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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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Filaggrin Is a Predominant Member of the Denaturation-Resistant Nickel-Binding Proteome of Human Epidermis

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TO THE EDITOR

Excessive nickel skin exposure may cause contact sensitization (Thyssen and Menné, 2010). There appears to be a link between the expression of the skin protein filaggrin and the propensity of becoming nickel sensitized. Approximately 10% of Northern Europeans carry at least one filaggrin null mutation, which is associated with ichthyosis vulgaris (Smith *et al.*, 2006), atopic dermatitis (Palmer *et al.*, 2006), and allergic nickel dermatitis (Novak *et al.*, 2007; Ross-Hansen *et al.*, 2011). Double-allele null mutations lead to a complete lack of filaggrin. The protein is expressed just below the outermost skin layer, stratum corneum (SC), as a large polyprotein, profilaggrin (Sandilands *et al.*, 2009). It consists of 10–12 equally sized filaggrin units that vary by up to 40% in amino-acid composition and are released by enzymatic processing (Thulin *et al.*, 1996; Sandilands *et al.*, 2009). In the SC, filaggrin is degraded to amino acids, which are major contributors to water binding (Kezic *et al.*, 2008). Hence, filaggrin molecules constitute a heterogeneous protein population. As filaggrin is an abundant histidine-rich protein (McKinley-Grant *et al.*, 1989), we hypothesized that it contributes to epidermal nickel binding. Consequently, lack of filaggrin could cause increased percutaneous nickel penetration and ultimately sensitization. We investigated whether filaggrin from human epidermal extracts bound nickel and evaluated the binding potential of other proteins. The full protein spectrum from *ex vivo* epidermal samples was visualized by SDS-PAGE (Figure 1, lane 2). Immunoblotting with monoclonal anti-filaggrin antibodies revealed the size range of filaggrin molecules with major reactivity at a molecular weight corresponding to a heterogeneous monomer population (Figure 1, lanes 8 and 10–12, arrowhead). Nondenaturing binding studies with column-packed nickel Sepharose showed that filaggrin among other proteins was enriched by immobilized metal affinity chromatography (IMAC) (Figure 1, lanes 4–6 and 10–12). To verify the specificity of the antibodies and identify other nickel-binding proteins, the protein contents of lane 4 (Figure 1) were identified by systematically cutting out gel slices and analyzing by tandem mass spectrometry (MS). This analysis gave a semiquantitative measure of the 20 most abundant nickel-binding proteins (Supplementary Table S1 online). Filaggrin was found in top 6, and most of the other proteins were previously reported to bind nickel and/or other divalent metals (Supplementary Table S1 online). Whether the proteins were captured on-column through protein interactions rather than direct nickel binding was studied by varying the washing stringency conditions. Increasing salt concentration (0.5–2 M NaCl) or urea gradient washing (2–8 M urea) did not result in filaggrin release, nor did it negate the binding of the other identified proteins (data not shown). Under denaturing and reducing conditions, i.e., by washing the column with 2% SDS and 0.1 M DTT (Figure 2a), most proteins including a low-molecular-weight filaggrin species were released (Figure 2a, lanes 6 and 14). The monomers and larger filaggrin molecules

Abbreviations: DTT, dithiothreitol; IMAC, immobilized metal affinity chromatography; MS, mass spectrometry; SC, stratum corneum

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retained their nickel-binding properties and were only eluted by EDTA (Figure 2a, lanes 8 and 16). Thus, filaggrin chelates nickel, and the finding corroborates that filaggrin is functioning as a natively unfolded protein. The relevance of nickel binding in terms of percutaneous penetration depends on the proteins' topographical location in the epidermis. Previously, *ex vivo* skin coloring has shown nickel accumulation in the SC following nickel skin contact (Wells, 1956). Recently, Kubo *et al.* (2013) demonstrated distinct Cr(III) barrier properties of mouse SC coinciding with filaggrin and its amino-acid derivatives. In filaggrin-deficient mice, Cr(III) permeated the SC. As the origin of filaggrin from epidermal extracts is unknown, we extracted proteins from superficial skin scrapings to evaluate the binding potential of SC-derived filaggrin. The samples were boiled in SDS and DTT to solubilize extractable proteins. A high-molecular-weight (55–60 kDa) filaggrin was detected in the EDTA fraction (Figure 2b, lanes 8–9). This protein was not present in the effluent or wash fractions (Figure 2b, lanes 6–7). The identity of filaggrin was verified by tandem MS, which revealed additional filaggrin corresponding to monomers (Figure 2b, arrowhead). Using polyclonal anti-filaggrin antibodies, both monomers (arrowhead) and high-weight filaggrin (asterisk) were detected (Figure 2b, lane 9). The larger species may be a product of isodipeptide crosslinking by transglutaminases, a feature conferring the extreme stability of SC cornified envelopes (Candi *et al.*, 2005). Whereas the major portion of filaggrin resides in the keratin-matrix interior of the cell, some of it is incorporated into the cornified envelope (Steinert and Marekov, 1995). Only a small fraction of filaggrin from the epidermis is substrate for *in vitro* transglutaminase crosslinking and may represent a subgroup accessible for modification or nonspecific processing (Simon *et al.*, 1996). The failure of monomer recognition by monoclonal antibodies in the SC extracts suggests that filaggrin modifications take place. Both filaggrin and keratin would be important for nickel binding in the SC as they are major constituents there. Even though keratins were also retained by IMAC under nondenaturing conditions, keratin

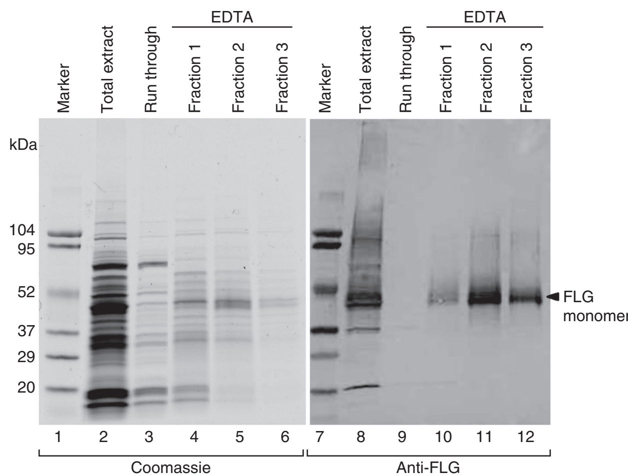

Figure 1. Filaggrin is enriched by nickel affinity chromatography. Epidermal extracts were incubated with Ni Sepharose and poured onto a column. Fractions were collected from effluent (run through) and after EDTA elution. The full protein spectrum was visualized by Coomassie stain after SDS-PAGE. Filaggrin was identified by blotting with monoclonal antibodies (anti-FLG). Arrowhead denotes filaggrin monomer population.

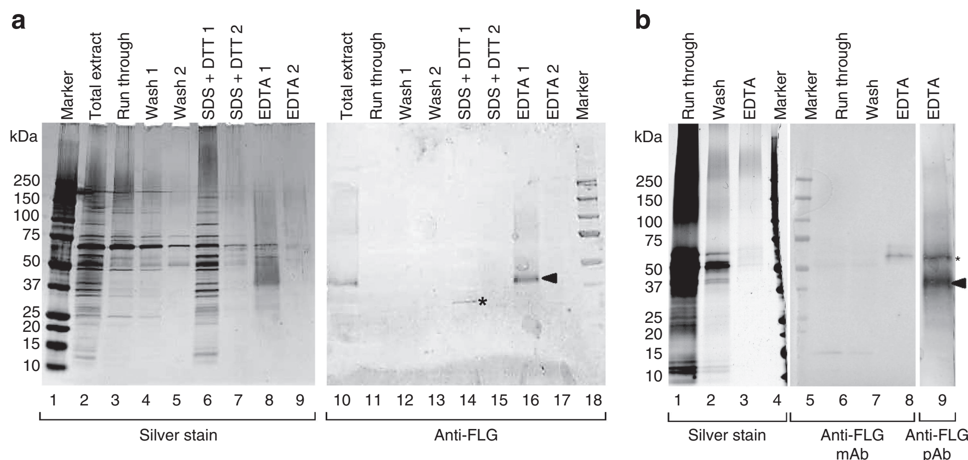

Figure 2. Filaggrin monomers resist elution from nickel Sepharose under reducing and denaturing conditions, and filaggrin from the stratum corneum (SC) binds to nickel. Epidermal extracts were incubated with Ni Sepharose and poured onto a column. Fractions were collected from the run-through, washings, and EDTA eluates. The full protein spectrum was visualized by silver staining after SDS-PAGE. (**a**) Filaggrin was identified by blotting with mAbs (anti-FLG). Arrowhead: filaggrin monomer population. Asterisk: low-molecular-weight filaggrin species. (**b**) The soluble proteins from SC were extracted under denaturing and reducing conditions. Filaggrin was identified by blotting with either mAbs or polyclonal antibodies (anti-FLG mAb and pAb, respectively). Arrowhead: filaggrin monomer population. Asterisk: high-molecular-weight filaggrin.

nickel binding has not been reported. Unfortunately, SC protein extraction must be performed under denaturing conditions, and the presence of resident proteins that need their secondary or tertiary structure for nickel binding could not be evaluated. Moreover, as the cornified envelope resists breakdown into its protein constituents, they are inaccessible for analysis. Finally, the setup did not allow for assessment of nickel binding by free amino acids. In summary, we demonstrate that filaggrin derived from both SC and full epidermis binds nickel. Other epidermal proteins may bind nickel, but filaggrin is a strong, denaturation-resistant chelator, and the relevance of the other proteins, e.g., for the accumulation of nickel in the SC, remains unclear. As filaggrin null mutations are associated with an increased risk of allergic nickel dermatitis in European populations, this study provides a possible link between genetics, protein expression, and function.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

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Evidence for an Alternatively Spliced MITF Exon 2 Variant

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TO THE EDITOR

Alternative splicing of exon 1 in the microphthalmia-associated transcription factor (*MITF*) gene gives rise to a family of transcription factors that differ only in exon 1 sequence (Steingrimsson *et al.*, 2004). Each MITF isoform is associated with a specific promoter and each unique promoter/isoform combination results in MITF expression in a different cell lineage. In the melanocytic cell lineage, MITF is expressed from the M-promoter, leading to a transcript containing exon 1 M; therefore, this isoform is termed MITF-M. The amplification of *MITF-M* occurs in 10–20% of all melanoma cases, and it has been suggested that MITF-M may be a prognostic marker for poor survival in melanoma (Garraway *et al.*, 2005; Ugurel *et al.*, 2007). Recently, a germline mutation in *MITF* (E318K) has also been shown to contribute to increased susceptibility to melanoma (Bertolotto *et al.*, 2011; Yokoyama *et al.*, 2011). The MITF signaling pathway has further been shown to contribute to melanoma susceptibility. A recent study identified frequent somatic mutations in both MITF and its regulator SOX10, with 14% of primary and 20% of metastatic melanoma containing mutations in these genes (Cronin *et al.*, 2009).

Recently, an isoform of MITF-M that is alternatively spliced at exons 2 and 6 has been reported. This shortened isoform, termed MITF-MDel, was expressed at a detectable level only in melanocytes

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