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The Amino-Terminal Part of Human Filaggrin-2 is a Component of Cornified Envelopes

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Running title: FLG2 is cross-linked to cornified envelopes

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Abbreviations: CE, cornified envelope; FLG2, filaggrin-2; TGase, transglutaminase.

TO THE EDITOR

Filaggrin-2 (FLG2), a member of the S100-fused type protein family, is a 250 kDa protein mainly expressed in differentiated keratinocytes of the epidermis (Wu et al, 2009; Hsu et al, 2011). Its expression is reduced in atopic dermatitis (Pellerin et al, 2013), and *FLG2* gene heterozygous non-sense mutations are associated with a more persistent form of the disease in Afro-American populations (Margolis et al, 2014). Loss of FLG2 due to homozygous mutations is responsible for peeling skin syndrome type A (Bolling et al, 2018; Mohamad et al, 2018). FLG2 consists of an S100-homologous NH₂-terminal domain, a spacer domain, a large central domain and a short COOH-tail (Figure 1a). The central domain is formed by two types of 75-77 amino-acids repeats: nine A-type repeats and 14 B-type repeats. During cornification FLG2 is proteolytically processed, the various domains being released. The COOH-tail exhibits anti-microbial activity (Hansmann et al, 2015). The B-type repeats are first located in the matrix of the lower corneocytes and degraded later (Hsu et al, 2011). The resulting amino-acids are suspected to be part of the natural moisturizing factor. The role of the amino-terminal part, including the A-type repeats, is not known. Here, we demonstrate that it is cross-linked to the cornified envelope (CE).

CEs were purified, as previously reported (Simon et al, 1996), from plantar stratum corneum obtained from two patients undergoing podiatry care but who had no history of skin diseases and had given their written informed consent. The CEs were then digested with endoproteinase V8. The resulting polypeptides were separated in SDS-gel, in-gel digested with trypsin, and identified using Nanoflow liquid chromatography-tandem mass spectrometry (Supplementary Materials online). One hundred and twenty-six proteins, with various types and cellular location, were identified. Some of them are well known CE components, including keratins, filaggrin, hornerin, desmosomal proteins, cystatin-alpha and transglutaminases (TGases) 1, 3 and 5 (Figure 1b-d and Supplementary Table S1. The

complete list is also available upon request to MS). Eleven unique peptides derived from FLG2 were also identified (Figure 1; Supplementary Table S2). They mostly corresponded to the S100, the spacer and the type-A repeat domains (3 peptides in each domain). In agreement with this result, two peptides corresponding to the spacer domain have been previously shown to be incorporated in CEs (Vermeij et al, 2011). However, peptides derived from hornerin, a known component of CEs (Henry et al, 2011; Hsu et al, 2017), were distributed all along the protein sequence (Supplementary Table S3). Further supporting the hypothesis that FLG2 A-type repeats are cross-linked to CEs, are their sequence homology with hornerin (50-75% identity), and their high content in glutamine which may act as amine acceptor in the formation of N ϵ (γ -glutamyl)-lysyl isopeptide bonds catalysed by TGases.

To confirm that the amino-terminal part of FLG2 is a component of CEs, the plantar envelopes (with two additional samples) and CEs purified from abdominal skin were stained with an antibody directed against the spacer domain (Wu et al, 2009). Abdominal skin samples, from three females without any history of skin diseases undergoing plastic surgery, was obtained from Genoskin (Toulouse, France), following written informed consent of the donors and as agreed by the French Ministry of Research (#AC-2017-2897). Using indirect immunofluorescence, the CEs were clearly labelled (Figure 2a). Roughly one out of two CEs was labelled, whatever the anatomic territory (see Supplementary Table S4). The purified CEs were then incubated for increasing periods of time with endoproteinase V8, as previously reported (Hsu et al., 2017), and the fragments produced were analyzed by immunoblotting (Figure 2b). As typically observed when CEs are proteolysed, multiple high molecular weight bands with a smearing pattern (that differed between plantar and abdominal CEs) were immunostained after the protease action.

We tested whether the FLG2 spacer domain was a substrate of TGases, as previously described (Hsu et al, 2017). A recombinant form of the spacer (FLG2 residues 96 to 235;

Figure 2c) was incubated with TGase-1 or TGase-3, in the presence of monodansylcadaverine. Samples were then separated by SDS-gel electrophoresis and gels observed under UV light (Figure 2d). TGase-1 and TGase-3 induced a time-dependent incorporation of monodansylcadaverine in the spacer and in high-molecular-weight cross-linked complexes (indicated by dots).

Finally, we showed that reduced amount of FLG2 induced a lower mechanical resistance of CEs purified from reconstructed human epidermis (Figure 2e).

In conclusion, our data demonstrate that FLG2 N-terminal part, including A-type repeats, is incorporated in CEs. Thus FLG2 and filaggrin have overlapping roles in the homeostasis of the epidermal barrier. Three non-exclusive possibilities may explain the stratum corneum fragility observed in the absence of FLG2, as in peeling skin syndrome type A patient skin: 1) an abnormal intermediate filament compaction, since mouse Flg2 has been shown to interact with and aggregate keratin filaments *in vitro* (Presland et al, 2006); 2) a reduced corneocyte resistance, as suggested by our data; 3) an induced defect in keratinocyte differentiation program, as seen in the patient skin and in reconstructed human epidermis (Pendaries et al, 2015).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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FIGURE LEGENDS

Figure 1. Position of filaggrin-2-derived peptides identified after mass spectrometry analysis of plantar cornified envelopes. (a) Schematic representation of human filaggrin-2 with the N- and C-terminus, the N-terminal S100-homologous domain, the spacer domain and the repetitive region containing A-type and B-type repeats. Numbers indicate amino acid residues. The position of the peptides identified after mass spectrometry analysis is indicated by black horizontal lines, that of the two peptides reported by Vermeij *et al.* by green lines, that of the recombinant form of filaggrin-2 used for both rabbit immunization and transglutaminase assays (aa 96-235) by a red line, and the mutated amino acids in peeling skin disease by vertical arrows. (b) List of some of the identified proteins after mass spectrometry. The complete raw data Data are available via ProteomeXchange with identifier PXD011833. (c and d) The 126 identified proteins were analyzed using Ingenuity Pathway Analysis for their location and types.

Figure 2. Filaggrin-2 spacer domain is cross-linked to cornified envelopes. (a) Cornified envelopes (CEs) purified from abdominal and plantar human skin were observed in dark field and immunodetected with the anti-filaggrin-2 antibody (red) or without primary antibody. Arrows in the upper panels indicate immunolabelled envelopes. A typical immunodetected envelope from each anatomical site is enlarged in the middle panels. Scale bar: 50 μm . (b) The CEs were analyzed by immunoblotting with the same antibody after incubations without (V8-) or with (V8+) V8 protease for 0 to 72 h, as indicated. (c) The recombinant filaggrin-2 spacer domain (rFLG2) was Coomassie blue stained after separation by SDS-gel electrophoresis. Although its theoretical molecular mass is 15.66 kDa, two bands around 27

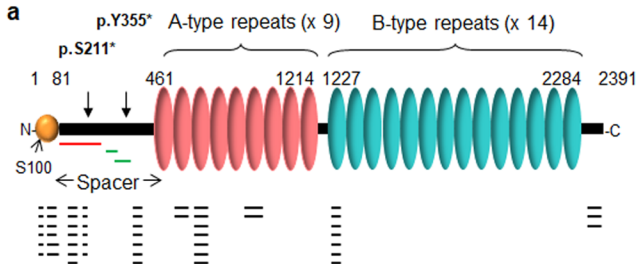
and 55 kDa were observed. (d) Casein, as a control, and rFLG2 were incubated with either transglutaminase 1 (TGase-1) or transglutaminase 3 (TGase-3) for 0, 1 and 4 hours in the presence of CaCl_2 and monodansylcadaverine. Samples were separated by SDS-gel electrophoresis, and incorporation of monodansylcadaverine was revealed by UV illumination.

- , high-molecular-weight cross-linked complexes. The position of molecular mass markers (m) is indicated on the right. (e) Reconstructed human epidermis were produced with keratinocytes from one donor and transduced with either control shRNA (shCo) or *FLG2*-specific shRNA (shFLG2). In each condition, CEs were purified from four pooled epidermis. The same amount of CEs were then sonicated for 5 seconds (n = 6 independent experiments), and the remained intact envelopes were observed under a dark field microscope and quantified.

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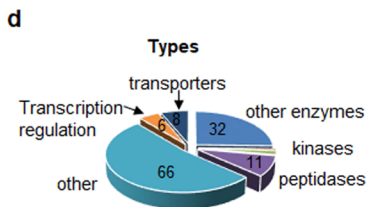
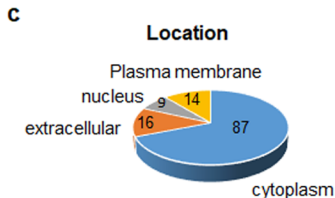
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b

access	symbol	description
P04264	KRT1	keratin 1
P35908	KRT2	keratin 2
P12035	KRT3	keratin 3
P19013	KRT4	keratin 4
P13647	KRT5	keratin 5
P02538	KRT6A	keratin 6A
P04259	KRT6B	keratin 6B
KRT6C	KRT6C	keratin 6C
P35527	KRT9	keratin 9
P13645	KRT10	keratin 10
P13646	KRT13	keratin 13
P02533	KRT14	keratin 14
P08779	KRT16	keratin 16
Q04695	KRT17	keratin 17
P08727	KRT19	keratin 19
Q9C075	KRT23	keratin 23
Q7Z3Z0	KRT25	keratin 25
Q7Z794	KRT77	keratin 77
Q8N1N4	KRT78	keratin 78
Q5XKE5	KRT79	keratin 79
Q6KB66	KRT80	keratin 80
Q9NSB4	KRT82	keratin 82



Q08554	DSC1	desmocollin 1
Q14574	DSC3	desmocollin 3
Q02413	DSG1	desmoglein 1
P15924	DSP	desmoplakin
P14923	JUP	junction plakoglobin
Q13835	PKP1	plakophilin 1
Q5T749	KPRP	Keratinocyte proline rich protein
P35321	SPRR1A	small proline rich protein 1A
P22528	SPRR1B	small proline rich protein 1B

