Filaggrin in atopic dermatitis

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The recent identification of loss-of-function mutations in the structural protein filaggrin as a widely replicated major risk factor for eczema sheds new light on disease mechanisms in eczema, a disease that had heretofore largely been considered to have a primarily immunologic etiopathogenesis. The filaggrin gene *(FLG)* mutation findings are consistent with a recently proposed unifying hypothesis that offers a mechanistic understanding of eczema pathogenesis synthesizing a heritable epithelial barrier defect and resultant diminished epidermal defense mechanisms to allergens and microbes, followed by polarized T_H2 lymphocyte responses with resultant chronic inflammation, including autoimmune mechanisms. Although compelling evidence from genetic studies on *FLG* implicates perturbed barrier function as a key player in the pathogenesis of eczema in many patients, much is still unknown about the sequence of biologic, physicochemical, and aberrant regulatory events that constitute the transition from an inherited barrier defect to clinical manifestations of inflammatory eczematous lesions and susceptibility to related atopic disorders. The exact contribution of *FLG* to the wider atopic story, factors modifying *FLG* expression, and the role of other barrier proteins remain to be delineated. In this review we highlight recent advances in our understanding of the *FLG* genetics in the cause of eczema and related complex diseases. (Reprinted from J Allergy Clin Immunol 2008;122:689-93.)

Key words: *atopic dermatitis, barrier function, cornified cell envelope, eczema, epidermal differentiation complex, filaggrin, ichthyosis vulgaris, natural moisturizing factor, pH, proteases, Staphylococcus aureus*

The epidermis provides an essential attribute of adaptation to terrestrial life, namely an occlusive interface barrier, restricting both water loss from the body and ingress of pathogens. This barrier is formed after a complex, integrated, and exquisitely regulated series of biochemical events culminating in a program of cell death by terminally differentiating keratinocytes.[1] Epithelial keratinocytes replace their plasma membrane with a tough, insoluble macromolecular layer called the cornified envelope (CE) to achieve and maintain this barrier.

Abbreviations used

CE: Cornified envelope
EDC: Epidermal differentiation complex
FLG: Filaggrin gene
IV: Ichthyosis vulgaris
KLK7: Kallikrein 7
NMF: Natural moisturizing factor
SC: Stratum corneum
SPINK5: Serine protease inhibitor Kazal type 5

Initial steps in the formation of the CE result in the sequential expression of several major protein products, but only certain proteins from a choice of more than 20 are used in the final stages of CE reinforcement to meet site-specific requirements, of which filaggrin is one of the final proteins to be incorporated.[1] These structural proteins are extensively cross-linked by transglutaminases and act as a scaffold for the attachment of a layer of lipids covalently bound to the extracellular surface, forming an outer lipid envelope. In response to deficiency, injury, or other environmental triggers, proteins forming the CE might be upregulated in an effort to compensate and maintain an effective barrier.[1] Cell differentiation, death, and desquamation occur sequentially, with recent convergent approaches affording insight into the molecular mechanisms and diseases associated with defects in the pathways of cornification.

Many of the key proteins involved in cornification are encoded for in a gene-dense locus on chromosome 1q21, which is termed the epidermal differentiation complex (EDC).[2] The EDC spans an area of 1.62 megabases containing more than 70 genes expressed during the late stages of terminal differentiation, and genome-wide screens have shown significant linkage colocalization with psoriasis, autoimmune diseases, and eczema, heightening interest in this locus.[3] Many EDC proteins share significant sequence similarities, and phylogenic data suggest that these proteins derived from a common ancestor, evolving to meet tissue-specific demands. These genes cluster within the EDC according to expression pattern and are in tight linkage disequilibrium, suggesting that they might also be coregulated. Genes found within this locus encode for proteins such as loricrin, involucrin, small proline-rich proteins, late envelope proteins, and the S100 calcium-binding proteins, of which filaggrin is a key member. There is considerable evidence for redundancy mechanisms in CE assembly, in that the absence of 1 CE reinforcement protein can be compensated by increased expression of others in experimental animal models.[4] This is exemplified by the loricrin$^{-/-}$ mouse, which displays mild epidermal erythema at birth that normalizes within days, in spite of the fact that loricrin typically comprises 70% to 85% of the protein content of CE. This phenotype is associated with the compensatory upregulation of the EDC structural proteins Sprr2D, Sprr2H, and repetin. Targeted ablation of the murine involucrin gene, a near-ubiquitous component of CE,

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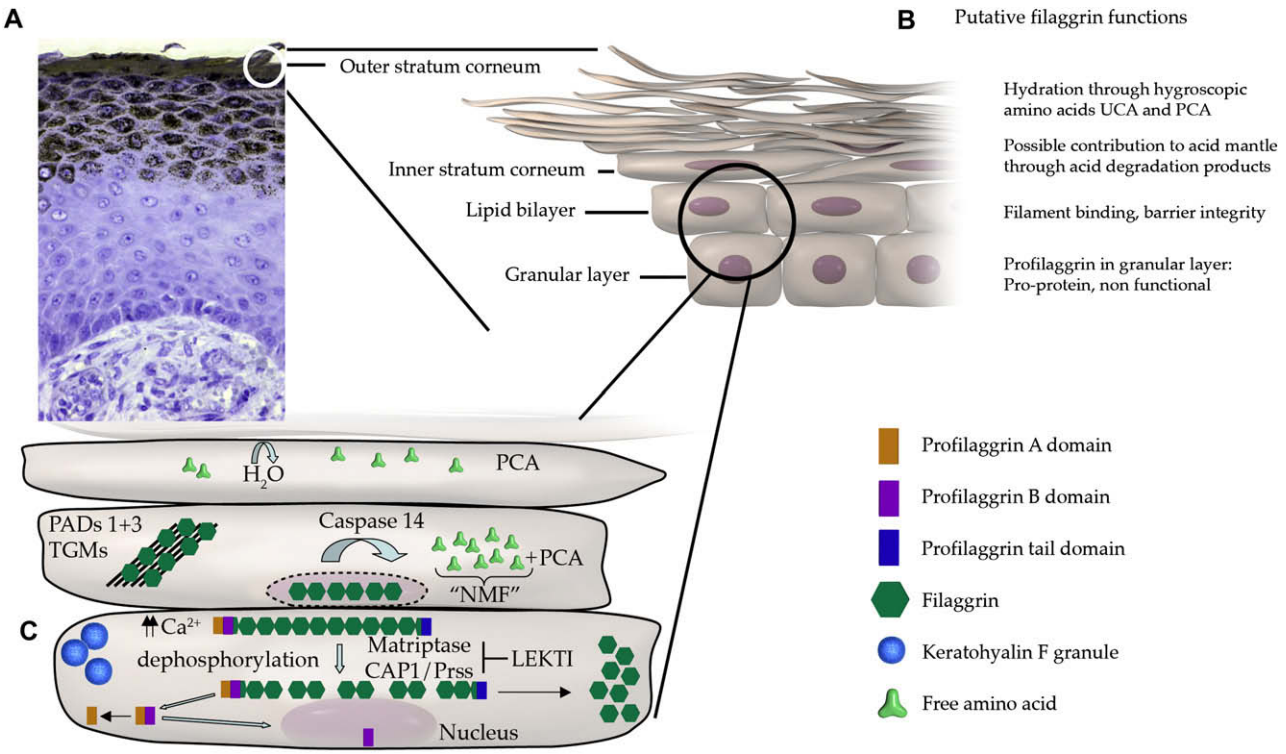

FIG 1. *FLG* expression and putative functions in the skin barrier: schematic summarizing the filaggrin expression pattern and putative functions. **A**, The precursor proprotein profilaggrin is strongly expressed within keratohyalin granules, tightly limited to and accounting for the typical appearance of the granular layer. The SC stains strongly positive for filaggrin. **B**, Filaggrin has several proposed site-specific functions under the influence of the epidermal terminal differentiation program through the outer granular layer (cleavage of profilaggrin to filaggrin) and the lipid bilayer of the inner SC (filament compaction, contribution to barrier integrity) and during desquamation of the outer SC (production of amino acid degradation products that contribute to the hydration of these outer layers and likely contribute to the "acid mantle"). **C**, Summary of current knowledge of molecular control of filaggrin homeostasis. Profilaggrin is dephosphorylated in conditions of increasing calcium concentration and is then proteolytically cleaved by the proteases matriptase (inhibited by the protease inhibitor *LETKI*) and CAP1/Prss. After proteolysis, the filaggrin B domain locates to the nucleus as part of the terminal differentiation process. Free filaggrin protein is cross-linked to keratin filaments by transglutaminases and subsequently deiminated by peptidylarginine deiminases *(PADs)* 1 and 3. Further posttranslational modification is undertaken by caspase 14 to produce the free amino acid hygroscopic degradation products urocanic acid *(UCA)* and pyrrolidone carboxylic acid (*PCA*; collectively known as NMF), which contribute to SC hydration. *TGMs*, Transglutaminases.

similarly lacks a discernable phenotype. The recent development of a composite triple-knockout mouse deficient in involucrin, envoplakin, and periplakin demonstrates delayed barrier formation during embryogenic development, defects in the assembled CE, and excessive accumulation of cornified layers throughout postnatal life, suggesting that these initiator CE proteins are critical for barrier acquisition.[4] Strikingly, reduced epidermal protease activity, as opposed to upregulation of structural proteins, rescues the phenotype from lethality, with a marked increase of the protease inhibitor serpina1b, resulting in a compensatory reduction in desquamation, with secondary downstream defects in defective filaggrin gene *(FLG)* processing.[4] For a more detailed consideration of the epidermal barrier in atopic dermatitis, please see an earlier article in this series by Elias et al.[5]

FLG EXPRESSION AND FUNCTION

The giant inactive precursor profilaggrin is a large, complex, highly phosphorylated polypeptide that is the main constituent of the keratohyalin F granules that are visible in the granular cell layer of the epidermis (Fig 1, *A*). During formation of the cornified cell envelope, profilaggrin is dephosphorylated and proteolytically cleaved by serine proteases, including channel-activating serine protease/Prss and matriptase/matriptase, to release multiple copies of the functional FLG repeat peptide units. Control of protease activity is balanced by a series of inhibitors, which are abundant and pivotal in epithelial differentiation, the most characterized of which is lymphoepithelial Kazal-type trypsin inhibitor, the polyvalent protein product encoded by serine protease inhibitor Kazal type 5 *(SPINK5)*. After cleavage, liberated filaggrin binds to and collapses the keratin cytoskeleton, resulting in a flattened squame aligned parallel to the outer surface of the epidermis. The cleaved N-terminal S100-like calcium-binding domain of profilaggrin enters the nucleus, where it is postulated to have an additional role in regulating terminal differentiation. Subsequently, within the stratum corneum (SC) itself, the filaggrin peptide is progressively degraded by posttranslational modification enzymes (including peptidylarginine deiminase 1 and 3 isoforms) into a pool of hydrophilic amino acids, including urocanic acid, pyrrolidone carboxylic acid, and alanine. This

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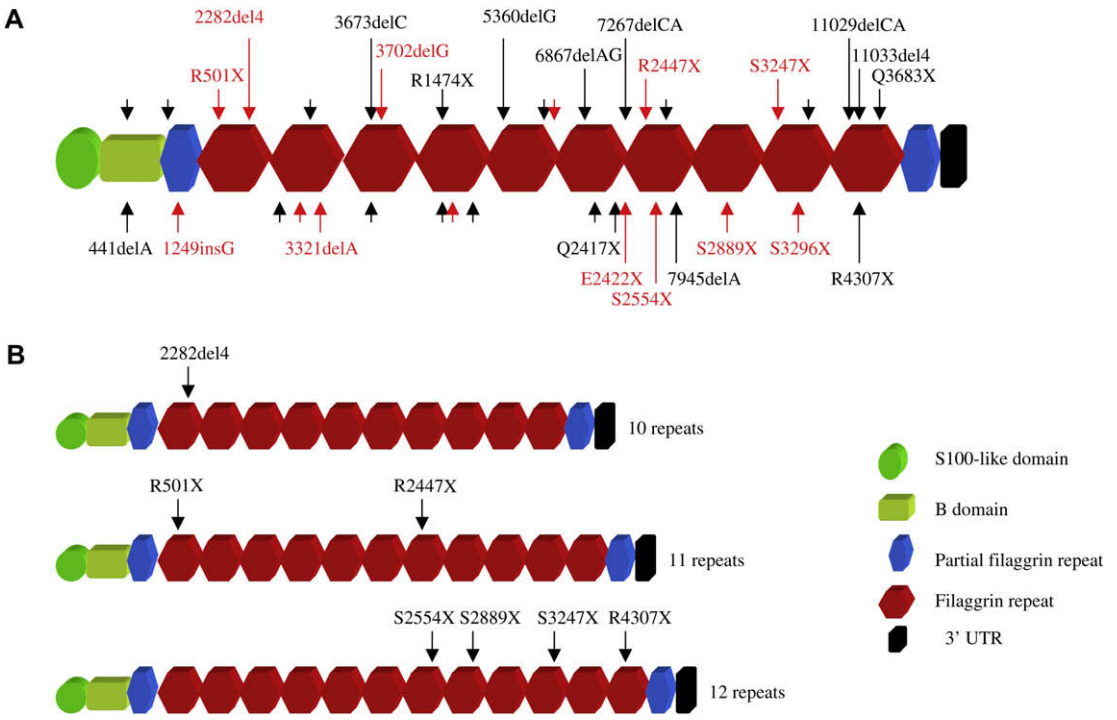

FIG 2. Protein organization and location of mutations. *FLG* is composed of a large transcript encoded by 3 exons, of which the third exon encodes the *FLG* protein repeats. The bulk of *FLG* protein sequences consist of a tandem array of repeating units of 35 kd separated by a 7- to 10-amino-acid linker peptide. There are 10 highly homologous *FLG* polypeptide units, with a variable number of *FLG*-repeat units consisting of 10, 11, or 12 units. The locations of 37 known mutations are shown. Reported mutations resulting in functional null alleles are numbered; positional locations of as-yet-unreported mutations are demonstrated by unmarked *arrows*. Prevalent mutations are indicated by *red font*, and family-specific mutations are in *black* **(A)**. Recurrent mutations can occur on the background of a 10-, 11-, or 12-repeat allele **(B)**. *UTR*, Untranslated region.

combined pool of amino acids, their metabolites, and various ions make up what is known as the natural moisturizing factor (NMF; Fig 1, *C*).[6] NMF is highly hygroscopic and plays a central role in maintaining hydration of the SC. NMF might additionally play a critical role in the maintenance of the pH of the skin, regulating key biochemical events, including protease activity, barrier permeability, and cutaneous antimicrobial defense, functions that are fundamentally linked and coregulated. The importance of filaggrin-derived breakdown products and the profound effect on barrier function in their absence is underscored by the remarkably short half-life of filaggrin, which exists for only 6 hours before full proteolysis. Expression of *FLG* and subsequent activation of hydrolysis of filaggrin peptides into NMF are additionally determined by the properties of the microenvironment, including local pH, external humidity, and transepidermal water loss.[6]

FLG MUTATIONS CONFER STRONG GENETIC SUSCEPTIBILITY TO ECZEMA

The discovery of the association of *FLG* mutations with atopic diseases followed insights into a common disorder of keratinization, ichthyosis vulgaris (IV). IV is the most common of the ichthyotic disorders, estimated to affect 1 in 250 individuals, and is characterized by generalized fine white scale, palmoplantar hyperlinearity, and keratosis pilaris. For more than 20 years, much indirect evidence pointed toward mutations in *FLG* as causative; however, many confounders delayed confirmation of this association. These included inconsistencies in the reported inheritance pattern, with apparent dominant and recessive inheritance, erroneously reported linkage, and a repetitive gene sequence limiting amplification. Two loss-of-function *FLG* mutations (R501X and 2282del4) were ultimately detected by using long-range sequencing and multiple alignment techniques, revealing a semidominant pattern of inheritance with incomplete penetrance.[7] The number of mutations identified has increased dramatically in the past 2 years, each predicting nonsense or out-of frame deletion/insertion mutations, with population-specific patterns emerging worldwide.

To date, the number of *FLG* mutations identified in European populations is 20, of which 6 are prevalent and 14 are of low frequency. In Asian populations an additional 17 mutations, of which 8 are prevalent and 9 are of low frequency, have been identified (Fig 2, *A*). Of note, more distal mutations allow limited expression of profilaggrin but no production of functional filaggrin subunits, implying a critical role of the C-terminus for *FLG* processing; there is also some early evidence of a trend toward reduced penetrance of more distal mutations.[8] The combined allele frequency of the initial mutations translates into a carrier frequency of almost 10% in individuals of European ancestry.[8] This unexpected finding combined with the known clinical association of IV with eczema and decreased expression of FLG in patients with eczema pointed to a possible association in the pathogenesis of eczema. This association has now been unambiguously established in a series of replication studies, making this one of the most robust gene associations thus far identified in complex trait genetics.[8-15]

Overall, between 18% and 48% of all eczema collections carry *FLG*-null alleles.[14] The relatively high allele frequency of several haplotypically independent null alleles in the population is intriguing and suggests that these have not arisen because of genetic drift alone but might be as a result of balanced selection caused by an as-yet-unclear evolutionary heterozygote advantage.[16]

The *FLG* mutation findings were corroborated in 2 recent, large, population-based studies on more than 6700 English children[17] and 3000 German children, in whom the 2 common *FLG* mutations R501X and 2282del4 and 3 rare variants were analyzed.[18] In the German study *FLG* variants increased the risk for eczema 3-fold (odds ratio, 3.12; 95% CI, 2.33-4.17; $P = 2.5 \times 10^{-14}$) with a population-attributable risk of 13.5%. Importantly, these mutations are highly associated with allergen sensitization and the subsequent development of asthma associated with eczema, an association that has been consistently reported.[14] At a population level *FLG* mutations appear to confer an overall risk of asthma of approximately 1.8 but only in the context of prior eczema.[19] Because *FLG* is not expressed in bronchial mucosa, transcutaneous sensitization is one suggested mechanistic possibility for *FLG* to confer asthma risk.[9,20]

FLG: EPISTATIC EFFECTS?

Other genetic associations within pathways that modulate filaggrin have been reported, including common maternally derived polymorphisms in the serine protease inhibitor *SPINK5* (particularly Glu420Lys) that have been shown to modify the risk of eczema, asthma, and IgE, suggesting that this pathway might lie in altered expression of environmental proteases. Pathologic loss-of-function mutations in *SPINK5*, as found in patients with Netherton syndrome, are associated with a profound barrier defect and severe atopic diathesis, resulting in unchecked proteolysis by processing enzymes, such as matriptase, and other serine proteases of an extracellular desmosomal component (corneodesmosin) and lipid-processing enzymes.[21] Gain-of-function polymorphisms in the kallikrein 7 gene *(KLK7)* encoding the protease stratum corneum chemotrypic enzyme, have been additionally reported to adversely affect barrier function, are postulated to affect the proteolytic processing of profilaggrin, and are potentially regulated by lymphoepithelial Kazal-type trypsin inhibitor. Recently, we studied these reported mutations in several large patient collections involving more than 2500 patients and 10,000 control subjects. We were able to confirm a role for maternally inherited *SPINK5* mutations in a German family cohort but could not replicate the *KLK7* findings. Neither *KLK7* nor *SPINK5* had any epistatic effects with *FLG*-null alleles.[22]

FLG AND ECZEMA PATHOGENESIS: MECHANISMS AND SPECULATIONS

Although the very strong genetic association of *FLG* mutations with eczema is now clear, the mechanistic pathways from inherited *FLG* haploinsufficiency to the typical inflammatory lesions of eczema requires further elucidation. FLG deficiency leads to reduced NMF,[23] which is likely a contributor to the xerotic phenotype seen in many patients with eczema. The initiation of the typical inflammatory response is of great interest, and with this in mind, one should remember that around 40% of all carriers of *FLG*-null alleles never have any signs of eczema.[17] The environmental and genetic modifiers (discussed above) of this risk are currently unclear, although recent evidence also indicates that filaggrin skin expression could be modulated by the atopic inflammatory response mediated by the cytokines IL-4 and IL-13,[24] thus providing a link between this structural molecule and the inflammatory response in eczema.

Other currently speculative mechanisms include the possibility that *FLG* haploinsufficiency might critically modify pH-related altered commensal bacteria expression, thus manipulating host immunity. Altered host immunity to bacterial infections is a notable feature of atopic dermatitis.[25] Growth of *Staphylococcus aureus* is facilitated by increased pH, and it colonizes the skin of more than 90% of patients with eczema. Exposure of a naive immune system to *S aureus* superantigens can trigger and establish a permanent T_H2 immune response through activation and amplification of innate immune responses. The neutralizing acid SC pH has also been shown to independently facilitate excessive protease activity and reduce the activity of key lipid-processing enzymes, resulting in the formation of defective lamellar membranes and a disrupted permeability barrier.

CONCLUSION

FLG mutations are the strongest and most widely replicated genetic risks for eczema identified to date. They have a clear permissive effect in the early inflammatory effects that characterize eczema and affect both priming of disease and chronicity. The identification of these mutations has enlivened the field of eczema genetics. Their identification raises the potential for targeted intervention and therapy and might lead to a consideration of a new molecular classification of eczema. The environmental and genetic interactions with *FLG*-null alleles that contribute to the pathogenesis of this distressing, fascinating, and complex disease will be of great interest in the next several years.

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