Comparative Analysis of Armadillo Family Proteins in the Regulation of A431 Epithelial Cell Junction Assembly, Adhesion and Migration

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p0071 is an armadillo family protein related to both the adherens junction protein p120ctn and to the desmosomal proteins plakophilins 1–3. p0071 assembles into both adherens junctions and desmosomes, suggesting that this protein may regulate the balance between adherens junction and desmosome formation. Furthermore, this subfamily of proteins may also regulate cell functions directly influenced by intercellular junctions, including the strength of cell adhesion and the ability of cells to migrate. These possibilities were tested by expressing exogenous p0071 in A431 epithelial cells and monitoring the effects on adhesive junction assembly in comparison to other closely related armadillo family proteins. In this model system, p0071 specifically enhanced adherens junction assembly but dramatically compromised desmosome assembly, resulting in keratin filament retraction from regions of cell–cell contact. Protein interaction studies revealed that p0071 bound to the first 160 amino-terminal residues of desmoplakin and also interacted directly with plakoglobin, suggesting that p0071 may regulate desmosome assembly by controlling plakoglobin availability. Using an *in vitro* assay to measure the strength of cell–cell contacts, both plakophilin-1 and p120ctn were found to increase the strength of adhesion. Interestingly, p0071 expression caused no overall changes in adhesive strength, but dramatically inhibited the ability of A431 cells to close an *in vitro* wound. These results suggest that p120ctn/plakophilin family proteins interact with intercellular junction binding partners to differentially modulate the adhesive and migratory behavior of epithelial cells.

Key words: adherens junction/armadillo/desmosome/plakophilin
J Invest Dermatol 123:426–433, 2004

The establishment and regulation of cell adhesion is fundamental to the development and maintenance of tissue organization in multicellular organisms. In the epidermis, cell–cell adhesion is mediated primarily through adherens junctions and desmosomes. Desmosomes function as plasma membrane attachment sites for the intermediate filament network (Kowalczyk *et al*, 1999a; Green and Gaudry, 2000; Getsios *et al*, 2004), whereas adherens junctions anchor the actin cytoskeleton to the plasma membrane (Wheelock and Johnson, 2003). In general, adherens junctions are thought to initiate cell–cell contact, whereas desmosomes reinforce and sustain adhesion (Perez-Moreno *et al*, 2003). For these reasons, desmosomes are particularly prevalent in organs subject to substantial mechanical stress, such as heart and skin. Because of the importance of cell–cell adhesion during development and in tissue function in adults, it is critical to understand the mechanisms by which cells regulate the assembly and disassembly of adhesive junctions.

Although adherens junctions and desmosomes are morphologically distinct, both types of junctions utilize a similar architectural design to couple extracellular adhesive interactions to the cytoskeletal network. In both adherens junctions and desmosomes, transmembane cadherins are coupled to the cytoskeleton through a series of cytoplasmic protein–protein interactions. The desmosomal cadherins, desmogleins and desmocollins, are required for normal epidermal cell–cell adhesion by engaging in what are thought to be heterophilic interactions (Garrod *et al*, 2002a, b). The cytoplasmic domains of the desmosomal cadherins promote the association of these cadherins with intermediate filament networks through interactions with plakoglobin, desmoplakin, and the plakophilins (Kowalczyk *et al*, 1999a; Green and Gaudry, 2000; Garrod *et al*, 2002a). Similarly, adherens junctions couple actin filaments to classical cadherins, such as E-cadherin, through interactions with β-catenin and α-catenin (Wheelock and Johnson, 2003).

Previous studies have established an important role for armadillo family proteins in coupling cadherins to various cytoskeletal filaments and in regulating junction assembly (Hatzfeld, 1999). The armadillo family is defined by the presence of a 42 amino acid repeated motif termed an armadillo domain (Peifer *et al*, 1994). Plakoglobin and β-catenin are among the original founding members of this gene family, and both proteins bind directly to the cytoplasmic

Abbreviation: DP-NTP, desmoplakin amino-terminal polypeptide

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domain cadherins (Kowalczyk *et al*, 1999a). In addition, intercellular junctions contain proteins from a larger subfamily of armadillo gene products that includes p120ctn-related proteins and the plakophilins (Hatzfeld, 1999; Anastasiadis and Reynolds, 2000). This group of proteins is characterized by 10 central armadillo repeats and includes p120ctn, p0071, ARVCF, δ-catenin, and the plakophilins 1–3. The specific functions of many of these armadillo family proteins remain unclear, although it is likely that cells utilize this extensive repertoire of armadillo proteins to assemble functionally distinct junctions. For example, p120ctn assembles into actin-associated adherens junctions and regulates cadherin turnover (Davis *et al*, 2003; Xiao *et al*, 2003), whereas the plakophilins are thought to assemble predominantly into intermediate filament-based desmosomes (Hatzfeld, 1999; South *et al*, 2003; South, 2004). Plakophilin-1 is assembled into desmosomes in the upper layers of stratified epithelia and may play a role in increasing desmosome size and number (South, 2004). Furthermore, a rare skin disease, skin fragility-ectodermal dysplasia syndrome, results from mutations in the plakophilin-1 gene (McGrath *et al*, 1997). Thus, a growing body of literature supports the contention that the p120ctn/plakophilin family of armadillo proteins represent key modulators of intercellular junctions in a wide range of cell types.

p0071 is the only member of the p120ctn subfamily found to assemble constitutively into both adherens junctions and desmosomes (Hatzfeld *et al*, 2003). p0071 (also known as plakophilin-4) is characterized by the presence of 10 arm repeats, a coiled coil region in the head domain and a PDZ binding motif in the carboxyl-terminal tail domain (Hatzfeld and Nachtsheim, 1996). The dual targeting of p0071 to both adherens junctions and desmosomes raises the possibility that p0071 plays a role in the molecular cross-talk that is thought to occur between different classes of intercellular junctions, thus influencing a range of cellular events that are regulated by either the actin or intermediate filament cytoskeleton. The following studies were undertaken to determine whether p0071 regulates the balance between desmosome and adherens junction assembly. Our findings indicate that expression of p0071 in epithelial cells causes disassembly of desmosomes, enhanced assembly of adherens junctions, and the inhibition of epithelial cell migration.

Results

p0071 localizes to both adherens junctions and desmosomes in A431 epithelial cells p0071 has been shown previously to assemble into the intercellular junctions of a variety of cultured epithelial cell lines, including HeLa, A431, HaCat (Hatzfeld and Nachtsheim, 1996; Hatzfeld *et al*, 2003) and primary cultures of vascular endothelial cells (Calkins *et al*, 2003). To characterize further the subcellular localization of p0071, an affinity purified antibody directed against a unique peptide from the p0071 head domain was used to determine the distribution of p0071 in A431 epithelial cells by immunofluorescence microscopy. Endogenous p0071 localized to cell–cell contacts, colocalizing with the desmosomal protein, desmoplakin (Fig 1*A–C*), as well as with the adherens junction protein, β-catenin (Fig 1*D–F*). This dual localization confirms that p0071 can assemble into both adherens junctions and desmosomes (Hatzfeld *et al*, 2003).

Expression of p0071 displaces desmoplakin from epithelial cell junctions To test the effects of p0071 on desmosomal and adherens junction assembly, p0071 levels were increased in A431 epithelial cells using an adenoviral expression system. Cells expressing increased levels of p0071 exhibited a striking decrease in junctional staining for desmoplakin, suggesting a loss of desmosome assembly or stability (Fig 2*A–C*). These results were in contrast to the effects of other closely related armadillo family member

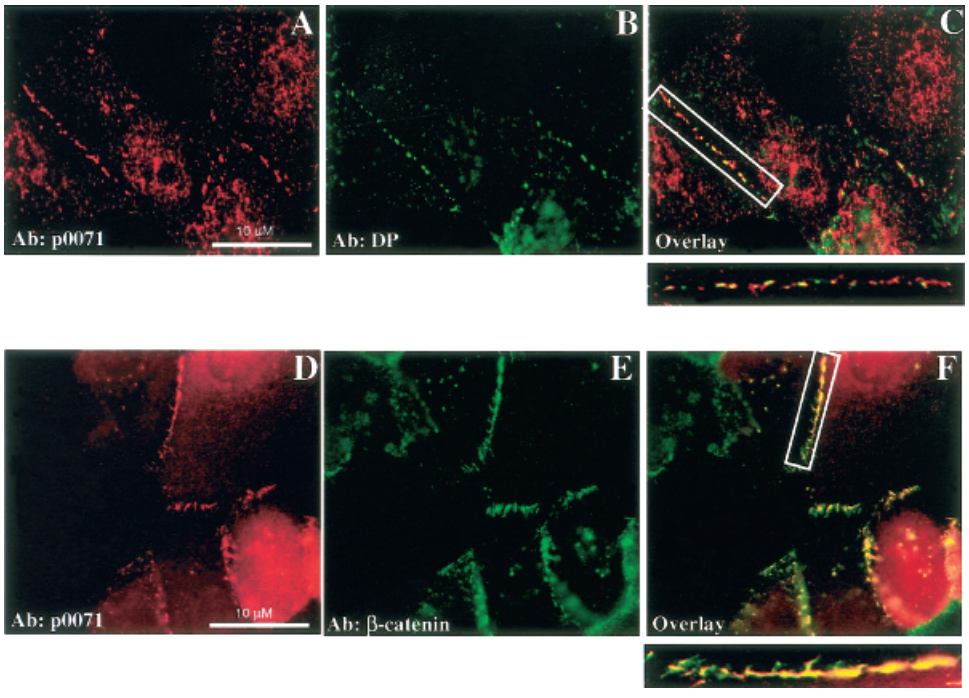

Figure 1
p0071 Localization in A431 cells. A431 cells were processed for immunofluorescence using a rabbit polyclonal antibody (Ab) directed against the head domain of p0071 and either desmoplakin (*A–C*) or β-catenin (*D–F*).

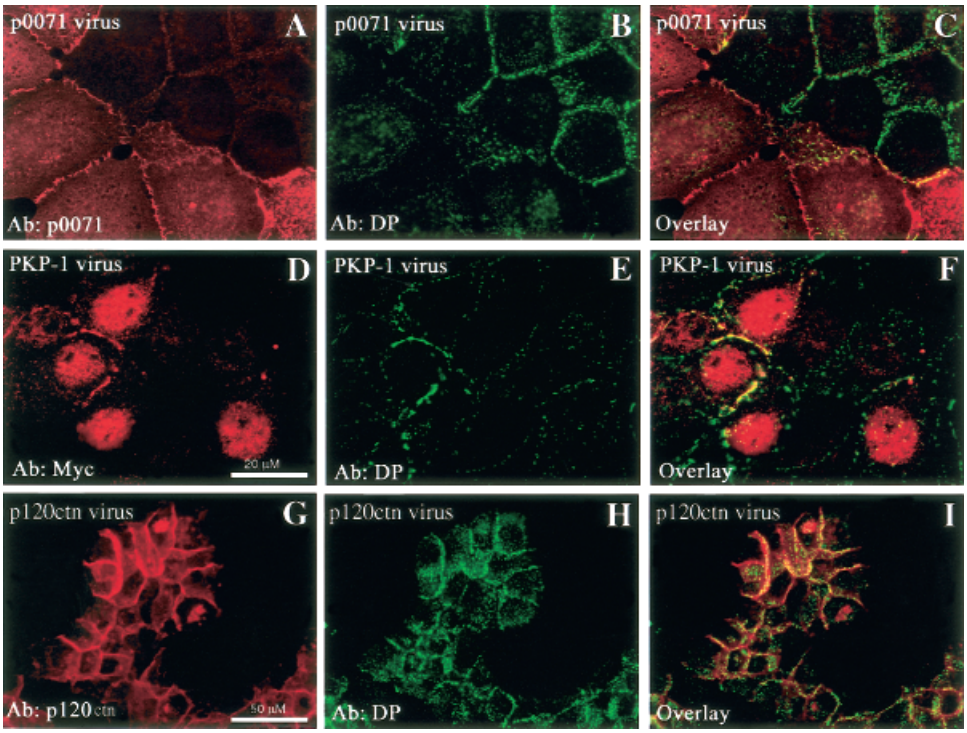

Figure 2
Enhanced expression of p0071 displaces desmoplakin from intercellular junctions. p0071 was expressed in A431 cells using an adenoviral delivery system and cells were examined 24 h post infection. p0071 expression resulted in the loss of desmoplakin from intercellular junctions (*A-C*). In contrast, desmoplakin localization at cell junctions was enhanced in cells expressing plakophilin-1 (*D–F*). Control adenoviral infection with p120ctn virus resulted in no change in desmoplakin localization at cell junctions (*G–I*).

proteins on A431 junction assembly. For example, plakophilin-1 expression caused a dramatic recruitment of desmoplakin to cell–cell junctions (Fig 2*D–F*) (Kowalczyk *et al*, 1999b; Hatzfeld *et al*, 2000; South *et al*, 2003), whereas p120ctn failed to cause any apparent change in junctional desmoplakin accumulation (Fig 2*G–I*). These results demonstrate that enhanced cellular levels of p0071 specifically disrupt desmosome assembly in A431 epithelial cells.

To further define the role of p0071 in desmosome assembly, the effects of p0071 expression on intercellular junction remodeling were examined in more detail. Expression of exogenous p0071 caused a dramatic loss of plakoglobin from regions of cell–cell contact (Fig 3*A–D*). Consistent with the loss of desmoplakin from intercellular junctions in cells expressing p0071 (Fig 2*A–C*) keratin filaments were retracted from regions of cell–cell contact where p0071 was concentrated (Fig 3*F–I*). In contrast to the effects of p0071 on desmosome assembly, p0071 expression enhanced junctional accumulation of E-cadherin (Fig 3*K–N*) and β-catenin (Fig 3*P–S*), suggesting that although desmosome assembly was inhibited, adherens junction assembly was promoted by p0071. Further characterization of the changes in A431 epithelial cell junction assembly in response to elevated levels of p0071 was performed using sequential detergent extraction. Western blot analysis demonstrated a shift in desmoplakin distribution from cytoskeletal- and membrane-associated fractions to cytosolic compartments (Fig 4). Conversely, β-catenin distribution shifted modestly from the membrane associated fraction to the cytoskeletal-associated fraction, consistent with increased β-catenin localization at intercellular junctions observed by immunofluorescence (Fig 3*Q–T*). Taken together, these data suggest that p0071 functions to promote adherens junction formation although acting as a negative regulator of desmosome assembly.

p0071 binds to amino acid residues 1–160 of the amino-terminal domain of desmoplakin To further investigate the mechanisms by which p0071 regulates junction assembly, p0071 interactions with other junctional components were examined. In previous studies, we and others found that the head domain of p0071 interacts directly with the desmoplakin amino-terminal domain (Calkins *et al*, 2003; Hatzfeld *et al*, 2003). To determine more precisely the range of amino acid residues within the desmoplakin amino-terminal region required for p0071 binding, several deletion constructs of the desmoplakin amino-terminal domain ($DP_{(1–160)}$, $DP_{(1–368)}$, and $DP_{(160–584)}$) were constructed and tested for interactions with p0071. In addition, these same regions of the desmoplakin amino–terminal domain were tested for interactions with plakoglobin. Using the yeast two-hybrid system, direct interactions between proteins were assayed by monitoring yeast colony growth in the absence of histidine and adenine (Fig 5*A*, *B*). Plakoglobin was found to interact with all three regions of the desmoplakin amino-terminal head domain, $DP_{(1–160)}$, $DP_{(1–368)}$, or $DP_{(160–584)}$ (Fig 5*B*). In contrast, p0071 bound to $DP_{(1–160)}$ and $DP_{(1–368)}$ but failed to bind to $DP_{(160–584)}$ (Fig 5*B*). Finally, p0071 also interacted directly with plakoglobin (Fig 5*B*). These results indicate that p0071 and plakoglobin bind to partially overlapping regions of desmoplakin, with p0071 interacting with the most amino-terminal region of the desmoplakin head domain (Fig 5*C*).

Effects of armadillo family proteins on the strength of A431 adhesion The mechanical strength of epithelial tissues depends upon the tethering of cytoskeletal networks to sites of intercellular adhesion. Previous work has demonstrated that expression of a desmoplakin dominant negative mutant, DP-NTP, leads to the uncoupling of keratin filaments from cell–cell contacts through the displacement of endo-

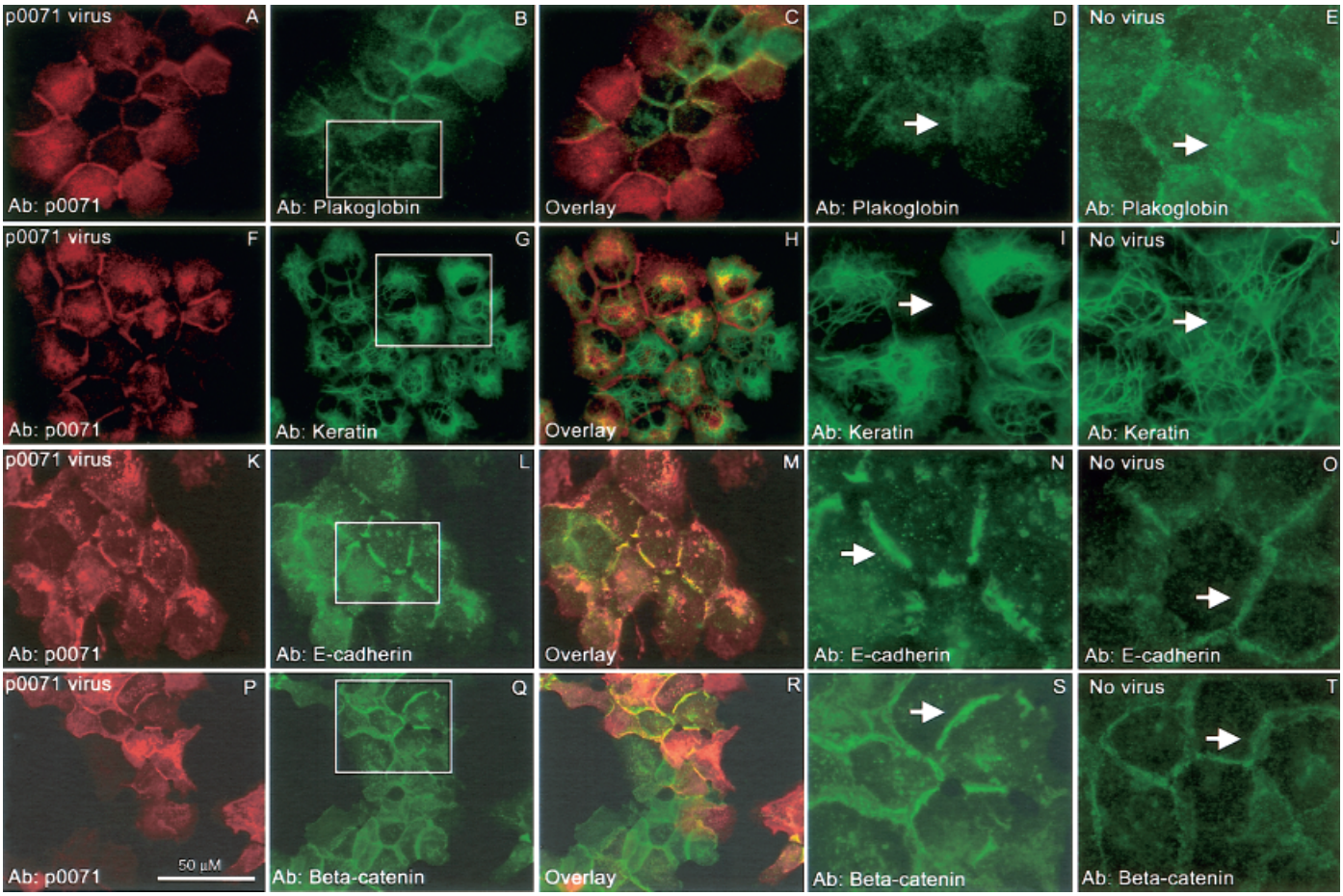

Figure 3
p0071 alters the localization of plakoglobin, keratin, β-catenin, and E-cadherin. A431 cells expressing elevated levels of p0071 were processed for immunofluorescence using antibodies directed against p0071, plakoglobin, cytokeratin, E-cadherin, and β-catenin. Elevated levels of p0071 displaced plakoglobin (*A–D*) from intercellular junctions. In addition keratin filaments are retracted from cell–cell contacts in cells with enhanced p0071 levels (*F–I*). Both E-cadherin (*K–N*) and β-catenin (*P–S*) are enhanced at cell junctions in cells expressing high levels of p0071. Higher magnification images of the boxed regions are shown in panels *D*, *I*, *N*, and *S*. For comparison, high magnification images of control cells lacking exogenous p0071 are shown in panels *E*, *J*, *O*, and *T*.

genous desmoplakin (Bornslaeger *et al*, 1996). Furthermore, the loss of desmoplakin leads to a dramatic decrease in the strength of adhesion between adjacent epithelial cells (Huen *et al*, 2002). As shown above, enhanced levels of p0071 displaced desmoplakin (Fig 2) and plakoglobin, and caused keratin filament retraction from sites of cell–cell contact (Fig 3), suggesting that p0071 may weaken epithelial cell–cell adhesion. Therefore, the adhesive strength of A431 cells expressing elevated levels of p0071 was tested (Fig 6). A431 cells with enhanced levels of p0071 were compared to cells with elevated levels of DP-NTP. For comparison, cells expressing elevated levels of either p120ctn or plakophilin-1 were also analyzed. As expected, A431 cells expressing DP-NTP demonstrated a significant decrease in adhesive strength, whereas both p120ctn and plakophilin-1 increased adhesive strength. Interestingly, cells expressing p0071 exhibited no change in adhesive strength relative to control cells. These results indicate that unlike DP-NTP, p0071 does not compromise overall strength of A431 cell–cell adhesion.

p0071 inhibits wound closure *in vitro* Many of the proteins associated with intercellular adhesion also have been implicated in cell motility (Anastasiadis and Reynolds, 2001), and modification of adhesive strength is thought to be a prerequisite for the initiation and maintenance of cell migration (Thiery, 2002). To assess whether p0071 alters A431 motility, we employed an *in vitro* scratch assay, in which a "wound" was introduced into confluent monolayers of A431 cells (Fig 7). Cells with elevated levels of p0071 were compared to cells with elevated levels of DP-NTP, p120ctn or plakophilin-1. Cells expressing the mutant desmoplakin polypeptide DP-NTP responded to the scratch by rapidly migrating into the wounded area. In contrast, cells with enhanced levels of either p120ctn or plakophilin-1 exhibited very little change in wound closure rates relative to control cells. Cells with enhanced levels of p0071, however, exhibited dramatically retarded ability to close the *in vitro* wound. Similar results were obtained when cells were treated with mitomycin C to prevent cell proliferation (not shown). These data suggest that disassembly of desmosomes by DP-NTP is associated with enhanced cell motility. In contrast, even though elevated levels of p0071 expression resulted in the disruption of desmosomes, cell motility was inhibited.

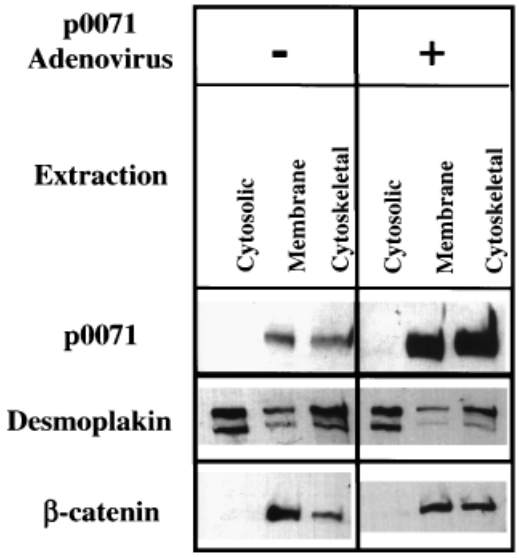

Figure 4
p0071 expression shifts desmoplakin from cytoskeletal to cytoplasmic pools. A431 cells infected with either p0071 virus or empty virus were subjected to sequential detergent extraction with saponin, Triton X-100, and SDS, separating the cellular components into cytosolic, membrane-associated and cytoskeletal-associated pools, respectively. Western blot analysis revealed that increased amounts of p0071 shifted desmoplakin from the cytoskeletal-associated pool to a cytoplasmic pool. In contrast, p0071 expression increased β-catenin accumulation in the cytoskeletal-associated pool.

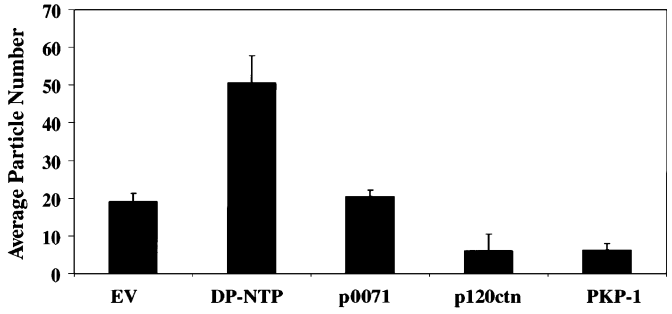

Figure 6
p120ctn and plakophilin-1 enhance adhesive strength of epithelial sheets. A431 cells were grown to confluence and infected with viruses carrying DP-NTP, p0071, p120ctn, or plakophilin-1. Intact monolayers of A431 cells were released from the culture dish via incubation with dispase and the degree of fragmentation of the monolayer was quantified after gentle rocking of the cell layer in a centrifuge tube. DP-NTP expression dramatically weakened A431 intercellular adhesion. In contrast, p120ctn and PKP-1 enhanced adhesive strength, whereas p0071 had no effect as compared to control cells expressing empty virus. Results from triplicate samples are shown with standard deviation and are representative of greater than five independently conducted experiments.

Discussion

The regulation of adhesion between epithelial cells is critical for normal tissue patterning, wound healing, and maintenance of adult tissue architecture. The loss of adhesion or a breakdown in the proper regulation of epithelial adhesion results in numerous human pathologies ranging from cancers to autoimmune bullous diseases of the skin (Thiery, 2002; Amagai, 2003; Wheelock and Johnson, 2003). The results of the present study suggest a key role for the armadillo family protein p0071 in regulating the balance between adherens junction and desmosome assembly. Furthermore, p0071 inhibited A431 cell migration into an *in vitro* wound, suggesting that the protein may play a role not only in modulating the nature of epithelial cell–cell contact, but also the motility characteristics of epithelial cells.

p0071 is the only member of the p120 family of armadillo proteins that has been found to constitutively associate with both adherens junctions and desmosomes (Fig 1*A–F*) (Hatzfeld and Nachtsheim, 1996; Hatzfeld *et al*, 2003). This property of p0071 is reminiscent of plakoglobin (Cowin *et al*, 1986; Cowin, 1994), suggesting that these two members of the armadillo family function specifically to regulate the cross-talk between adherens junctions and desmosomes.

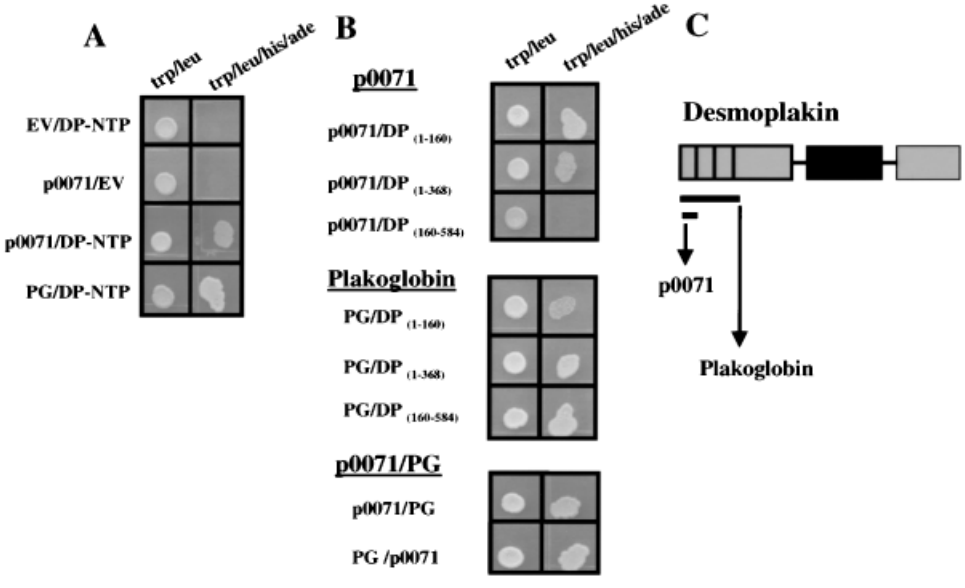

Figure 5
p0071 binds the first 160 amino acid residues of the amino-terminal region of desmoplakin. p0071 polypeptides were expressed as fusions with the Gal4 DNA-binding domain and tested for interactions either with the empty activation domain (AD) or with desmoplakin amino-terminal domain polypeptides fused to the Gal4 transactivation domain. Transformation of both the DNA-binding domain and the activation domain vectors was confirmed by growth in the absence of tryptophan and leucine. Yeast colony growth in the absence of histidine and adenine was used as a readout for protein interactions. (*A*) The first 584 amino acids of desmoplakin were found to interact with both plakoglobin and p0071. (*B*) Plakoglobin bound to all desmoplakin amino-terminal polypeptides tested, whereas p0071 bound only to the most amino-terminal region of desmoplakin. Furthermore, p0071 also bound directly to plakoglobin (*C*) Schematic summary of protein interactions with the desmoplakin amino-terminal domain observed using two hybrid analysis. PG, plakoglobin. EV, empty virus.

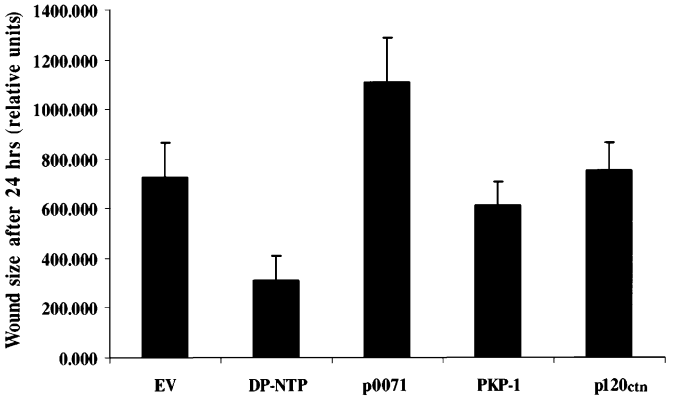

Figure 7
p0071 inhibits migration of A431 cells. Confluent monolayers expressing either DP-NTP, p0071, p120ctn, or plakophilin-1 (PKP-1) were wounded by a single linear pass across the monolayer with a sterile pipette tip. The initial size of the wound was determined and the degree of wound closure after 24 h was determined at multiple independent points along the wound edge (>15 measurements/wound). DP-NTP expressing cells demonstrated the highest rate of migration into the wound. p120ctn and PKP-1 did not cause observable differences in A431 migration relative to control cells, whereas p0071 inhibited migration of cells into the wound. Error bars represent standard deviation and results are representative of more than three independently conducted experiments.

Previous studies have demonstrated that plakoglobin, in a complex with E-cadherin, is required for the initiation of desmosome assembly in A431 cells (Lewis *et al*, 1997). In an analysis of the domains of p0071 that target the protein to adherens junctions or desmosomes, Hatzfeld *et al* (2003) suggested that high levels of p0071 in MCF-7 cells may favor adherens junction assembly over desmosome formation. In the current study, we tested this possibility and compared p0071 effects on junction assembly to the related proteins plakophilin-1 and p120ctn. Our findings strongly suggest that p0071 functions as a negative regulator of desmosome assembly. Interestingly, endogenous p0071 exhibited partial co-localization with desmosomes, but localized more strongly with adherens junction components (Fig 1). One explanation for this observation may be that as p0071 levels increase locally near intercellular junctions, adherens junction formation would be favored while desmosome formation would be hindered. Consistent with this hypothesis, increased p0071 expression in A431 epithelial cells resulted in the loss of membrane associated desmoplakin (Fig 2), similar to results reported in MCF-7 cells (Hatzfeld *et al*, 2003). A more comprehensive analysis of p0071 effects on junction assembly revealed that plakoglobin also was displaced from intercellular junctions, and intermediate filaments retracted from regions of cell–cell contact (Fig 3). These results demonstrate a dramatic disassembly of desmosomes and reorganization of keratin filaments in response to p0071. This function of p0071 is in striking contrast to plakophilin-1, which enhances desmoplakin association with desmosomes (Fig 2) (Kowalczyk *et al*, 1999b; Hatzfeld *et al*, 2000; South *et al*, 2003; South, 2004). Importantly, our results also indicate that p0071 does not globally inhibit cell–cell adhesion. p0071 expression enhanced junctional immunostaining of E-cadherin and β-catenin (Fig 3), indicating that p0071 promotes the assembly or stability of adherens junctions. The preferential assembly of adherens junctions with an associated loss of desmosomes occurred specifically in response to p0071 and not in response to other closely related armadillo family proteins.

The mechanisms whereby p0071 promotes adherens junction assembly while simultaneously disassembling desmosomes are not well understood. In the desmosome, plakoglobin binds the amino-terminal region of desmoplakin (Kowalczyk *et al*, 1997; Smith and Fuchs, 1998). This interaction is likely to be important in targeting desmoplakin to desmosomes (Stappenbeck *et al*, 1993). Based on this important interaction and the ability of p0071 to displace plakoglobin from cell–cell junctions, we directly compared the ability of p0071 and plakoglobin to interact with specific regions of the desmoplakin amino-terminal domain using yeast two-hybrid analysis. Plakoglobin interacted with a broad region of the desmoplakin amino-terminal head domain, whereas p0071 bound to the first 160 amino acids of desmoplakin (Fig 5). Thus, the p0071 and plakoglobin binding sites on desmoplakin only partially overlap. Furthermore, similar to Hatzfeld *et al* (2003), we found that p0071 binds directly to plakoglobin (Fig 5). Based on these analyses of interactions between p0071 and various desmosomal binding partners, it is likely that p0071 regulates desmosomal assembly either by sequestering plakoglobin from the desmosome or by interfering with the plakoglobin–desmoplakin interaction.

The potential functional effects of a cellular shift in junction assembly from desmosomes toward adherens junctions in response to p0071 expression was examined using a dispase cell–cell adhesion assay and a wound-healing migration assay. Surprisingly, although p0071 disrupted desmosomes, the overall adhesive properties of A431 cells remained unchanged (Fig 6). As reported previously (Bornslaeger *et al*, 1996; Huen *et al*, 2002), the disruption of desmosomes by the mutant desmoplakin polypeptide DP-NTP resulted in a significant loss of adhesion between A431 cells (Fig 6). In contrast to p0071, p120ctn, and plakophilin-1 both increased the strength of adhesion. Collectively, these results likely reflect the combined effects of both adherens junctions and desmosomes on overall adhesive strength. For example, DP-NTP causes loss of desmosomes, but does not increase adherens junction assembly (Huen *et al*, 2002). Conversely, both p120ctn and plakophilin-1 increase assembly or stability of either adherens junctions or desmosomes, respectively, resulting in a net increase in strength of adhesion. p0071 has dual effects on these junctions causing the loss of desmosomes while simultaneously increasing adherens junction assembly. It is possible that the lack of any observable effect of p0071 on overall adhesion strength is due in part to the compensating effects of increased adherens junction assembly in the face of desmosomal disassembly.

Analysis of migration indicated that p0071 inhibits migration of A431 epithelial cells. p120ctn and plakophilin-1 expression caused no detectable change in A431 cell shape or wound closure. This result was somewhat surprising since p120 has been implicated previously in the regulation of cell migration (Noren *et al*, 2000; Grosheva *et al*, 2001). In contrast to p0071, the disruption of desmosomes by DP-NTP resulted in dramatically increased migration of cells into the wounded area (Fig 7). These results are consistent

with the notion that desmosomes function as a brake on cell migration, and that DP-NTP can be used to release this brake and stimulate migration. In an apparent paradox, p0071 disassembled desmosomes and caused keratin filament retraction, but simultaneously reduced migration of A431 cells into the wound. It is possible that p0071 inhibits cell motility by enhancing the stability of adherens junctions. It is also possible, however, that p0071 directly alters actin cytoskeletal dynamics outside of the context of intercellular junction assembly. Similar to p120ctn, p0071 expression at high levels causes cell shape changes (Calkins, unpublished observations) that have been attributed to the regulation of Rho family GTPases (Anastasiadis *et al*, 2000; Noren *et al*, 2000; Grosheva *et al*, 2001; Cozzolino *et al*, 2003). Interestingly, Papagerakis *et al* (2003) recently demonstrated that the loss of p0071 correlated with increased tumor size in primary oropharyngeal carcinomas. Although the mechanisms by which p0071 may influence tumor progression are largely unknown, the findings in the present study suggest that alterations in p0071 expression patterns may be of significance not only in tumor progression but also in other epithelial functions dependent upon dynamic alterations in cell adhesion and motility.

Materials and Methods

Cell lines The human epidermoid carcinoma cell line A431 was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Hyclone Laboratories, Logan, Utah) and penicillin/streptomycin/amphotericin B (PSF, Invitrogen, Carlsbad, California). For adenovirus production, a human embryonic kidney cell line QBI-HEK 293A (Qbiogene, Carlsbad, California) was routinely cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin/amphotericin B. Cells were typically fixed and processed for immunofluorescence or biochemical analysis after 24–48 h.

cDNA Constructs and transfections A series of desmoplakin deletion mutants including $DP_{(1–160)}$, $DP_{(1–368)}$, and $DP_{(160–584)}$ was generated as follows. DP-NTP (Bornslaeger *et al*, 1996; Kowalczyk *et al*, 1997), which encodes the first 584 amino acids of the desmoplakin amino-terminal domain, was digested with *Eco*RI/*Xho*I to generate $DP_{(1–160)}$, or digested with *Xho*I/*Hin*dIII to generate $DP_{(160–584)}$. $DP_{(1–368)}$ was generated via PCR using a 5′-primer (5′-CGG GAA TTC ATC GAT GCC GCC ATG GTT AGC TGC AAC GGA), which generates an *Eco*RI site (underlined) at the 5′-end of the construct, and the 3′-primer (5′-CCC AAG CTT CTA CTT ATC GTC GTC ATC CTT GTA ATC AAT GCA CTA GGT GAT CTG AAG), which generates a *Hin*dIII site (underlined) at the 3′-end of the construct. These constructs were subcloned into donor vectors of the Creator system (Clontech, Palo Alto, California). All constructs were verified by sequencing.

Adenovirus production Full-length p0071 (Calkins *et al*, 2003), a myc-tagged plakophilin-1 (Bornslaeger *et al*, 2001) and FLAG tagged DP-NTP were subcloned into the pAd-Track vector, which coexpresses GFP with the cDNA of interest (He *et al*, 1998). p120ctn adenovirus was generated as described previously (Xiao *et al*, 2003). Adenoviruses were produced using the pAdeasy adenovirus-packaging system and high titer virus was generated as described elsewhere (Xiao *et al*, 2003). Infection rates for immunofluorescence analysis averaged 50%–60%, while infection rates used for western blotting were 80%–90%. Rates of infection were determined visually using GFP, which is co-expressed with the protein of interest.

Immunofluorescence A431 cells cultured on glass coverslips were fixed in methanol on ice for 5 min or 3.7% paraformaldehyde on ice for 5 min followed by extraction in ice-cold 0.5% Triton X-100 for 5 min. Endogenous p0071 was detected using 104PKP-4, a rabbit polyclonal antibody raised against a peptide from the p0071 head domain: "N"-RVSDAVQPNNYLIRTEPRQ-"C" (Bethyl Laboratories, Montgomery, Texas). A chicken polyclonal antibody, Chicken-7, was raised against a GST-fusion protein containing human p0071 amino acid residues 1–508 including a C-terminal FLAG tag (Aves Laboratories, Tigard, Oregon) and was used for western blot analysis. Localization of junctional proteins utilized the following antibodies: mouse anti-desmoplakin 1 and 2, mouse anti-p120ctn, mouse anti-plakoglobin, and mouse anti-E-cadherin (all from Transduction Laboratories, San Diego, California). Cytokeratin was detected using a mouse anti-cytokeratin antibody (Immunotech, Marseille, France). Myc-tagged plakophilin-1 was detected using a rabbit anti-myc antibody (Bethyl Labs). Secondary antibodies conjugated to various Alexa Fluors (Molecular Probes, Eugene, Oregon) were used for dual label immunofluorescence. Microscopy was performed using a fluorescence microscope (Leica DMR-E Leica, Wetzlar, Germany) equipped with narrow band pass filters and a Hamamatsu Orca digital camera (Orca, Bridgewater, NJ). Images were captured, pseudo-colored, and processed using Open Lab software (Improvision, Lexington, Massachusetts). The cells were fixed and processed for immunofluorescence analysis 24 h after adenoviral infection.

Yeast two-hybrid assays Yeast two-hybrid vectors encoding the GAL4 DNA binding (pLP-GBK) or transcription activation domain (pLP-GAD) were purchased from BD Biosciences, Clontech (Palo Alto, CA). The desmoplakin deletion constructs described above ($DP_{(1–160)}$, $DP_{(1–368)}$, and $DP_{(160–584)}$) were subcloned from the Creator system donor vector into either of the Creator system acceptor vectors (pLP-GBK and pLP-GAD) and then verified by sequencing. p0071, plakoglobin, and the desmoplakin deletion constructs were generated as described previously (Calkins *et al*, 2003). p120ctn cDNA was kindly provided by A.B. Reynolds (Vanderbilt School of Medicine, Nashville, Tennessee). To assay interactions between proteins, 5–10 μg plasmid DNA was transformed into the yeast strain AH109 (Clontech) using standard LiAc transformation. Double transformants were selected by growth on plates lacking leucine and tryptophan. Expression of the *HIS* and *ADE* reporter genes were analyzed as a reporter for protein interactions by monitoring colony growth on plates lacking histidine, adenine, leucine, and tryptophan.

Sequential detergent extraction and western blot analysis Sequential detergent extraction was performed as described (Palka and Green, 1997) with the following exceptions. Cells were grown to confluence on 100 mm plates and extracted for 10 min on ice in 400 μL saponin buffer (0.01% saponin, 10 mM Tris, pH 7.5, 140 mM NaCl, 5 mM EDTA, 2 mM EGTA, 1 mM PMSF, 1 μg per mL leupeptin, 1 μg per mL pepstatin A). Cells were scraped using a rubber policeman and centrifuged at approximately 14,000 × *g* for 30 min at 4°C. After centrifugation, the saponin-soluble pool was transferred to a fresh tube. The amount of buffer used at each step was adjusted so that the final volume of each pool was 500 μL. The remaining pellet was extracted in 500 μL cold Triton X-100 buffer (1% Triton X-100, 10 mM Tris, pH 7.5, 140 mM NaCl, 5 mM EDTA, 2 mM EGTA, 1 mM PMSF, 1 μg per mL leupeptin, 1 μg per mL pepstatin A). Samples were vortexed for 30 s and centrifuged at 14,000 × *g* for 30 min at 4°C. The Triton X-100-soluble pool was transferred to a fresh tube. The Triton-insoluble proteins were solublized in 50 μL SDS buffer (1% SDS, 10 mM Tris, pH 7.5, 140 mM NaCl, 5 mM EDTA, 2 mM EGTA, 1 mM PMSF, 1 μg per mL leupeptin, 1 μg per mL pepstatin A). The samples were analyzed by SDS-PAGE followed by immunoblot analysis using chemiluminescence (ECL, Amersham Pharmacia Biotech, Piscataway, NJ).

Dispase-based dissociation assay A dispase dissociation assay was performed as described previously (Huen *et al*, 2002). Briefly,

A431 cells were seeded in triplicate onto 35 mm dishes at confluent densities. At 24 h post seed, cells were treated with adenovirus expressing p0071, DP-NTP, plakophilin-1, or p120ctn. 24 h post infection, cultures were washed twice in phosphate-buffered saline and then incubated in 1 mL of 1 U per mL dispase (Roche Chemical, Indianapolis, Indiana) for 30 min at room temperature. Released monolayers were transferred to 15 ml centrifuge tubes and the volume brought to 5 mL with DPBS. Samples were placed on a platform rocker and subjected to 50 tilt cycles at a 45° angle. Monolayer fragments were counted using a dissection microscope.

Wound-healing assay To determine cell motility A431 cells were seeded onto 35 mm dishes and grown to confluence. Cells were infected with various adenoviruses 24 h after seeding. The confluent monolayer was wounded using a sterile disposable pipette tip with aspiration 24 h post infection. The degree of wound closure over time was measured using Open Lab image analysis software using images obtained immediately after scraping and then again at 24 h post wounding. All experiments were done in triplicate.

The authors are grateful to Drs A. Reynolds and M. Hatzfeld for sharing cDNA reagents. Funding for this study was provided by National Institutes of Health grants to A.K. (R01 AR048266) and K.J.G. (P01DE012328, Project 4). S.V.S. was supported by NIH T32AR007587. Additional funding was provided by the Emory Skin Diseases Research Center (NIH P30AR042687).

DOI: 10.1111/j.0022-202X.2004.23319.x

Manuscript received March 18, 2004; revised May 7, 2004; accepted for publication May 12, 2004

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