Interaction of Plectin with Keratins 5 and 14: Dependence on Several Plectin Domains and Keratin Quaternary Structure

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Plectin, a cytolinker of the plakin family, anchors the intermediate filament (IF) network formed by keratins 5 and 14 (K5/K14) to hemidesmosomes, junctional adhesion complexes in basal keratinocytes. Genetic alterations of these proteins cause epidermolysis bullosa simplex (EBS) characterized by disturbed cytoarchitecture and cell fragility. The mechanisms through which mutations located after the documented plectin IF-binding site, composed of the plakin-repeat domain (PRD) B5 and the linker, as well as mutations in K5 or K14, lead to EBS remain unclear. We investigated the interaction of plectin C terminus, encompassing four domains, the PRD B5, the linker, the PRD C, and the C extremity, with K5/K14 using different approaches, including a rapid and sensitive fluorescent protein–binding assay, based on enhanced green fluorescent protein-tagged proteins (FluoBACE). Our results demonstrate that all four plectin C-terminal domains contribute to its association with K5/K14 and act synergistically to ensure efficient IF binding. The plectin C terminus predominantly interacted with the K5/K14 coil 1 domain and bound more extensively to K5/K14 filaments compared with monomeric keratins or IF assembly intermediates. These findings indicate a multimodular association of plectin with K5/K14 filaments and give insights into the molecular basis of EBS associated with pathogenic mutations in plectin, K5, or K14 genes.

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INTRODUCTION

Plectin is a large ubiquitous cytolinker protein of the plakin family (Sonnenberg and Liem, 2007; Winter and Wiche, 2013; Bouameur *et al.*, 2014). Several plectin isoforms with distinct N termini exist. They exhibit specific tissue expression and subcellular distribution. In basal keratinocytes, plectin and the epithelial isoform of BPAG1 (BPAG1e), another plakin family member, anchor the intermediate filament (IF) network formed by keratins 5 and 14 (K5/K14) to the basal cell membrane through their association with transmembrane α6β4 integrin and BP180 (also called bullous pemphigoid antigen 2 or collagen type XVII) (Guo *et al.*, 1995; Andrä *et al.*, 1997; Borradori and Sonnenberg, 1999; Walko *et al.*, 2011). In striated muscle, plectin has a key role in tethering the desmin IF network to the Z-discs, between the peripheral myofibrils and the sarcolemma, as well as to the intercalated discs (Andrä *et al.*, 1997; Konieczny *et al.*, 2008). Structurally, all plectin variants possess the same C-terminal tail composed of a succession of six plakin-repeat domains (PRDs) linked by connecting segments and ending with a C extremity (E) containing glycine-serine-arginine repeats (see Figure 1). A 50-amino-acid stretch immediately following the fifth PRD B (B5), in the so-called ''linker'' (L), is important for the binding of plectin to various IF types (Nikolic *et al.*, 1996; Favre *et al.*, 2011; Karashima *et al.*, 2012) (see Figure 1). Recent studies have shown that plectin specifically binds to the central rod domain of type III IF proteins vimentin and desmin (Favre *et al.*, 2011).

IFs are involved in a variety of cell functions, including maintenance of cell resilience and cytoarchitecture (Herrmann *et al.*, 2009; Ramms *et al.*, 2013; Seltmann *et al.*, 2013). This family comprises six types of protein whose expression is cell-type specific and depends on tissue differentiation (Herrmann and Aebi, 2004; Szeverenyi *et al.*, 2008). Structurally, they consist of a central α-helical rod domain flanked by N- and C-terminal ''head'' and ''tail'' domains, respectively. Their dimerization is mediated by their rod, whereas their subsequent stepwise assembly in

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Abbreviations: EGFP, enhanced green fluorescent protein; HA, hemagglutinin; HA-PL, HA-tagged full-length plectin 1c; IF, intermediate filament; PRD, plakin-repeat domain; Y3H, yeast three-hybrid

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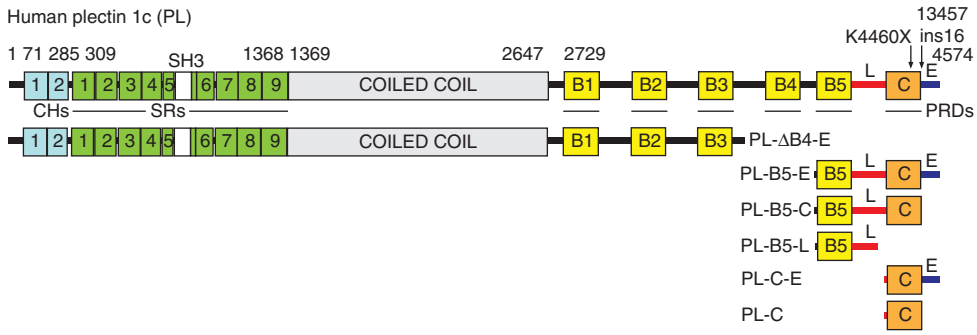

Figure 1. Schematic of the structure of human plectin 1c and of truncation mutants used in this study. The actin-binding domain consists of two calponin homology domains (CHs, pale blue). The plakin domain is composed of the spectrin repeats (SRs, green) 3 to 9, with an atypical SH3 domain (white) embedded in the fifth SR. The C-tail comprises plakin-repeat domains (PRDs, yellow: type B, orange: type C) and connecting segments including the linker (L, red) and the C extremity (E, violet). Residue numbers and the location of two truncating mutations found in patients suffering from epidermolysis bullosa simplex-muscular dystrophy are also indicated. The N terminus of the two biggest constructs was hemagglutinin-tagged and that of the smaller constructs was fused to either enhanced green fluorescent protein (EGFP) or H6-EGFP.

unit-length filaments and in long insoluble filaments requires their head. Their final radial compaction is enabled by their tail. Keratin IFs are obligatory heteropolymers assembled from type I and II keratin heterodimers that are mostly expressed in epithelial cells, whereas type III vimentin and desmin IFs are homopolymers expressed in mesenchymal and muscular tissues, respectively (Steinert and Roop, 1988). Genetic defects in humans, as well as gene-targeted elimination of K5, K14, plectin, or BPAG1e in mice, cause cytoskeletal disorganization and loss of cytokeratin anchorage to hemidesmosomes, resulting in an epidermolysis bullosa simplex phenotype (Guo *et al.*, 1995; Andrä *et al.*, 1997; Coulombe and Omary, 2002; Porter and Lane, 2003; Intong and Murrell, 2012; Liu *et al.*, 2012; Winter and Wiche, 2013). Plectin or desmin gene defects also result in muscular dystrophy (Andrä *et al.*, 1997; Winter and Wiche, 2013), accompanied by disorganization of the desmin IF network and malposition of mitochondria (Capetanaki *et al.*, 2007). These findings unequivocally demonstrate the importance of the interplay between IFs and plakins for the maintenance of the cytoarchitecture in the skin and in skeletal muscle.

A surprising cause of epidermolysis bullosa simplex-muscular dystrophy is the truncating mutations in the plectin's last PRD (PRD C) or even in the C extremity (Schröder *et al.*, 2002; Winter and Wiche, 2013), as these domains are not crucial for plectin binding to IFs in contrast to the preceding linker region (Nikolic *et al.*, 1996) (see Figure 1). There is some evidence that other sequences within the plectin C terminus are involved in its binding to IFs, but their exact contribution is still unclear (Nikolic *et al.*, 1996; Favre *et al.*, 2011; Karashima *et al.*, 2012; Bouameur *et al.*, 2013). Last but not least, distinct binding sites for plakins have been identified on various IF proteins (Kouklis *et al.*, 1994; Meng *et al.*, 1997; Fontao *et al.*, 2003), although the involvement of a common conserved region within IF proteins in their binding to distinct plakins seems more plausible on the basis of recent findings (Lapouge *et al.*, 2006; Favre *et al.*, 2011).

Here, we have characterized the interaction of K5/K14 with plectin by using several independent methods. Grouped together, our findings provide insights into the association of plectin with IFs, as well as a better understanding of the molecular basis of inherited diseases associated with plectin and cytokeratin defects.

RESULTS AND DISCUSSION

The IF-binding domain of plectin interacts preferentially with the type I keratins K14, K10, and K18

The plectin C terminus was found to colocalize with cytokeratins in transfected cells (Nikolic *et al.*, 1996) and to interact with K14 but not with K5 in yeast two-hybrid assays (Geerts *et al.*, 1999). However, the preferential binding to K14 was not confirmed in a separate study using biochemical binding assays with a recombinant plectin encompassing the PRD B5 and the linker region (PL-B5-L). Furthermore, the interaction of this recombinant protein with K5/K14 polymers was modest compared with monomeric keratins (Steinbock *et al.*, 2000). To better characterize the interaction of plectin with type I and type II keratins *in vitro,* we expressed in bacteria a recombinant PL-B5-L protein tagged at its N terminus with H6-enhanced green fluorescent protein (EGFP) (H6-EGFP-PL-B5-L) (see Figure 1). Recombinant H6-EGFP-PL-B5-L and EGFP-H6, the latter used as control and standard to determine the concentration of EGFP-tagged proteins in solution, were purified from soluble bacterial extracts in nondenaturing conditions (Supplementary Figure S1a–c online) and their interaction with various recombinant IF proteins (see Supplementary Figure S2 online) was analyzed. In our binding assays, the IF proteins were (1) directly spotted onto nitrocellulose membrane, (2) subjected to SDS-PAGE and then transferred onto nitrocellulose membrane, or (3) immobilized on the plastic surface of ELISA-microtiter plate wells (see Supplementary Materials and Methods online), whereas H6-EGFP-PL-B5-L was used as the liquid-phase ligand (fluorescent overlay or sorbent assays, respectively). H6-EGFP-PL-B5-L preferentially interacted with monomeric K14 rather than with monomeric K5, in agreement with yeast two-hybrid results (Geerts *et al.*, 1999), and even better with dimeric K5/K14 with a Kd of 135 nM ± 36 (mean ± SD from five independent

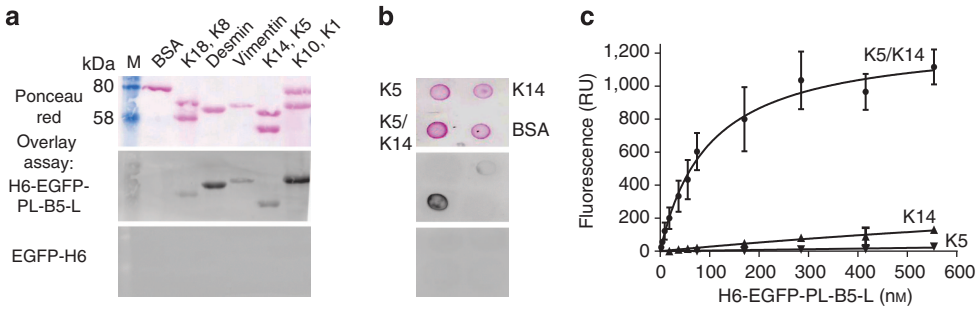

Figure 2. Recombinant H6-EGFP-PL-B5-L protein preferentially binds to type I keratins and dimeric K5/K14. (**a**) Fluorescent overlay assays; proteins (0.5 μg each) were separated on 10% SDS-PAGE, transferred onto nitrocellulose membranes, and stained with Ponceau red (type I keratins K10, K14, and K18 are smaller than their type II partners K1, K5, and K8, respectively). Membranes were then overlaid with pure recombinant H6-EGFP-PL-B5-L or EGFP-H6 (0.5 μM each) and scanned for fluorescence. (**b**) As for (**a**), but proteins (K5, K14, dimeric K5/K14, and BSA) were spotted onto nitrocellulose membranes (3.5 pmol protein per spot). Quantification of overlay assays is presented in Supplementary Figure S3 online. (**c**) Fluorescent-sorbent binding assays; K5 and K14 (4 pmol) were immobilized as monomers or mixtures (K5/K14) in wells of ELISA-microtiter plates and incubated with serial dilutions of pure recombinant H6-EGFP-PL-B5-L. Its binding to intermediate filament proteins was measured with a fluorometer (RU: relative unit); each point represents the mean ± SD of four independent experiments. Values of EGFP-H6 were similar to the blank values (data not shown).

experiments) (Figure 2a–c, Supplementary Figure S3 online). H6-EGFP-PL-B5-L bound also more to K10 and K18 than to K1 and K8, respectively (Figure 2a and Supplementary Figure S3 online), indicating a preferential interaction with monomeric type I keratins rather than with type II keratins. A possible explanation for the discrepancies between our findings and a previous study (Steinbock *et al.*, 2000) is that these authors used immobilized H6-PL-B5-L, purified from inclusion bodies (Nikolic *et al.*, 1996), and randomly europium-labeled keratins as fluid-phase ligands.

As the production of plectin fragments as soluble and active proteins in bacteria is often ineffective (Nikolic *et al.*, 1996; Spurny *et al.*, 2007) (our own unpublished data), we transfected mammalian cells, which ensure better protein folding compared with bacteria (Hartl *et al.*, 2011), to produce several EGFP-tagged recombinant forms of plectin (Supplementary Figure S1d–f online). The soluble extracts from transfected HEK 293T cells were used as the liquid phase in overlay assays (FluoBACE) by analogy with the single-molecule pull-down method (Jain *et al.*, 2011). Both qualitatively and quantitatively, the results obtained with soluble EGFP-PL-B5-L expressed in 293T cells (Figure 3a and b, Figure 4a, Supplementary Figure S4a–d online) or in bacteria (Figure 2 and Supplementary Figure S3 online) were similar.

Identification of a second IF-binding site, independent of the linker, in the plectin C terminus

We took advantage of the simple and powerful FluoBACE method to further characterize the IF-binding properties of the plectin C terminus. The longer recombinant protein EGFP-PL-B5-E (see Figure 1) exhibited qualitatively similar IF-binding properties to EGFP-PL-B5-L, but its relative binding to various IF proteins was different, suggesting that the PRD C and/or the C extremity contribute to the total binding of PL-B5-E (Figure 3a and b, Supplementary Figure S4a–h online). EGFP-PL-C-E could indeed interact on its own with IFs but much more weakly than plectin constructs containing the linker, and it did not show a clear preference for type I keratins or dimeric K5/K14 (Figure 3a and b, Supplementary Figure S4i–l online). To confirm that PL-C-E binds to K5/K14, we performed an immunofluorescence study in transfected cells and yeast three-hybrid (Y3H) assays. EGFP-PL-C-E partially colocalized with the K5/K14 IF network in 87% of transfected HaCat keratinocytes (100 analyzed cells), whereas EGFP-PL-C exhibited a diffuse pattern in all transfected cells (Figure 3c). In Y3H, GAL4-DB-PL-C-E was also able to promote the growth of yeast cells co-transformed with GAL4-AD-K5 and with GAL4-AD-K14, reflecting the interaction between PL-C-E and K5/K14 (Figure 5a). These results univocally indicate that other sequences within plectin can bind to cytokeratins besides those of the linker region. Hence, plectin and desmoplakin, a plakin showing high homology to plectin (see Figure 1 in (Fontao *et al.*, 2003)), contain distinct sequences within their C-terminal portion that can independently interact with specific IFs with different selectivity (this study and (Fontao *et al.*, 2003)).

To confirm that the results obtained with recombinant plectin C-terminal constructs are relevant to full-length plectin, we tested the IF-binding properties of a hemagglutinin (HA)-tagged full-length plectin 1c (HA-PL) and those of a plectin 1c recombinant protein lacking the C-terminal domains from the PRD B4 to the C extremity, HA-PL-ΔB4-E (see Figure 1). Both proteins were expressed in 293 T cells (Supplementary Figure S5a online). As expected from previous studies that mapped the IF-binding domain to the plectin C terminus (Wiche *et al.*, 1993; Nikolic *et al.*, 1996), HA-PL, but not HA-PL-ΔB4-E, interacted with monomeric IF proteins and dimeric K5/K14 (Supplementary Figure S5b and c online). In three independent experiments, we found a better binding of HA-PL to monomeric K14, K10, and K18 than to their type II partners K5, K1, and K8, respectively, as well as a better binding to dimeric K5/K14 than to monomeric K14. Hence, the IF-binding properties of full-length plectin 1c and its C terminus seem similar.

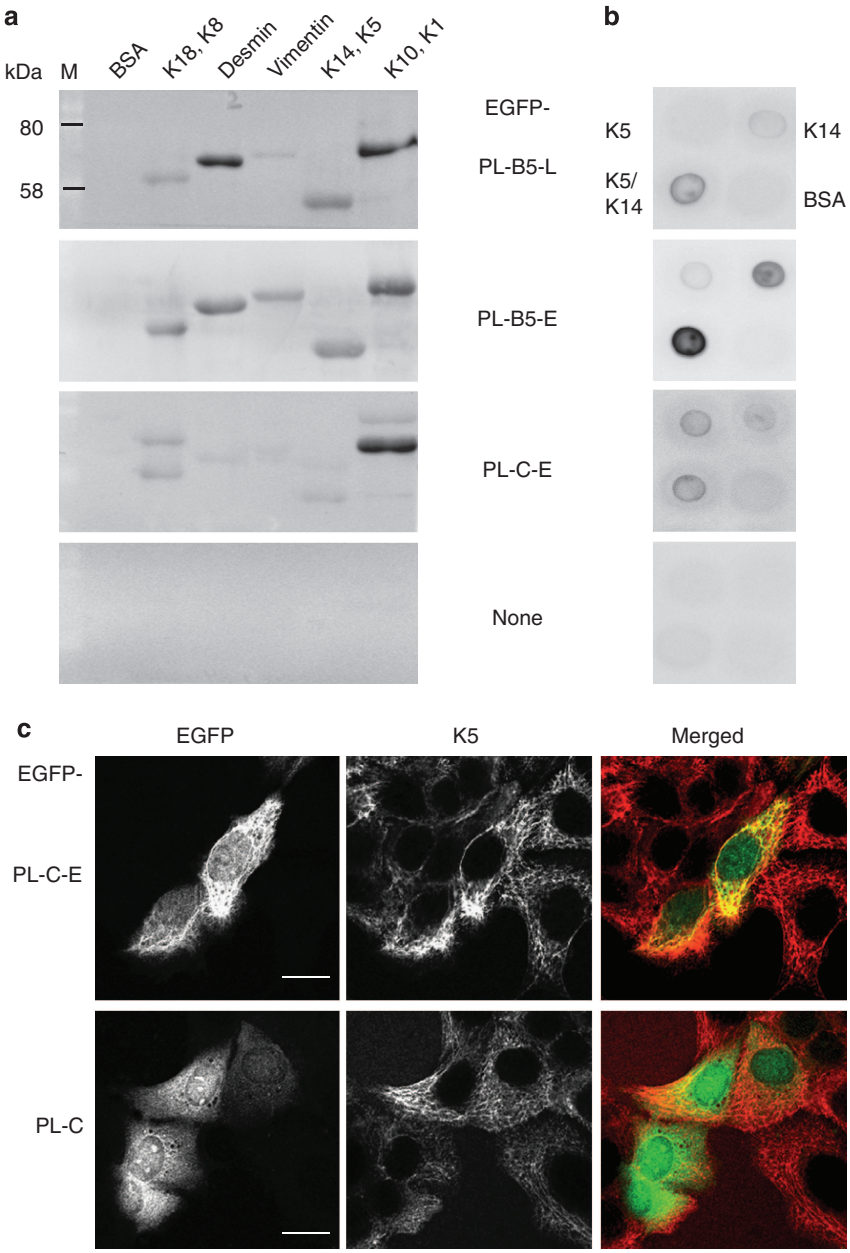

Figure 3. The C terminus of plectin bears two separate intermediate filament-binding domains, B5-L and C-E, exhibiting different specificities. (**a**, **b**) FluoBACE-overlay assays; membranes, prepared as for Figure 2a and b, were overlaid with soluble extracts of transiently transfected 293T cells expressing enhanced green fluorescent protein (EGFP)-tagged PL-B5-L (230 nM), PL-B5-E (140 nM), PL-C-E (340 nM), or EGFP (160 nM) and scanned for fluorescence. Quantification of overlay assays is presented in Supplementary Figure S4 online. (**c**) Confocal laser scanning microscope pictures of transiently transfected HaCat keratinocytes expressing EGFP-PL-C-E or EGFP-C (green), fixed with methanol. Keratins were revealed with the mouse mAb MNF116 (red). Bar = 10 μm.

Multimodular binding of plectin B5-E to IFs

To gain insight into the primary mechanisms underlying epidermolysis bullosa simplex-muscular dystrophy in patients carrying truncating mutations in the plectin gene close to the end of the coding sequence (Schröder *et al.*, 2002; Winter and Wiche, 2013) (see Figure 1), we investigated the K5/K14-binding properties of C-terminal deletion mutants of PL-B5-E with FluoBACE. Optimal interaction was observed with PL-B5-E. Removal of the C extremity from PL-B5-E (PL-B5-C) significantly reduced its binding to K5/K14. Analysis of the binding parameters revealed that the apparent Bmax value (dependent on the number of binding sites) was decreased, whereas the apparent Kd was unchanged (Figure 4). Truncation of the C extremity form PL-C-E (PL-C) abolished its weak binding to K5/K14 (data not shown). Removal of the PRD C from PL-B5-C (PL-B5-L) affected the apparent Kd, but not the Bmax value (Figure 4), an observation suggesting a complex interplay between the different plectin domains. Therefore, all

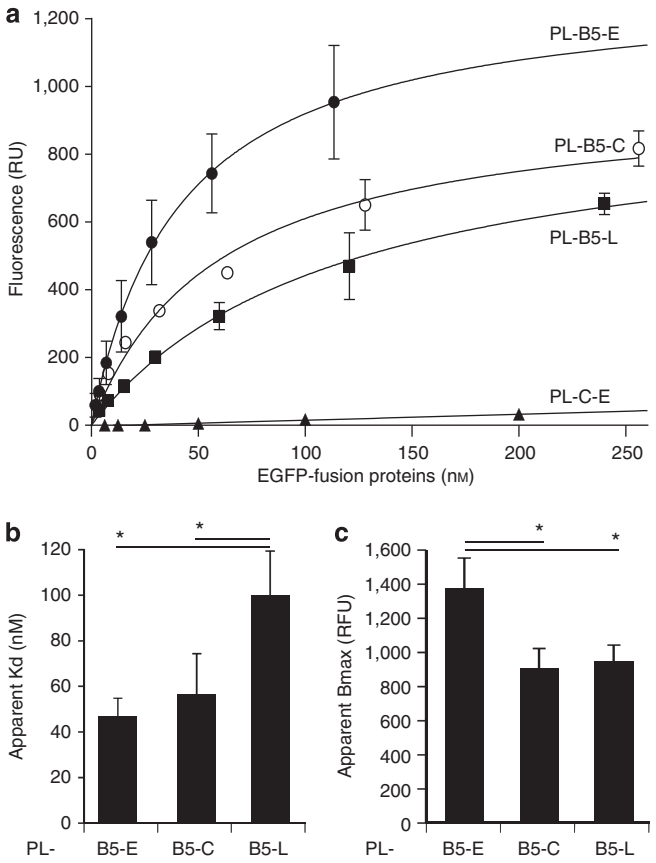

Figure 4. All domains of PL-B5-E contribute to its efficient binding to K5/K14. (**a**) Fluorescent-sorbent binding assays; K5/K14 (4 pmol), immobilized in wells of ELISA-microtiter plates, were incubated with serial dilutions of soluble extracts from 293T cells expressing various enhanced green fluorescent protein (EGFP)-tagged plectin recombinant proteins (three independent experiments, four for EGFP-PL-B5-E); data represent means ± SD. Values of EGFP-PL-C (up to 400 nM) or EGFP (up to 200 nM) were similar to the blank values (data not shown). (**b**, **c**) Effects of C-terminal truncations of EGFP-PL-B5-E on the apparent Kd and Bmax calculated from each individual binding curves; *t*-test $*P<0.05$.

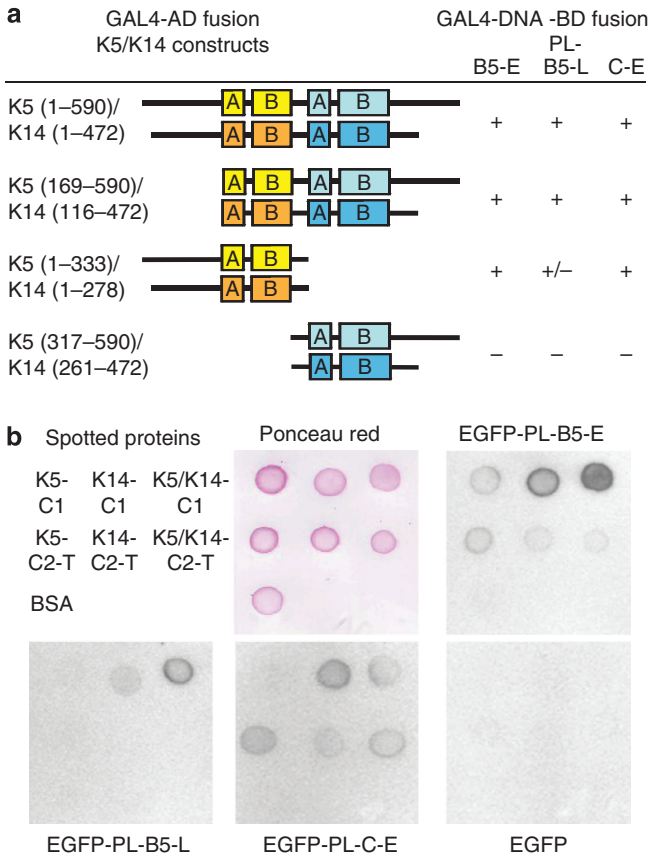

Figure 5. The C terminus of plectin mainly interacts with the coil 1 subdomains of K5/K14. (**a**) Yeast three-hybrid analysis of the interaction site(s) of various plectin constructs on K5/K14 by testing several deletion mutants of K5/K14 (keratin residue numbers are indicated in brackets): −, +/−, and + mean no growth, exclusive growth on His selection medium, and growth on both His and adenine selection media, respectively. (**b**) FluoBACE-overlay assays of various plectin constructs on coil 1 (C1) or coil 2 to tail (C2-T) of K5, K14, and K5/K14 or BSA (0.2 µg per spot). For quantification of binding results, see Supplementary Figure S6 online.

subdomains within PL-B5-E contribute to its binding to K5/K14. Similar results were obtained with other IFs, such as desmin, vimentin, and K8/K18 (data not shown).

Plectin preferentially binds to the coiled-coil 1 of K5/K14

To define regions within K5/K14, which are functionally important for their binding to plectin, we carried out Y3H assays using various deletion mutants of K5/K14. The results show that all tested plectin constructs could promote the growth of yeast cells co-transformed with the coiled-coil 1 domain constructs but not with constructs encoding the coiled-coil 2 and tail domains of K5/K14 (Figure 5a). FluoBACE on filter plates (see Supplementary Materials and Methods online) confirmed PL-B5-E binding to the rod domain and to the coil 1 of K5/K14 (data not shown). Preferential binding of both EGFP-PL-B5-L and EGFP-PL-B5-E to the coil 1 versus to the coil 2-tail of K5/K14 was also found in FluoBACE-overlay assays (Figure 5b). Nevertheless, EGFP-PL-B5-E could also weakly interact with the coil 2-tail of K5 and K5/K14, apparently via the contribution of the C-E domains (Figure 5b). This interaction was not revealed in Y3H assays, probably because it was too weak. The different binding sites on K5/K14 for PL-C-E or PL-B5-L could explain the higher binding capacity of PL-B5-E for K5/K14 compared with smaller recombinant proteins (see Figure 4). These findings and those of a previous study showing that plectin specifically associates with the coil 1B domain of desmin and vimentin (Favre *et al.*, 2011) (our own unpublished data) suggest that the association of plectin with IF proteins involves a similar set of sequences among cytokeratins and type III IF proteins. It is tempting to sponsor this idea for other IFs, as plectin is able to interact with a large number of them (Winter and Wiche, 2013).

The quaternary structure of K5/K14 filaments positively affects their association with plectin

As our results (Figures 2 and 3, Supplementary Figures S3 and S4 online) indicated that dimerization of K5 and K14 affects their binding to plectin, a phenomenon previously observed

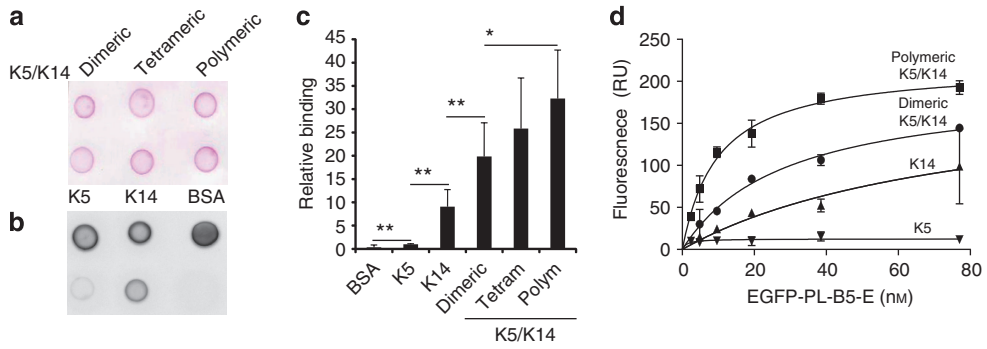

Figure 6. The binding of PL-B5-E to K5/K14 is modulated by the quaternary state of K5/K14 filaments. FluoBACE-overlay assays: (**a**) K5 and K14 were spotted onto nitrocellulose membranes as monomers or in different oligomeric forms (see Supplementary Data online, FluoBACE-overlay assay, for exact conditions) (0.2 μg protein per spot). Spotting quality was controlled by Ponceau red staining. (**b**) Fluorescence scan of a membrane prepared as in **a** overlaid with EGFP-PL-B5-E (88 nM). (**c**) Quantification of **b** (eight independent experiments), fluorescence values were normalized to K5 (per picomol protein); data represent means ± SD, *t*-test, $*P = 0.01$, $**P < 0.005$. (**d**) As for **c**, with serial dilutions of soluble extracts from 293T cells expressing EGFP-PL-B5-E (two independent experiments); data represent means ± SD.

with desmoplakin (Fontao *et al.*, 2003), we overlaid K5 and K14 proteins, spotted in different assembly stages, with EGFP-PL-B5- E (see Figure 1 and experimental conditions in Supplementary Materials and Methods online: FluoBACE-overlay assay) (Bousquet *et al.*, 2001; Yamada *et al.*, 2002; Herrmann and Aebi, 2004). As inferred from the signal intensities, the assembly stage of cytokeratins affected their binding to EGFP-PL-B5-E (Figure 6b). Indeed, recombinant plectin preferentially associated with assembled K5/K14 filaments, when compared with dimeric or tetrameric K5/K14 and monomeric K14 (Figure 6c). On the basis of the results of serial dilution studies, the different binding ability was mainly due to a variable number of binding sites for recombinant plectin on K5/K14 filaments (Bmax values) (Figure 6d). The increased number of binding sites for PL-B5-E on K5/K14 filaments may be because of either the formation of new binding sites on assembled IFs or to the oligomerization state of the recombinant plectin protein via their PRDs (Janda *et al.*, 2001). In line with these ideas, PL-B5-L is able to induce either aggregation or depolymerization of IFs depending on its concentration (Steinbock *et al.*, 2000). Furthermore, the plectin PRDs undergo homodimerization and heterodimerization in oxidative conditions (Spurny *et al.*, 2007), and the dimeric crystal structure of desmoplakin PRD B appears to also favor the existence of interactions among PRDs (Choi *et al.*, 2002).

CONCLUSION

We have dissected the binding properties of plectin to epidermal cytokeratins using several approaches, including FluoBACE, which is a rapid and sensitive fluorescent protein–binding assay. Our results reveal a much more complex interaction mode between plectin and cytokeratins than previously reported. Beyond the linker region, plectin contains additional domains within its C terminus contributing to and directly mediating interaction with IF proteins. Our results provide a better understanding of the molecular effects of pathogenic mutations within the plectin C terminus on IF anchorage and tethering. Although the cytokeratins coil 1 is the major binding site for plectin, polymerization of keratins increases their binding capacity to plectin. The latter observation implies that the quaternary structure of IFs is important for proper binding to plectin and for their anchorage to membrane sites, such as hemidesmosomes. Hence, it is most likely that mutations in cytokeratin genes affecting the formation of an extended IF network have an as yet unrecognized impact on the binding of plectin and by this means contribute to the cytoarchitecture disorganization typically observed in epidermolysis bullosa simplex.

MATERIALS AND METHODS

Cell culture and transfection

Human epithelial kidney 293T and HaCat cells were cultured in DMEM (Invitrogen, Zug, Switzerland) supplemented with 10% newborn calf serum (Sigma, St Louis, MO), 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin. HEK 293T cells were transfected by a standard calcium phosphate method, whereas HaCat keratinocytes were transfected with lipofectamine 2000 (Invitrogen) (Deyrieux and Wilson, 2007).

Complementary DNA constructs

EGFP was cloned from pEGFP-C3 (Clontech, Saint-Germain-en-Laye, France) into pET28 (Novagen, Madison, WI), generating the plasmid p1354 to express EGFP-H6 in *Escherichia coli*. An adaptor coding for MGSSHHHHHHSSGLVPRGSHMLDSGGGS was cloned in frame with the 5′end of the EGFP-H6 sequence in p1354 generating p1454. Mouse plectin 1c fragments (PL-B5-E, residues 3954–4572; PL-B, res. 3954–4133; PL-B5-L, res. 3954–4266; PL-B5-C, res. 3954–4502; PL-C-E, res. 4292–4572; PL-C, res. 4289–4506) were cloned into bacteria p1454, yeast pAS2-1, and mammalian pEGFP-C vectors. Human HA-plectin 1c in pcDNA3 (HA-PL) was obtained from Sonnenberg A (NKI, Amsterdam, The Netherlands) (Koster *et al.*, 2003). HA-PL-ΔB4-E plasmid encodes plectin 1c without the last 964 residues. Human IF complementary DNAs, their mutants, or fragments were cloned into yeast pAS2.1 and pACT2 (Clontech) or pACT2-URA (Fontao *et al.*, 2003), and bacterial pET15b or pET23 (Novagen). K5 and K14 complementary DNAs were given by Birgit Lane (IMB,

Singapore). Constructs were generated by direct subcloning or after PCR. Correctness of amplified constructs was verified by sequencing.

Antibodies

The following antibodies were used: anti-GFP (B-2, Santa Cruz Biotechnology, Heidelberg, Germany), anti-H6 (HIS-1, Sigma), anti-HA (Y-11, Santa Cruz), anti-cytokeratins (MNF116, Dako, Baar, Switzerland), and anti-vimentin (V9, Sigma). The following secondary antibodies were used: goat anti-mouse or anti-rabbit-Alexa 488, anti-Alexa 568 (Invitrogen), or anti-IRDye 800cw (LI-COR, Bad-Homburg, Germany).

SDS-PAGE and western blotting

SDS-PAGE and western blotting were performed as previously described (Fontao *et al.*, 2003), except for the systematic use of secondary antibodies conjugated to IRDye 800cw. Western blot membranes were scanned with Odyssey (LI-COR). For in-gel detection of fluorescent proteins, samples were not boiled before loading and gels were scanned with a Typhoon 9400 (GE-Healthcare, Glattbrugg, Switzerland).

Yeast two- and three-hybrid assays

Yeast two- and three-hybrid experiments were performed exactly as previously described (Fontao *et al.*, 2003). For each keratin pair used in Y3H, their ability to heterodimerize was first verified in yeast two-hybrid (data not shown) by cloning one of the partners in pAS2.1. For PL-C-E (see Figure 5a), the mutant S4530G was used to prevent phosphorylation affecting IF binding (Bouameur *et al.*, 2013).

Recombinant proteins

The *E. coli* strain BL21 (DE3) was used to produce IF proteins and plectin mutants from pET vectors (Novagen). Protein expression was induced with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (Promega, Dübendorf, Switzerland) for 3 h at 37 °C or overnight at 22 °C. Tag-less keratins were purified as previously described (Fontao *et al.*, 2003). For H6-tagged K10 or keratin fragments, bacteria were lysed by sonication in 20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, 8 M urea, pH 7.4, and centrifuged at 21,000*g* for 30 min. Proteins were purified with a His-Trap-FF column according to the manufacturer's protocol (GE-Healthcare) and dialyzed against phosphate-buffered saline, 6 M urea, and 2 mM dithiothreitol. Recombinant EGFP-H6 and H6-EGFP-PL-B5-L were affinity-purified as for H6 keratins but without urea. For EGFP-H6, eluted fractions were dialyzed against 20 mM TrisHCl, pH 8.0, and refined on a Hi-Trap-Q-FF column (GE-Healthcare) equilibrated in dialysis buffer. EGFP-H6 was eluted with 1 M NaCl linear gradient and finally dialyzed against phosphate-buffered saline-dithiothreitol.

FluoBACE interaction assays

FluoBACE assays and related protocols are detailed in Supplementary Materials and Methods online (Aebi *et al.*, 1983; Stappenbeck *et al.*, 1993; Steinert *et al.*, 1999). Graphics were generated with GraphPad software (GraphPad Software, La Jolla, CA), and apparent binding parameters calculated from the simplified assumption of one binding site.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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