Plectin-Isoform-Specific Rescue of Hemidesmosomal Defects in Plectin (–/–) Keratinocytes

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The various plectin isoforms are among the major crosslinking elements of the cytoskeleton. The importance of plectin in epithelia is convincingly supported by the severe skin blistering observed in plectin-deficient humans and mice. Here, we identified plectin 1a (> 500 kDa), a full length plectin variant containing the sequence encoded by the alternative first exon 1a, as the isoform most prominently expressed in human and mouse keratinocytes. In skin sections and cultured keratinocytes, plectin 1a was shown to colocalize with hemidesmosomal structures. In contrast, a second isoform expressed in epithelia, plectin 1c, differing from 1a merely by a short N-terminal sequence, colocalized with microtubules. Expression of plectin 1a, but not of its N-terminal fragment alone, or of a third alternative full length isoform (plectin 1), restored the reduced number of hemidesmosome-like stable anchoring contacts in cultured plectin-null keratinocytes. Our results show for the first time that different isoforms of a cytolinker protein expressed in one cell type perform distinct functions. Moreover, the identification of plectin 1a as the isoform defects in which cause skin blistering in plectin-related genetic diseases, such as epidermolysis bullosa simplex MD and epidermolysis bullosa simplex Ogna, could have implications for the future development of clinical therapies for patients. ***Key words: cytolinkers/epidermolysis bullosa simplex/muscular dystrophy/plakins/plectin isoforms. J Invest Dermatol 120:189–197, 2003***

Plectin, one of the major crosslinking elements of the cytoskeleton, belongs to a family of structurally and in part functionally related proteins referred to as plakins or cytolinkers (for review see Wiche, 1998; Fuchs and Karakesisoglou, 2001; Leung *et al*, 2002). Plectin mediates network formation of intermediate filaments (IFs), the interlinking of IFs with microtubules and microfilaments, as well as the anchoring of IFs to the plasma and nuclear membrane. A novel important role of plectin as regulator of actin cytoskeleton dynamics has recently been demonstrated (Andrä *et al*, 1998).

There is growing evidence for an essential role of plectin in epithelial tissues. In stratified epithelia the protein has been localized at basal cell surface membranes and in peripheral areas of epithelial cells in all cell layers (Wiche *et al*, 1984). It is found associated with hemidesmosomes and cytokeratin filaments in the basal epithelial compartment of skin, and with desmosomal structures in polarized simple epithelial cells. Direct interactions of plectin with the hemidesmosomal integrin subunit β4 at multiple molecular sites (Rezniczek *et al*, 1998; Nievers *et al*, 2000) and with desmoplakin (Eger *et al*, 1997) have been demonstrated.

The importance of plectin in epithelia has also been demonstrated convincingly in plectin knock-out mice. These animals exhibit severe skin blistering combined with a reduction in the number of hemidesmosomes, and in addition they show abnormalities in skeletal and heart muscle (Andrä *et al*, 1997). Similar disorders were found in patients suffering from epidermolysis bullosa simplex (EBS) MD. This autosomal recessive disease, caused by mutations in the plectin gene, is characterized by intraepidermal blistering at the level of basal keratinocytes associated with late-onset muscular dystrophy (for review see Rouan *et al*, 2000). A dominant form of the disease, EBS-Ogna, leads to skin changes in the absence of muscular symptoms in the patients (Koss-Harnes *et al*, 2002).

Cloning and sequencing of rat, human, and mouse plectin cDNA (Liu *et al*, 1996; McLean *et al*, 1996; Fuchs *et al*, 1999) revealed a polypeptide chain sequence that is consistent with a three-domain structural model, where a long central rod domain, having an α-helical coiled-coil conformation, is flanked by globular N- and C-terminal domains. The N-terminal domain of plectin contains an actin-binding domain (ABD) shared by a large superfamily of actin-binding proteins (Stradal *et al*, 1998). The ABD of plectin has recently been found to be multifunctional, binding not only to actin but also to the integrin subunit β4 (Rezniczek *et al*, 1998; Geerts *et al*, 1999) and to itself (Fontao *et al*, 2001); another integrin β4 binding site has been mapped to the C-terminal domain of plectin (Rezniczek *et al*, 1998). In addition, the C-terminal part of the protein harbors an IF-binding site that was shown to mediate the interaction of plectin with vimentin and cytokeratins (Nikolic *et al*, 1996; Steinböck *et al*, 2000). A detailed analysis of the genomic exon-intron organization of the murine plectin gene revealed an unusual 5′ transcript complexity of plectin isoforms, with a total of 11 alternatively spliced first exons upstream of their common exon 2 (Fuchs *et al*, 1999).

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Abbreviations: ABD, actin-binding domain; FAC, focal adhesion contact; IF, intermediate filament; SAC, stable anchoring contact.

In this study we identified plectin 1a, the isoform containing sequences encoded by the alternative first exon 1a, as the isoform most prominently expressed in human keratinocytes. Using isoform-specific antibodies, plectin 1a was shown to localize specifically to hemidesmosomal structures in mouse skin sections and cultured mouse keratinocytes, in contrast to the other major isoform expressed in keratinocytes, plectin 1c. Only full length versions of plectin 1a but not of a third alternative isoform (plectin 1) were found sufficient to restore the reduced number of hemidesmosome-like stable anchoring contacts (SACs), a feature characteristic of plectin-deficient keratinocytes. This is the first time that different isoforms of plectin, and of cytolinkers in general, are shown to have distinct subcellular localization and presumably functions within one cell type (keratinocytes). We suggest that plectin 1a is the specific isoform responsible for the skin blistering symptom observed in plectin-related hereditary diseases.

MATERIALS AND METHODS

Cell culture Immortalized plectin (+/+) and (−/−) mouse keratinocyte cell cultures were established from plectin (+/+)/p53 (−/−) and plectin (−/−)/p53 (−/−) mice, which were obtained from plectin (+/−)/p53 (−/−) intercrosses (Metz *et al*, 1995). This method generates a defined mutation (absence of p53) that facilitates immortalization of cells that otherwise are difficult to immortalize. Mice carrying a mutated allele of plectin were generated as described previously (Andrä *et al*, 1997), and p53 (−/−) blastocysts were kindly provided by E. Wagner (IMP, Vienna, Austria). Keratinocyte cell lines were established from 1-d-old mice and routinely cultured as described previously (Troy and Turksen, 1999). Human primary keratinocytes were kindly provided by I. Schuster (Novartis, Vienna, Austria) and E. Tschachler (AKH, Vienna, Austria).

Antibodies The following primary immunoreagents were used: rabbit antiserum (no. 9) raised against a recombinant rat plectin protein fragment corresponding to exons 9–12 (N-term.), and mouse antiserum (123C) to a recombinant fragment corresponding to the C-terminal repeat 5 domain of rat plectin (C-term.), both prepared and kindly provided by B. Nikolic (this laboratory); isoform-specific affinity-purified rabbit antisera to an exon 1a peptide (anti-E1a) (Rezniczek *et al*, 1998), and to a GST-fusion protein containing the N-terminal sequence M_1–S_{17} of plectin 1c (12); rabbit antiplectin antiserum (no. 46) (Wiche *et al*, 1984); rat monoclonal antihuman integrin β4 antibody (346–11 A) kindly provided by S.J. Kennel, Oak Ridge Laboratories, TN; rat monoclonal antihuman integrin α6 (GoH3, PharMingen, San Diego, CA); mouse monoclonal antivinculin (clone vin 11–5) and antitubulin (clone B-5-1-2) antibodies (both from Sigma, St. Louis, MO); and undiluted hybridoma supernatant of monoclonal antimyc 1–9E10.2 antibody (ATCC Manassas, VA). As secondary antibodies horseradish-peroxidase-labeled goat antimouse and goat antirabbit IgGs (Bio-Rad, Hercules, CA) were used for immunoblotting, and Texas Red conjugated antibodies (Jackson Immuno-Research Laboratories, West Grove, PA) and Alexa Fluor™ 488 antibodies (Molecular Probes, Eugene, OR) were used for immunofluorescence microscopy.

RNase protection assay Isolation of total RNA, RNase protection analysis, and quantification of relative signal intensities were performed as described previously (Fuchs *et al*, 1999). Probes specific to human exons 1a (GenBank accession no AF330791) were derived from plectin genomic clones HPG24, HPG25, and HPG29 (Liu *et al*, 1996) by degenerative polymerase chain reaction (PCR). A probe specific to exon 1 was subcloned from human plectin cDNA clones pCGL25 and pCGL53 (Liu *et al*, 1996). The E1c-specific riboprobe was PCR-amplified from human brain QUICK-Clone™ cDNA (Clontech Laboratories, Palo Alto, CA). A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) specific probe (gift from M. Busslinger, IMP, Vienna, Austria) was truncated to produce a signal short enough to serve as loading control.

Immunoblotting Cells were lyzed in 20 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 2.5 mM ethylenediamine tetraacetic acid, 2.5 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, and protease inhibitors. Samples were subjected to sodium dodecyl sulfate 4% polyacrylamide gel electrophoresis; transfer and detection of proteins were performed as described previously (Eger *et al*, 1997).

Transient transfection experiments Transfections of immortalized plectin (−/−) keratinocytes were performed using LIPOFECTAMINE™ reagent (Life Technologies, San Diego, CA) according to the manufacturer's instructions. Plasmids pBK23 and pGR99 encoding plectin fragments corresponding to exons 1a–14 (Andrä *et al*, 1998) and exons 2–15, respectively, and pIK25, pIK26, and pPF50, each encoding full length versions of rat plectin starting with exon 1a-, exon 1-, and exon 1c-specific sequences, respectively, were used for transfections. pIK25 was generated by excision of a full length (13.7 kb) rat plectin cDNA fragment from plasmid pIK14 (obtained by combining PCR-amplified and plectin cDNA fragments; I. Kornacker, 2000, PhD thesis, University of Vienna) and cloning into expression vector pIK23. pIK23 was derived from a pBluescript®II KS + vector (Stratagene, La Jolla, CP), which contained the human K14 promoter, the generic intron, and the human growth hormone 3′ UTR/polyadenylation site of vector pK14iA (Kucera *et al*, 1996). pIK26 was generated by excision of full length (14.1 kb) rat plectin isoform 1 cDNA from plasmid pBN13 (I. Kornacker, 2000), and cloning into pIK23. The cDNA sequence encoding full length plectin 1c was PCR-cloned using mouse cDNA (Marathon-Ready, Clontech). pPF50 was generated by subcloning this sequence into a modified expression plasmid pEGFPN2 (Clontech) in which the CMV promoter was removed and replaced by the human K14 promoter used in pIK23. All expression plasmid inserts were fully sequenced.

Immunofluorescence microscopy Cells and 2 μm cryosections of murine skin were processed for immunofluorescence microscopy as described previously (Rezniczek *et al*, 1998). Specimens were viewed in an LSM 510 laser scanning microscope (Zeiss, Oberkochen, Germany).

RESULTS AND DISCUSSION

The major isoforms of plectin expressed in primary keratinocytes are full length versions of plectin 1a and plectin 1c In a recent study on plectin isoform expression in mouse tissues, a dominance of exon-1a-containing isoforms was observed in epithelial tissues (Fuchs *et al*, 1999). Confirming this, we found, using quantitative RNase protection assays, that the predominant plectin isoforms expressed in primary human keratinocytes contained exon 1a sequences (71% of all transcripts detected), followed by variants containing exon 1c (22%), exon 1 (4%), and exon 1b (1%) sequences (**Fig 1*A, B***). To distinguish the two major isoforms plectin 1a and plectin 1c, and dissect their possible functions *in vivo*, we generated plectin-1c-specific antibodies (anti-E1c) to be used along with previously prepared antibodies (Rezniczek *et al*, 1998) specifically recognizing isoform 1a (anti-E1a). First we assessed whether plectin 1a and plectin 1c were expressed in mouse keratinocytes as full length versions of the protein. In analysing cell lysates from immortalized plectin (+/+) and plectin (−/−) keratinocytes we used two antisera (N-term. and C-term.), recognizing epitopes in N- or C-terminal domains of plectin that are common to all plectin isoforms, in addition to the isoform-specific antibodies. All four antisera detected proteins with an apparent molecular mass of ≈500 kDa in plectin (+/+) but not plectin (−/−) cells (**Fig 1*C***). This clearly demonstrated that in keratinocytes both isoforms were expressed as full length versions of the protein. Proteins of smaller molecular mass were not detected on these blots.

Differential distribution of plectin isoforms 1a and 1c in keratinocytes: association of plectin 1a with hemidesmosomal structures On cryosections of plectin wild-type mouse skin, antiplectin 1a (E1a) antibodies conspicuously stained the basement membrane of the epidermis (**Fig 2*A***). Intriguingly, a punctated nature of this staining was revealed on grazing sections of the basal keratinocyte cell surface at high magnification (**Fig 2*D***). Similar results were obtained when antiserum no. 46 with immunoreactivity predominantly towards the rod domain of plectin was used (**Fig 2*B, E***). Additionally, a weak plectin-specific staining of suprabasal epithelial cell layers was observed. In contrast, plectin 1c was prominently expressed at the periphery of cells in the suprabasal layers of the epidermis (**Fig 2*C***) and was detected only at low levels in basal keratinocytes. The punctated staining pattern at the basal cell surface characteristic of plectin 1a was not detectable with

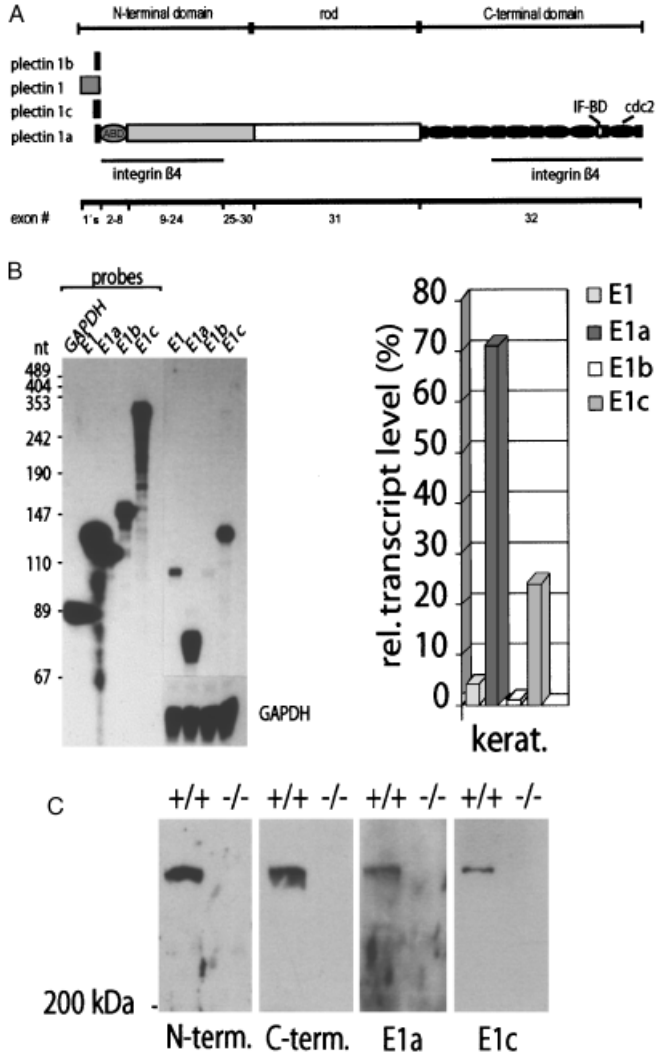

Figure 1. Expression of plectin transcripts and full length plectin isoforms 1a and 1c in keratinocytes. (*A*) Schematic representation of plectin subdomains, isoforms, and exon allocation. Segments to which functions have been assigned are indicated: ABD, actin binding domain, integrin β4 binding sites; IF-BD, intermediate filament binding site; cdc2, phosphorylation site for $p34^{cdc2}$. (*B*) RNase protection analyses of plectin transcripts containing distinct first coding exons in keratinocytes. Total RNA (10 μg) was analyzed using riboprobes specific to exons 1 (E1), 1a (E1a), 1b (E1b), and 1c (E1c). The GAPDH-specific riboprobe was used to control RNA loading. Riboprobes and protected fragments are shown. Length of protected fragments: E1, 108 nt; E1a, 87 nt; E1b, 115 nt; E1c, 149 nt; GAPDH, 53 nt (*left panel*). Autoradiography signals were quantified using a PhosphorImager. Values obtained for radiation levels over background were normalized for RNA loading and the radioactive guanine content of the riboprobes. A transcript level of 100% refers to the sum of values obtained for exons 1-, 1a-, 1b-, and 1c-containing transcripts (*right panel*). nt, nucleotides; rel., relative; kerat., primary keratinocytes. (*C*) Western blotting of cell lysates of immortalized plectin (+/+) and plectin (−/−) mouse keratinocytes using antisera to the N-terminal (N-term.; no. 9), the C-terminal (C-term.; 123C), and the exon-1a- (E1a) and exon-1c-encoded (E1c) domains of plectin.

anti-E1c antibodies (**Fig 2*F***). As the staining patterns observed with antiserum 46 showed the features of both isoforms combined, these data suggested that isoforms 1a and 1c both contain the rod domain of the protein, but can be distinguished by their distinct N-termini. Plectin (−/−) control keratinocytes were negative in all cases (**Fig 2*G–I***). The immunolocalization of plectin 1a on cryosections of mouse skin was consistent with immunogold electron microscopy data revealing plectin 1a

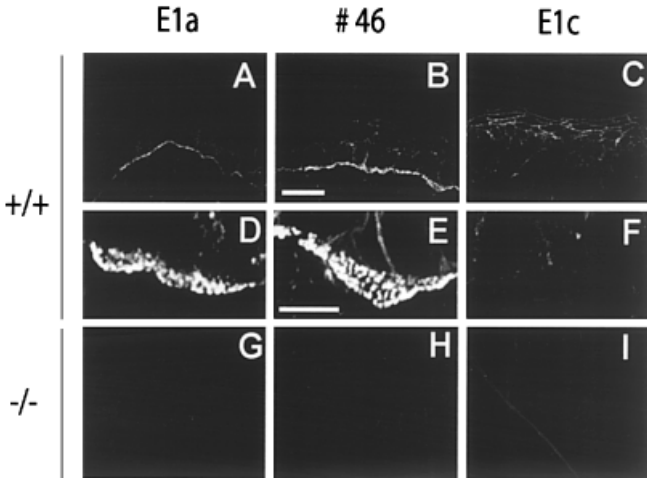

Figure 2. Immunofluorescence microscopy of cryosections of murine plectin (+/+) and (−/−) skin using anti-E1a, anti-E1c, and anti-full length plectin (no. 46) antisera. *Bars* in (*B*) (representative of *A–C* and *G–I*) and (*E*) (representative of *D–F*), 20 and 5 μm, respectively.

epitopes at hemidesmosomal structures and in association with cytokeratin filaments in the basal epithelial compartment of rat skin. Plectin 1a is probably targeted to these structures via its integrin β4 binding site(s) and anchored to keratin filaments via its terminal IF-binding site (Rezniczek *et al*, 1998).

To examine the intracellular localization of plectin isoforms 1a and 1c in epithelial cells in more detail, we performed immunofluorescence microscopy of immortalized plectin (+/+) and plectin (−/−) mouse keratinocytes. Plectin was found to colocalize with arrays of circular structures that were 1–4 μm in diameter and varied in numbers depending on cell size (**Fig 3**). These structures resembled SACs, which have been shown to bear functional and compositional similarities to skin hemidesmosomes (Carter *et al,* 1990). Indeed, using antiplectin 1a antibodies in combination with antibodies to the hemidesmosomal integrin subunit proteins β4 and α6, we observed colocalization of plectin 1a with both of these proteins (**Fig 3*A–C*, *D–F***, respectively). This colocalization appeared to be partial, possibly reflecting a heterogeneity of SACs and/or a dependence of the recruitment of plectin 1a on the assembly state or stability of these structures. SAC structures were also assembled in plectin-deficient cells (**Fig 3*H***); however, a statistical analysis in cell cultures of the same passage number revealed that only 12% of plectin (−/−) keratinocytes had developed these structures, compared to 50% of plectin (+/+) keratinocytes (**Fig 3*J***). The expression levels of integrin β4, as assessed by immunoblotting of cell lysates, were unchanged in plectin (−/−) compared to keratinocytes (data not shown). This indicated that the loss of plectin does not affect the expression or stability of other integral SAC components, but affects SAC formation.

A previous comparison of fibroblasts derived from plectin (+/+) and (−/−) mice and of keratinocytes from normal individuals and patients suffering from EBS-MD (Andrä *et al*, 1998; Nievers *et al*, 2000) had revealed an augmentation of focal adhesion contacts (FACs) in the plectin-deficient cells. Similarly, we found an increase in the number of vinculin-positive FACs in immortalized mouse plectin (−/−) keratinocytes compared to (+/+) control cells (**Fig 4*G***; compare also ***B*** and ***E***). The generation of more FACs in plectin (−/−) keratinocytes might result from a compensatory mechanism that cells use to cope with a reduced adhesion to the substratum elicited by the decrease or absence of SACs. Interestingly, double staining of plectin 1a and vinculin revealed that plectin 1a hardly colocalized with FACs (**Fig 4*A–C***). This suggested that the association of plectin 1a with SACs in cultured keratinocytes was specific.

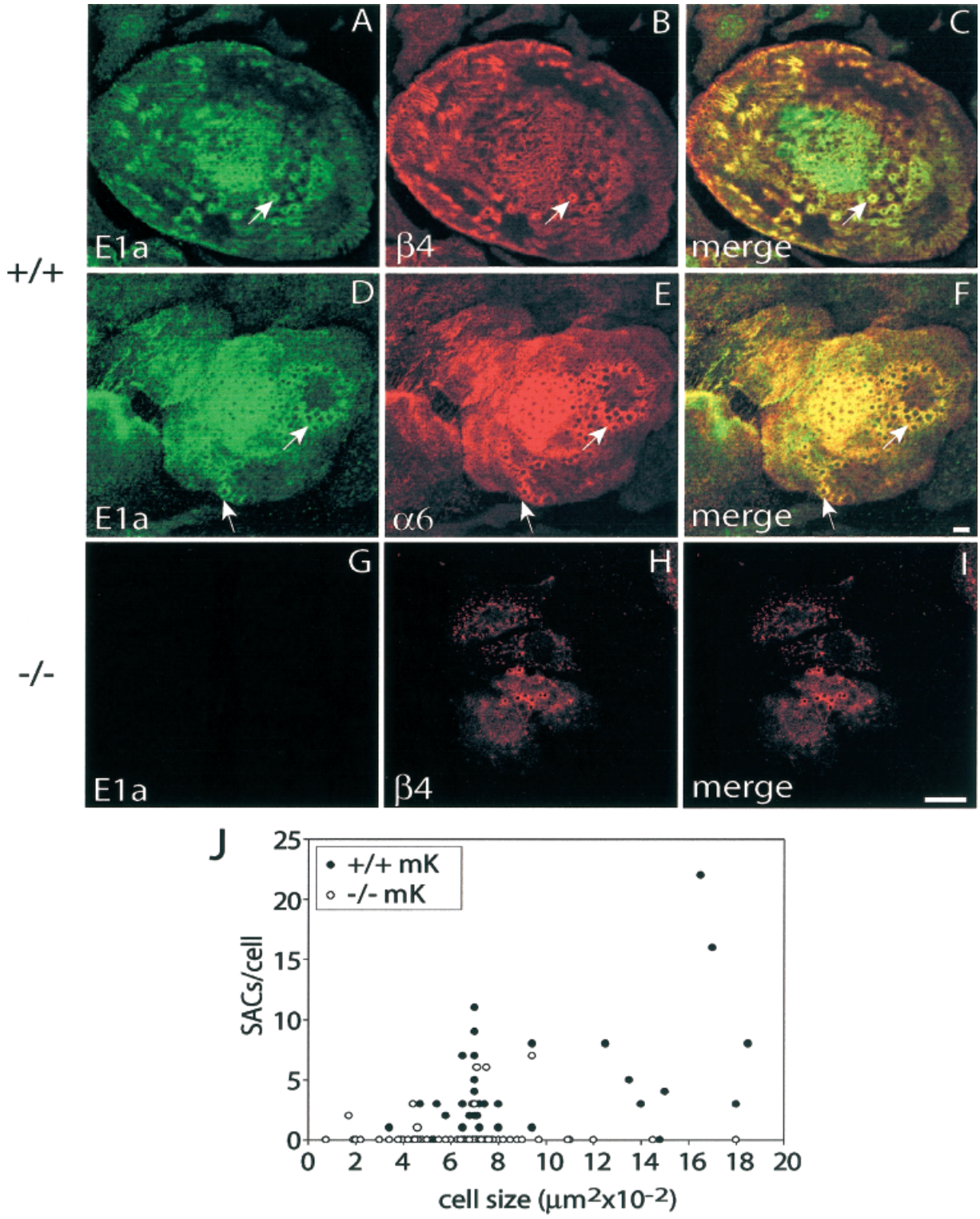

Figure 3. Localization of plectin 1a at SACs. (*A*)–(*I*) Immortalized plectin (+/+) and (−/−) keratinocytes were processed for double immunofluorescence microscopy as described in the text. Anti-E1a antiserum (E1a), anti-integrin β4 (β4), and anti-integrin α6 (α6) antibodies were used. *Bars* in (*F*) (representative of *A–F*) and in (*I*) (representative of *G–I*), 10 μm. Note that plectin 1a colocalized with integrin β4 and integrin α6 at SACs (*arrows* in *A–F*) and that plectin (−/−) cells display a few SACs (*H*, *I*). (*J*) Numerical analysis of SACs in plectin (+/+) and (−/−) mouse keratinocytes (mK). Data points represent individual cells. Sixty cells of each type were analyzed. Note that the number of SACs per cell varied with cell size and was reduced in plectin (−/−) compared to (+/+) keratinocytes.

Unlike plectin 1a, in plectin (+/+) keratinocytes plectin 1c was found associated with cytoskeletal filaments, which could be identified as microtubules by double immunofluorescence microscopy using isoform-specific antibodies (E1c) in combination with antibodies to α-tubulin (**Fig 5*A–C***). A localization of plectin 1c in hemidesmosomal structures was never observed. The filamentous staining pattern detected with plectin-1c-specific antibodies was absent in plectin (−/−) cells as expected, and the microtubule networks displayed no major changes (**Fig 5*D–F***), except for a possible tendency to

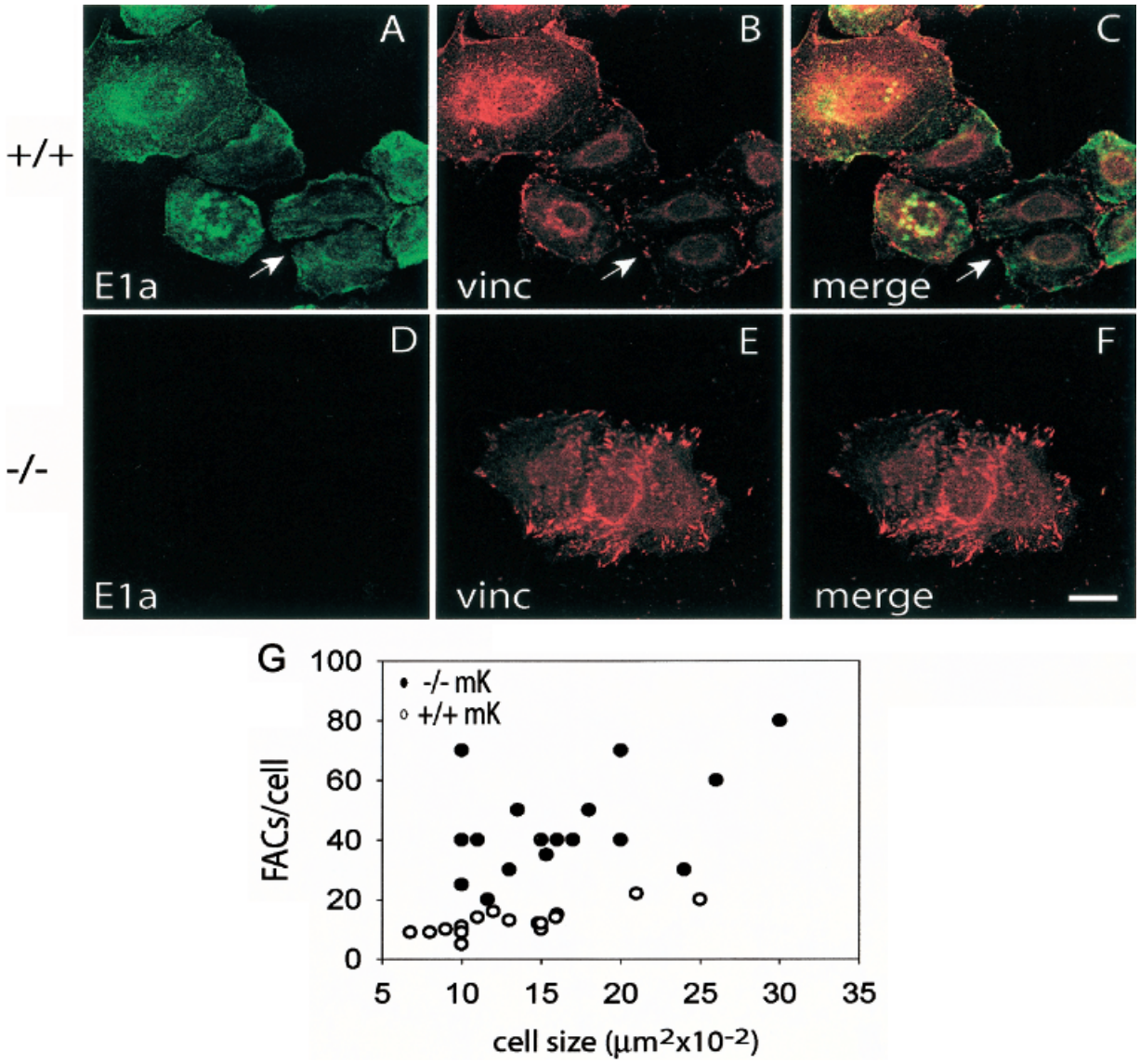

Figure 4. Differential distribution of plectin 1a and FACs. (*A*)–(*F*) Double immunofluorescence microscopy of immortalized plectin (+/+) and (−/−) keratinocytes, using anti-E1a antiserum (E1a) and antivinculin (vinc) antibodies. Note that plectin 1a does not colocalize with FACs (*arrows* in *A–C*), but shows partial colocalization with vinculin in FACs-free perinuclear regions; also the number of FACs was increased and FAC structures were more scattered over the cells in plectin (−/−) keratinocytes (*E*) compared to (+/+) keratinocytes (*B*). *Bar*: 10 µm. (*G*) Numerical analysis of FACs in plectin (+/+) and (−/−) mouse keratinocytes (mK). Data points represent individual cells.

higher microtubule density in plectin (−/−) cells. The immunoreactivity occasionally observed in the nucleus of both plectin (+/+) and plectin (−/−) keratinocytes was assumed to be nonspecific.

Functional distinction of plectin isoforms due to distinct rescue potentials in plectin-deficient keratinocytes The differential distribution of plectin variants and the specific association of plectin 1a with hemidesmosomal structures suggested a keratinocyte-specific function of this isoform. Therefore, the potential of plectin 1a to reverse the phenotypes of plectin-deficient keratinocytes was studied by transient transfection experiments. First an N-terminal fragment of rat plectin 1a (plectin 1a-14), which contained plectin's ABD and had previously been shown to restore the actin/FAC phenotype of fibroblasts (Andrä *et al,* 1998), was expressed in plectin (−/−) cells. Double immunofluorescence microscopy using plectin 1a- and vinculin-specific antibodies showed that the expression of this fragment in plectin (−/−) keratinocytes led to a reduction of FACs (**Fig 6*A–C***). Almost no FACs could be seen in plectin-1a-14-transfected cells compared to numerous vinculin-positive FACs at the periphery of untransfected cells (**Fig 6*B***). As documented by statistical analysis of cells randomly chosen from the same culture dishes, nontransfected plectin (−/−) keratinocytes showed up to 160 FACs per cell, whereas in transfected cells the number of FACs rarely exceeded 25 (**Fig 6*G***). Furthermore, contrary to mock-transfected cells or cells expressing another N-terminal plectin fragment that lacks the domain corresponding to exon 1a (data not shown), about 90% of E1a-14-transfected cells exhibited cellular protrusions (**Fig 6*A, D***, *arrows*), in which plectin 1a-14 was found to be accumulated. This N-terminal fragment of plectin 1a was never found associated with SACs, however, clearly demonstrating that this part of the molecule, although being sufficient to reduce the number of FACs per cell, does not colocalize with and has no effect on hemidesmosome-like structures. This is astonishing because the N-terminal fragment comprised the ABD, which is known to harbor one of plectin's integrin β4 binding sites (Rezniczek *et al*, 1998; Nievers *et al*, 2000). Thus, the localization of plectin 1a to hemidesmosomes may require the additional

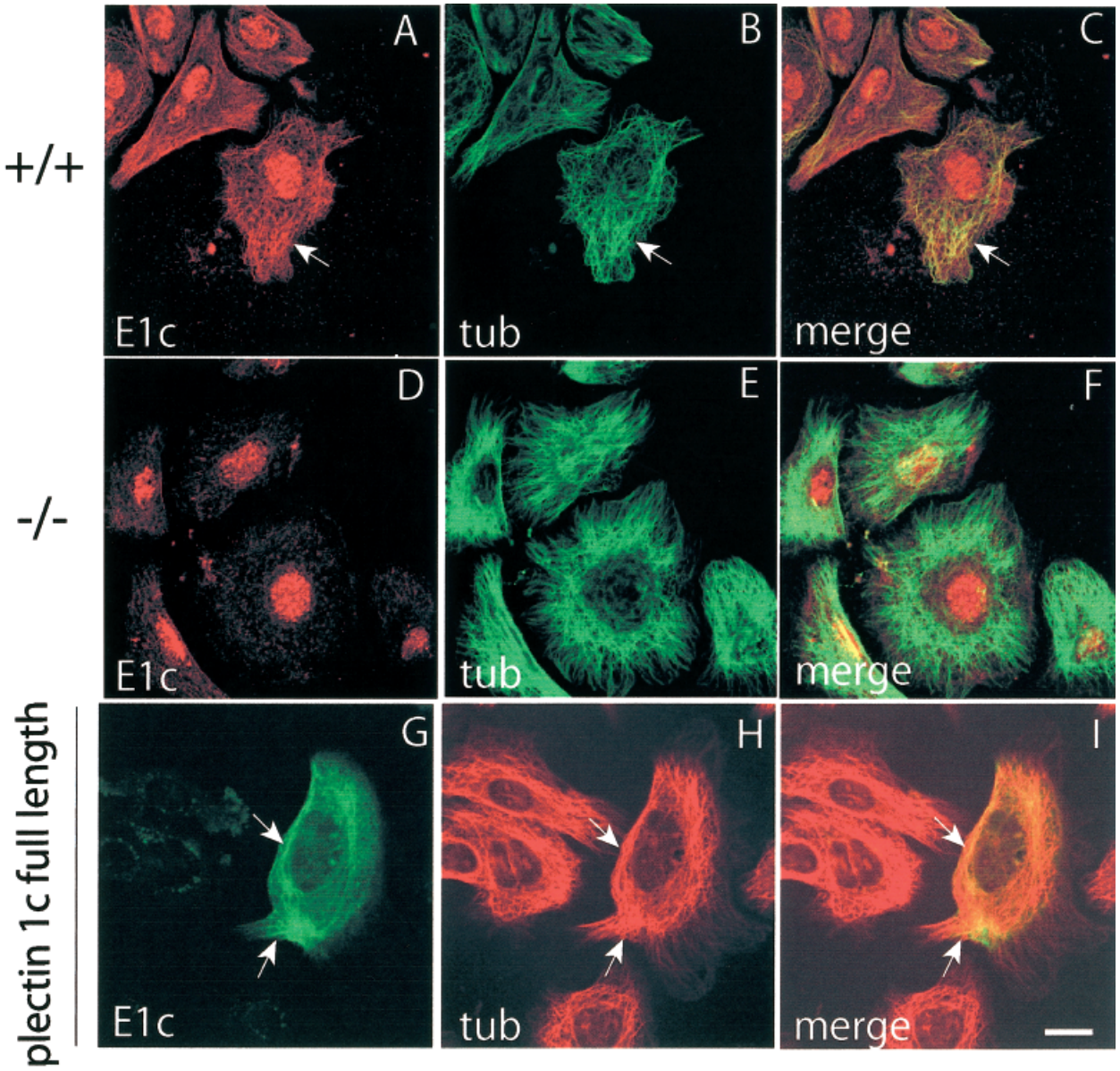

Figure 5. Colocalization of plectin 1c with microtubules in mouse keratinocytes. (*A*)–(*F*) Double immunofluorescence was carried out using anti-E1c (E1c) antiserum, and antitubulin (tub) monoclonal antibodies. Note colocalization of plectin 1c and microtubules (*A–C, arrows*) in wild-type (+/+) keratinocytes and in plectin-deficient (−/−) keratinocytes transfected with full length plectin 1c cDNA (*G–I, arrows*). Also note fully developed microtubule networks in plectin (−/−) keratinocytes (*E*). *Bar*: 10 μm.

integrin β4 binding site identified in its C-terminus or other functions provided by this domain, such as binding to hemidesmosome-associated proteins of unknown identity.

In contrast to the N-terminal fragment, full length plectin 1a expressed in plectin (−/−) keratinocytes showed association with SACs in the vast majority of transfected cells (**Fig 7*A–C***, *arrows*). Interestingly, statistical analyses revealed that the number of SACs detectable in these transfected (−/−) cells (8–52 SACs per cell) was significantly increased compared to nontransfected cells (0–2 SACs per cell; **Fig 7*G***). Moreover, in some cases the number of SACs per cell even exceeded the level of SACs in nontransfected plectin (+/+) cells (0–22 SACs per cell; **Figs 7*G***, **3*J***). The vast majority of SAC-positive transfected cells exhibited a spherical shape (**Fig 7*A***) consistent with the observation that none of these cells showed a significant number of vinculin-positive FACs. This indicated that expression of the full length version of plectin 1a was required for rescuing the SAC phenotype of plectin (−/−) keratinocytes.

To assess whether the SAC rescue potential of plectin 1a was specific for this isoform, immortalized plectin-deficient keratinocytes were transiently transfected with an expression plasmid encoding a full length version of the alternative isoform plectin 1c. Similar to endogenous plectin 1c of wild-type cells (**Fig 5*A***), plectin 1c expressed in transfected (−/−) cells was found associated with filamentous structures, and double staining (E1c/α-tubulin) showed partial codistribution with microtubules (**Fig 5*G–I***); association with SACs or cytokeratin 14 (data not shown) was not observed. Likewise, when plectin (−/−) keratinocytes were transfected with a full length version of plectin 1, a third isoform of plectin expressed in epithelial cells (see **Fig 1**), an association with structures resembling SACs was not detected; instead, the nuclear region was intensively stained and an association of plectin 1 with filamentous structures became apparent in perinuclear regions (**Fig 7*D–F***). Furthermore, in contrast to the accumulation of integrin β4 in SACs of plectin-1a-expressing cells, transfection of cells with plectin 1 cDNA resulted in a rather diffuse distribution of integrin β4 (compare **Fig 7*B, E***). Not a single plectin-1-transfected cell was found in which SACs could be detected (**Fig 7*I***), clearly indicating that only plectin 1a was able to

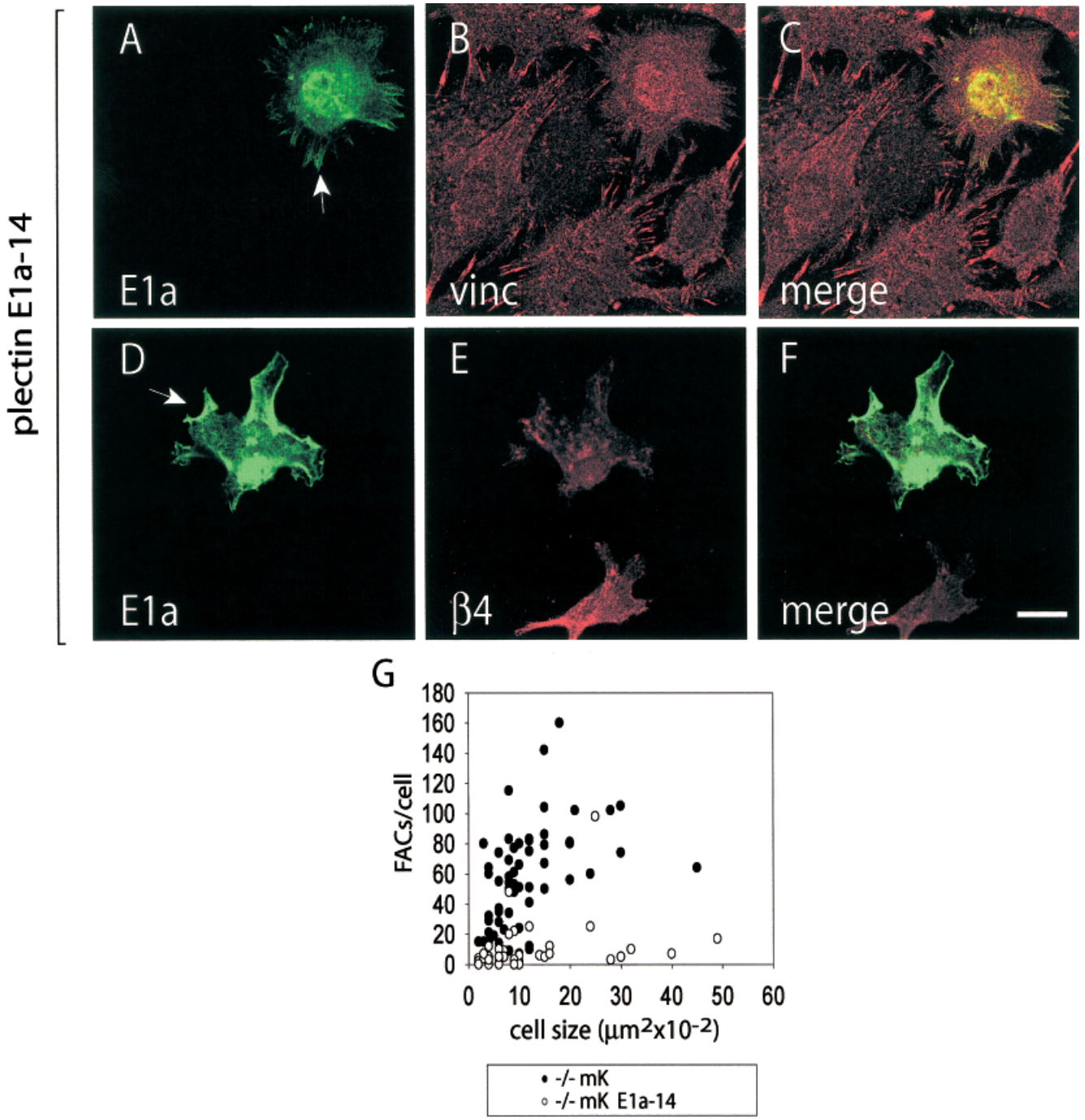

Figure 6. Transfection of cDNAs encoding N-terminal fragment plectin 1a-14 into immortalized plectin (–/–) mouse keratinocytes. (*A*)–(*F*) Double immunofluorescence microscopy was carried out using anti-exon 1a (E1a) antiserum, anti-integrin β4 (β4), and antivinculin (vinc) as primary immunoreagents. Note cellular protrusions in plectin (–/–) keratinocytes expressing plectin 1a-14 (*arrows* in *A* and *D*). Also, note partial colocalization of plectin 1a-14 and vinculin in perinuclear regions (compare to **Fig 4*A–C***). *Bar*: 25 μm. (*G*) Numerical analysis of FACs in transfected (–/– mk E1a-14) and nontransfected (–/– mk) immortalized plectin (–/–) keratinocytes. Data points represent individual cells. Note the reduction of FACs per cell upon transfection of the N-terminal fragment E1a-14.

restore the SAC phenotype of plectin-deficient keratinocytes. Similar to plectin 1a and plectin 1a-14, however, plectin 1 was found to reduce the number of FACs in plectin (–/–) keratinocytes (**Fig 7*H***). This suggests a regulatory mechanism for the generation of FACs involving N-terminal sequences other than those encoded by exon 1a, a notion supported by full length plectin 1a's absence from FACs. It should be noted that all isoforms studied were expressed under the control of the K14 promoter over similar transfection periods with similar transfection efficiency, ensuring comparable expression levels. In summary, these data showed that plectin 1a was specifically involved in the formation and/or stabilization of SACs, whereas plectin 1, plectin 1c, and plectin 1a-14 had no effects on SACs.

The function of the 37 amino acid long exon 1a-encoded N-terminal sequence unique to plectin 1a remains currently unclear. It is unlikely that it plays a role as a direct hemidesmosomal targeting signal considering that neither the fragment plectin 1a-14 (**Fig 6*A, D***) nor a GFP-fusion protein containing just the 1a-specific sequence (data not shown) colocalized with

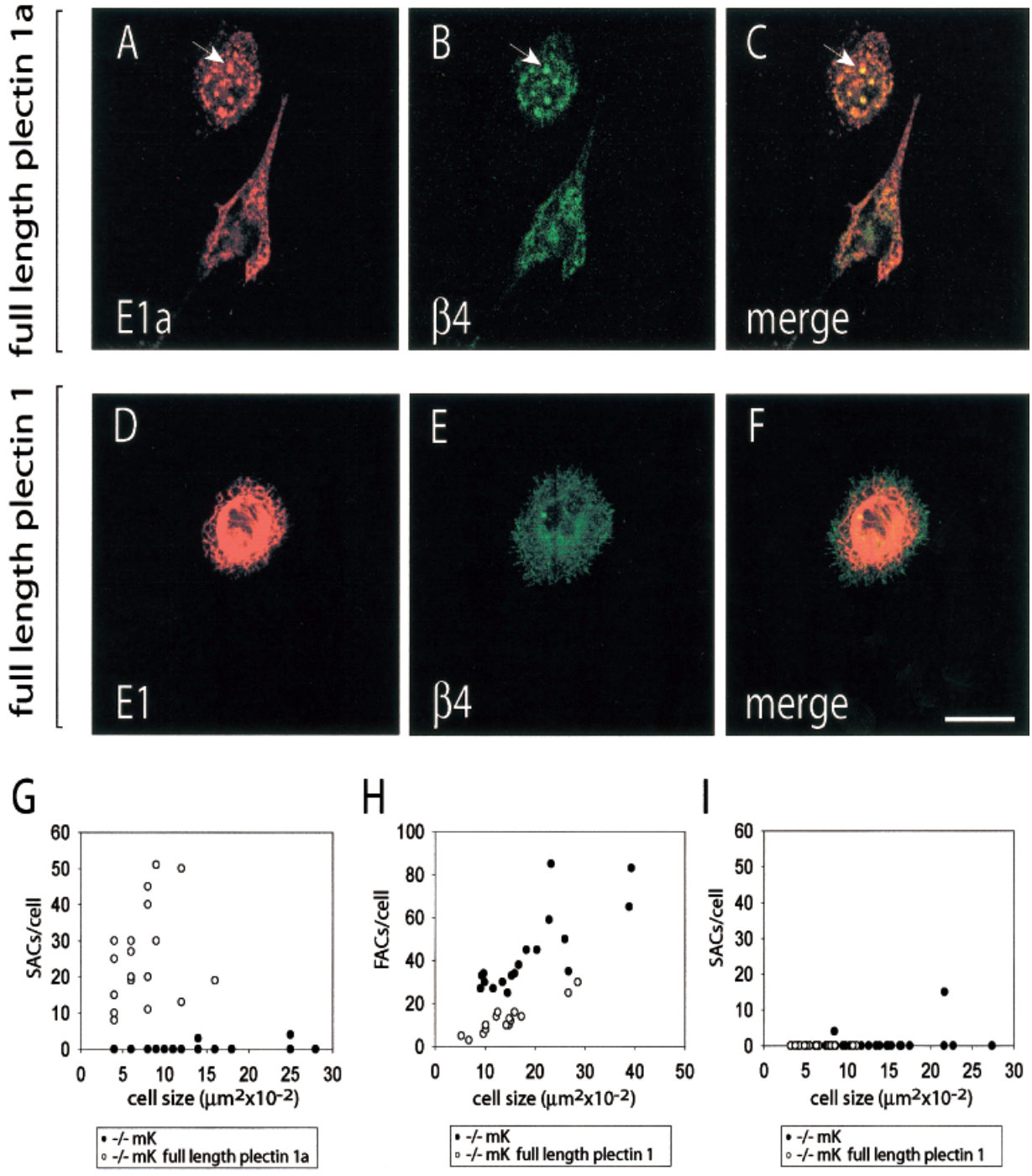

Figure 7. Transfection of cDNAs encoding full length plectin 1a (*A–C*) and full length plectin 1 (*D–F*) into immortalized plectin (–/–) mouse keratinocytes. (*A–F*) Double immunofluorescence microscopy was carried out using anti-exon 1a (E1a) antiserum, anti-integrin β4 (β4), and antimyc (E1) monoclonal antibodies as primary immunoreagents. Overexpressed full length plectin 1a colocalized with integrin β4 at SACs (*arrows* in *A–C*). In cells transfected with full length plectin 1 no SACs were detected (*D–F*); note filamentous staining pattern in perinuclear regions. *Bar*: 20 μm. (*G–I*) Numerical analyses of FACs (*H*) and SACs (*G, I*) in transfected and nontransfected (–/– mk) immortalized plectin (–/–) keratinocytes. Data points represent individual cells. Note the reduction of FACs per cell upon transfection of full length plectin 1, and the increase in SACs per cell upon transfection of full length plectin 1a, but not upon transfection of full length plectin 1.

hemidesmosomes in transfection assays. A more likely alternative would be that this sequence affects functions of plectin that involve other molecular domains, such as those serving as binding sites for plectin's various interaction partners. Such sites may reside in close vicinity to the 1a-specific sequence, such as in the immediately succeeding ABD, or in more remote sequence regions that become affected due to the folding of the polypeptide chain. The latter would be consistent with our preliminary observation that N-terminal recombinant fragments containing the exon 1a- or exon 1c-encoded sequences and the succeeding ABD showed equally effective binding to the cytoplasmic domain of integrin β4 when subjected to *in vitro* binding assays (unpublished data). It is conceivable that affected sites may even be part of C-terminal plectin domains, as there is evidence for intramolecular as well as intermolecular head-to-tail interactions of plectin molecules. Furthermore, interactions

between N- and C-terminal domains may also occur in oligomers formed by the lateral association of coiled-coil dimers in an antiparallel fashion (Foisner and Wiche, 1987). This complexity is likely to make it a challenging task for future studies to unravel the molecular mechanisms of how plectin 1a-specific and other isoform-specific N-terminal sequences determine the different functions of plectin variants.

Because plectin (−/−) mice and keratinocytes derived from these mice have a decreased number of hemidesmosomal structures (Andrä *et al*, 1997), plectin was proposed to function as hemidesmosomal stabilizer (Rezniczek *et al*, 1998). In transient transfection experiments in which full length plectin 1a was expressed in plectin (−/−) keratinocytes, the hemidesmosomal defect of these cells, which is believed to cause skin blistering in EBS-MD patients, was rescued. Plectin 1a is therefore most likely the plectin isoform, the defects in which are responsible for the skin blistering observed in EBS-MD. This is further supported by the observations that (i) neither full length plectin 1 nor 1c were able to rescue the hemidesmosomal phenotype of plectin (−/−) keratinocytes and (ii) endogenous plectin 1c was not associated with hemidesmosomal structures.

In conclusion, our results suggest that defects in plectin 1a, the major epithelial isoform, are responsible for the skin blistering of plectin-deficient humans and mice. These findings might be a first step towards understanding the function of plectin's unusual isoform diversity and could have implications for the development of clinical therapies for EBS-MD patients in the future.

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