-striated muscles (Schröder et al.,

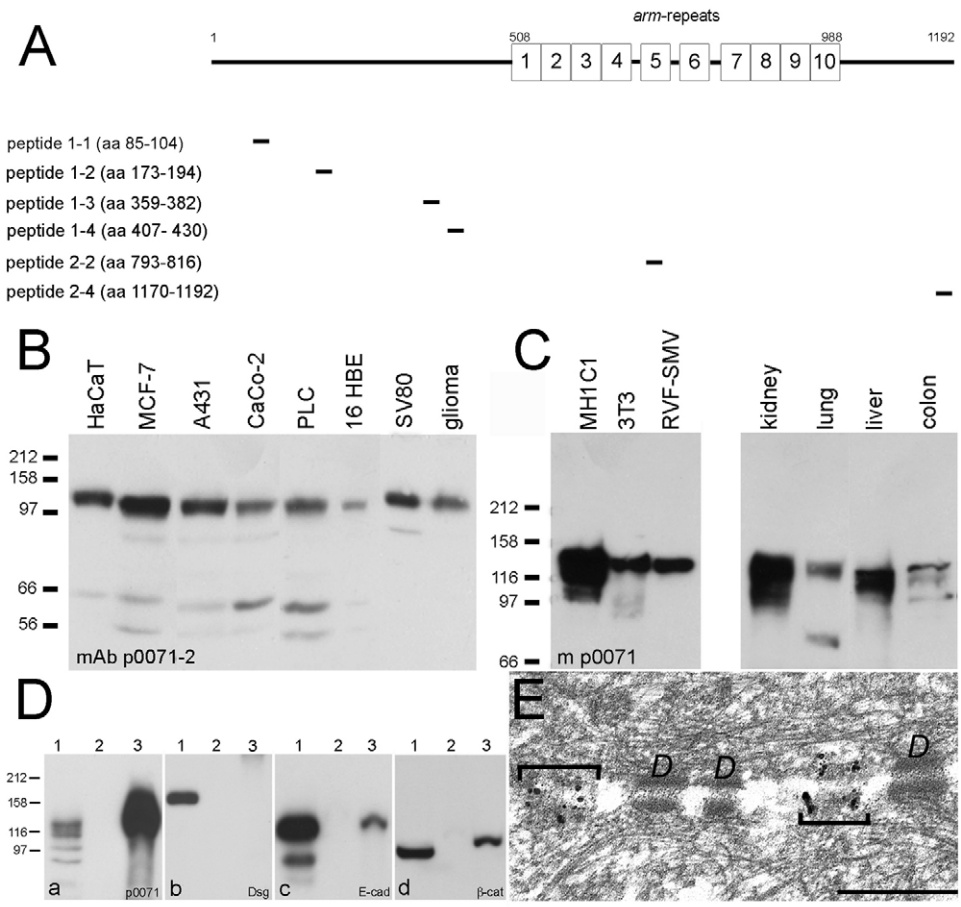

Fig. 1. (A) Schematic presentation of the domain structure of protein p0071 and the position of the amino acid (aa) sequences selected for the synthesis of oligopeptides used for immunization. (B,C) Detection of protein p0071 in (B) total lysates of human, (C, left panel) total lysates of rodent cell lines, and (C, right panel) total extracts of murine tissues. Cell lysates of the human cell lines HaCaT, MCF-7, A431, CaCo-2, PLC, 16 HBE14o, SV80 and glioma U333 (B) were processed for SDS-PAGE, and western blotted using mAb clone p0071-2. Notice the reactive polypeptide bands at ~135 kDa (all lanes) that can also be seen in the western blot analysis of the rodent cell line cultures MH1C1 (rat liver hepatocytes), 3T3 (mouse embryonal mesenchyme) and RVF-SM. (D) Immunoprecipitation experiments using total MCF-7 cell lysates and antibodies specific for protein p0071. Lanes 1, total cell lysates; lanes 2, negative controls for unspecific binding; lanes 3, immunoprecipitates. Western blotting using antibodies against (a) p0071, (b) desmoglein 1 and 2 (Dsg), (c) E-cadherin (E-cad) and (d) β-catenin (β-cat). Notice that in lanes 3, anti-p0071-1 immunoprecipitates were seen as an intense reaction for p0071 (a) and the complete absence of reaction for desmoglein (b); distinct amounts of p0071 were found in complexes of p0071 with E-cadherin (c) and β-catenin (d). Molecular mass markers are given at the left in kDa. (E) Immunoelectron microscopy image showing the localization of protein p0071 (immuno-gold particles enhanced by silver staining) to puncta adhaerentia in junctions (square brackets) of MCF-7 cells, and its absence from desmosomes (*D*) and bundles of intermediate-sized filaments. In panels A-E the following specific antibodies were used: mAb clone p0071-2 (B), guinea pig anti-mp0071 (C), mAb clone p0071-1 (D, a), mAb DG3.10 (D, b; Progen Biotechnik), monoclonal anti-E-cadherin (D, c; BD Biosciences Pharmingen), rabbit anti-β-catenin (D, d; from Sigma), guinea pig anti-hp0071 (E). Bar, 500 nm.

2000). Therefore, we examined this unexpected localization using our antibodies in cardiac and skeletal muscle tissues from human, bovine and rat. As shown for the sarcomeric muscles of rat and human hearts in Fig. C-D″, protein p0071 does only occur in the plaques of the composite junctions (area compositae) of the cardiac intercalated disks [for the specific molecular composition of this type of junction plaque see Borrmann et al. (Borrmann et al., 2006)] in wide-spread colocalization with both desmoplakin (Fig. 2D-D″) and N-cadherin (Fig. 2C-C″), whereas the sarcomeres were negative.

The most important result of our study is that, in contrast to previous reports, protein p0071 does not occur at detectable levels in normal desmosomes, i.e. in cell-cell adhering junctions formed by specifc cadherins of both the desmocollin and desmoglein group, or by a distinct set of plaque proteins – desmoplakin and plakophilin (for reviews, see Getsios et al., 2004; Green and Gaudry, 2000). The reported accumulation of genuine p0071 in natural desmosomes (Hatzfeld and Nachtsheim, 1996; Hatzfeld et al., 2003) could not be confirmed by us for any of the epithelial, carcinoma and fetal myocardial cells of tissues and in cell cultures. Reports of accumulation of p0071 at desmosmal plaques that occurred upon transfections of certain cultured cells with constructs that express the p0071 gene (Hatzfeld et al., 2003) are, of course, not necessarily contradictory to our findings in non-transfected cells. However, essentially in agreement with several previous reports (Borrmann et al., 2006) we demonstrated that protein p0071 is a major constituent of the myocardial composite junctions and of the diverse morphological forms of adherens junctions, in which it forms stable complexes with specific catenins and ‘classic’ cadherins (see also Calkins et al., 2003; Hatzfeld et al., 2003). It is obvious that the precise immunolocalization of p0071 that is now possible, as well as its precipitation, will significantly widen the application of junctional markers for cell biological research and for diagnostics in pathology.

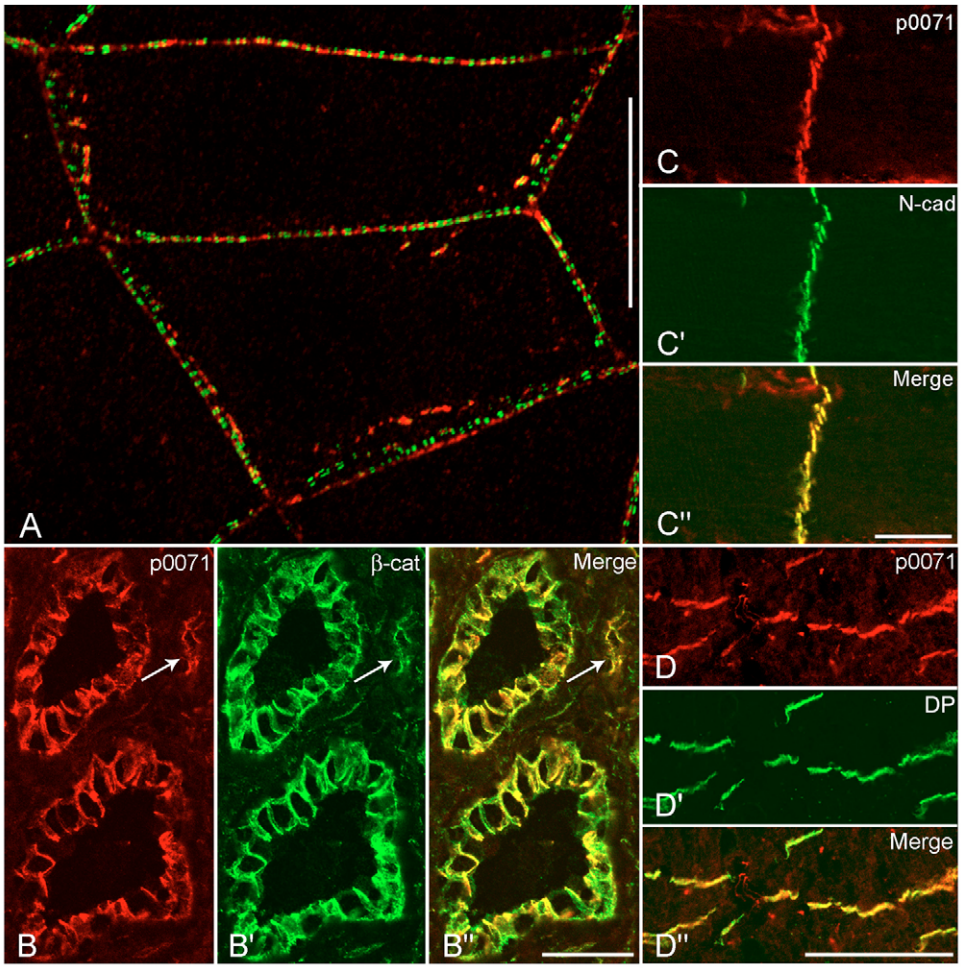

Fig. 2. (A-D″) Double-labeling immunolocalization experiments using (A-A″) cultured cells or (B-D″) tissues. (A) Laser-scanning confocal immunofluorescence micrographs showing the results of a typical double-labeling experiment using a monolayer culture of human epithelial MCF-7 cells at high density. Immunolocalization of p0071 using mAb clone p0071-3 (red) is compared with that of demsoplakin (green). Notice that the small puncta adhaerentia that contain p0071 are distinct from the desmosomes that contain desmoplakin. (B,B′) Localization of p0071 in the lactating bovine mammary gland. Confocal laser-scanning micrograph showing the immunolocalization of (B) p0071 (red; using mAb clone p0071-4) compared with that of (B′) β-catenin (green). (B″) Merged image. Arrows denote a small blood capillary with an endothelium that stains positive for p0071. (C-D″) Localization of p0071 in (C-C″) rat and (D-D″) human hearts. Micrographs were taken using a laser-scanning confocal microscope; location of p0071 was established using mAb clone p0071-4 (red) was compared with that of (D′) desmoplakin (green; DP) or N-cadherin (C′, green; N-cad). (C″,D″) Merged color images. Bars, 50 μm (B-B″, C-C″), 20 μm (A, D-D″).

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