Ichthyosis, Follicular Atrophoderma, and Hypotrichosis Caused by Mutations in *ST14* Is Associated with Impaired Profilaggrin Processing

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Congenital ichthyosis encompasses a heterogeneous group of disorders of cornification. Isolated forms and syndromic ichthyosis can be differentiated. We have analyzed two consanguineous families from the United Arab Emirates and Turkey with an autosomal recessive syndrome of diffuse congenital ichthyosis, patchy follicular atrophoderma, generalized and diffuse nonscarring hypotrichosis, marked hypohidrosis, and woolly hair (OMIM 602400). By genome-wide analysis, we found a homozygous interval on chromosome 11q24–q25 and obtained a LOD score of 4.0 at D11S910. We identified a homozygous splice-site mutation in the Arab patients and a frame-shift deletion in the Turkish patient in the gene suppression of tumorigenicity-14 (*ST14*). The product of *ST14*, matriptase, is a type II transmembrane serine protease synthesized in most human epithelia. Two missense mutations in *ST14* were recently described in patients with a phenotype of ichthyosis and hypotrichosis, indicating diverse activities of matriptase in the epidermis and hair follicles. Here we have further demonstrated the loss of matriptase in differentiated patient keratinocytes, reduced proteolytic activation of prostasin, and disturbed processing of profilaggrin. As filaggrin monomers play a pivotal role in epidermal barrier formation, these findings reveal the link between congenital disorders of keratinization and filaggrin processing in the human skin.

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INTRODUCTION

The epidermal barrier function is crucial for protecting the organism against the environment and to preventing the body exposed to the air from dehydrating by excessive transepidermal water loss. The barrier function is mainly localized to the outer layers of the epidermis, in which the keratinocytes undergo terminal differentiation finally resulting in the formation of the cornified cell envelope and horny lamellae characteristic of the stratum corneum. Recent studies have demonstrated that disturbance of the epidermal barrier function is involved in various genetic cornification disorders, particularly autosomal recessive congenital ichthyoses (OMIM 242300, 601277, 604777, 606545). Autosomal recessive congenital ichthyosis, characterized by generalized scaling of the skin and erythema (Traupe, 1989), is clinically and genetically heterogeneous and can be caused by mutations in more than six different genes, including *TGM1*, *ALOX12B*, and *ALOXE3*. Transglutaminase 1, encoded by *TGM1*, is involved in the formation of the cornified cell envelope. The lipoxygenases encoded by *ALOX12B* and *ALOXE3* are components of the epidermal 12-lipoxygenase pathway, their biological role for the epidermal barrier formation, however, remains to be clarified. Mouse models revealed that both transglutaminase-1 and 12*R*-lipoxygenase deficiencies caused ichthyosiform skin and severe barrier dysfunction (Matsuki *et al.*, 1998; Kuramoto *et al.*, 2002; Epp *et al.*, 2007; Moran *et al.*, 2007). Congenital ichthyosis may also be associated with hair abnormalities, as in Netherton syndrome (OMIM 256500) caused by mutations in *SPINK5*, which codes for the (lymphoepithelial kazal-type-related) serine protease inhibitor LEKTI (Chavanas *et al.*, 2000). Mouse lines with a *Spink5* knockout presented a lethal ichthyosis phenotype and an impairment of barrier function demonstrating the important role of LEKTI as a regulator of protease activity in the epidermis (Descargues *et al.*, 2005; Hewett *et al.*, 2005).

Recently, two missense mutations in the suppression of tumorigenicity-14 (matriptase) gene (*ST14*) were identified in

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Abbreviations: ARIH, autosomal recessive ichthyosis with hypotrichosis; IFAH, congenital ichthyosis, follicular atrophoderma, hypotrichosis and hypohidrosis; ST14, suppression of tumorigenicity-14

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two families with autosomal recessive ichthyosis with hypotrichosis (ARIH, OMIM 610765; Basel-Vanagaite *et al.*, 2007; Avrahami *et al.*, 2008). Matriptase is a member of the type II transmembrane serine proteases (List *et al.*, 2006a; Uhland, 2006). It is specifically synthesized in terminally differentiating keratinocytes and in matrix, precortex, and cortex cells and the shaft of the anagen hair. It is characterized by a complex life cycle, including autoactivation by proteolytic cleavage, shedding from the membrane, and regulation through the hepatocyte growth factor activator inhibitor-1. *St14* hypomorphic mice demonstrated low activity of matriptase and a phenotype resembling ARIH (List *et al.*, 2007a). Analysis of their epidermis showed reduced prostasin activation and profilaggrin processing.

Here we describe the analysis of two families with congenital ichthyosis, follicular atrophoderma, hypotrichosis, and hypohidrosis (IFAH, OMIM 602400) (Lestringant *et al.*, 1998; Tursen *et al.*, 2002). After a whole-genome linkage scan, we identified ablating mutations in *ST14* as the cause for the disease. The mutations resulted in an impairment of profilaggrin processing, demonstrating the link between congenital ichthyosis and loss of epidermal barrier function, characterized by the lack of filaggrin units.

RESULTS

Phenotype

The first family was a consanguineous family of Bedouin ancestry from the United Arab Emirates with five affected siblings (Figure 2a). The patients have been described earlier by one of us (Lestringant *et al.*, 1998), and a follow-up is given here with particular respect to the phenotype of ARIH published recently (Basel-Vanagaite *et al.*, 2007). The patients presented with congenital ichthyosis, follicular atrophoderma, hypotrichosis, and hypohidrosis. They ranged in age from 4 to 18 years at the time of first examination. Ichthyosis was present at birth, and there were no collodion babies. In all cases scaling was diffuse and involved the great flexures and scalp (Figure 1a). It spared face, elbows, knees, hands, and feet. Keratosis was always severe with possible episodes of transient variations: It might present either with large, light or dark brown adherent plate-like scales, up to 20 mm in diameter, occupying a shallow depression of the skin and of variable thickness, ranging from thin epidermal films to 3–4 mm thick keratotic ''plugs'', or with an intense powdery scaling, especially on the trunk and flexor aspects of the limbs. The scales were mostly distributed following a random linear pattern. In no case was there erythema or itching.

Follicular atrophoderma, a rare skin anomaly consisting of enlarged funnel-shaped depressions of the pilosebaceous orifices, was congenital and present in all patients (Figure 1b). These ''ice-pick'' marks appeared in patches on the dorsal aspects of hands and feet, first phalanges of fingers and toes, wrists, and around elbows and knees. In all patients, the facial skin was involved by ill-defined pitting giving a fine vermiculate or ''orange peel'' appearance. Around the wrists, elbows, and knees, there was a zone of ichthyosis progressively transformed into follicular atrophoderma.

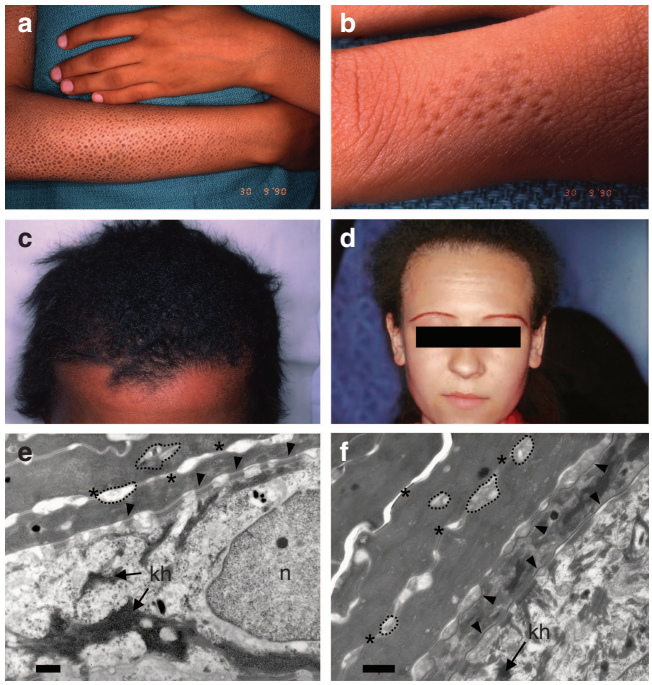

Figure 1. Clinical picture and ultrastructural analysis of patients with IFAH. (**a**–**c**) Patients from the Arab IFAH family. (**a**) Dark brown diffuse scaling on the right forearm and follicular atrophoderma on the wrist, hand, and fingers of IV:1 (Figure 2a). (**b**) Close-up of the phalanx dorsum showing follicular atrophoderma. (**c**) Unruly, curly scalp hair with the typical receding frontal hairline (IV:3). (**d**) The Turkish patient IV:1 (Figure 2b) showing light, woolly hair, hypotrichosis of the eyebrows, and the receding frontal hairline. (**e**–**f**) Ultrastructural analysis of the upper stratum granulosum and the lower stratum corneum. (**e**) Patient IV:1 from the Arab family, (**f**) patient IV:1 from the Turkish family. Dotted lines mark inter- and intralamellar deposits of lamellar body contents in the lower lamellae of the stratum corneum. Amounts of keratohyalin (kh) were reduced. Asterisks denote corneodesmosomes, arrow heads marginal bands of corneocytes (cornified envelopes); n, nucleus; scale bars = 1 μm.

Generalized, diffuse and nonscarring hypotrichosis was the third constant finding. It was present at birth, and boys, who presented with little head hair and next to no eyelashes and eyebrows, were more affected than girls. Hypotrichosis was improving with age. At the time of first examination, the younger siblings IV:3, IV:5, and IV:7 presented with sparse, unruly, and lusterless hair on the scalp, bald patches, and recessing frontal hair line (Figure 1c), whereas the two oldest siblings had nearly normal head hair with recessing frontal hairline only. Similarly eyebrows, at first wiry and limited to the very inner region, were progressively straightening and extending outward, and eyelashes, at first sparse and limited to the upper eyelids, were progressively growing on the lower ones. Facial and body hair were absent in IV:3, IV:5, and IV:7 but IV:1 had developed few hair on both ends of her upper lip and in her armpits at 18 years of age, and IV:2 at 22 years had sparse hair on his upper lip, with a tuft at both ends of it and on his whiskers. There were occasional vellus and crooked hair growing out of a pit of follicular atrophoderma.

Hypohidrosis was assessed clinically in IV:1, IV:2, and IV:3. Sweating could be seen only on the nose, eyebrows, palms, and soles even at 45 °C ambient temperature. There

had been no episodes of unexplained fever or fits in the siblings. At the time of first examination, there was no obvious photophobia in any sibling. Pingueculum was fully developed in IV:2 and was starting in IV:3. The boys were reviewed 8 years later. IV:3, then 19 years of age, presented with fully developed pingueculum and photophobia. His scalp hair was normal. The older brother (IV:2), 22, had marked photophobia and corneal opacities. Incidentally he had developed ichthyosis on the dorsum of his right hand and on his knees, where he used to have follicular atrophoderma. The rest of the physical findings were normal and there was no history of skin malignancy regarding the previously observed facial nevoid papules.

The second family, with one affected and one unaffected sibling, was of Turkish origin (Figure 2b; Tursen *et al.*, 2002). Parents and paternal grandparents were first cousins. Briefly, the female patient showed congenital ichthyosis with diffuse scaling, sparing the major flexures and the face. There was no history of a collodion baby. Follicular atrophoderma was mainly present on the backs of the hands. She showed patchy nonscarring hypotrichosis and a characteristic receding frontal hairline (Figure 1d). The hair was normal in length but light brown in color and woolly, in contrast to all other family members with straight black hair. The skin was clearly hypohidrotic. Detailed ophthalmological, neurological, and audiometric investigations were without findings.

Ultrastructural analysis

Upon histopathological examination, the epidermis was of normal thickness with regular differentiation of keratinocytes in the spinous layer. The granular layer was thinned, and the stratum corneum orthohyperkeratotic with a compact to basket-woven structure. The thickness of the stratum corneum correlated mostly with the reduction of the granular layer. Hair follicle epithelium was thinned, hair infundibulum showed hyperkeratosis and a very thin stratum granulosum. Perifollicular epidermis seemed to be depressed. Electron microscopy confirmed the very thin stratum granulosum in line with reduced amounts of keratohyalin spreading around keratin filaments in one or two layers only. The lower lamellae of the stratum corneum appeared only loosely connected laterally and contained intra- and interlamellar deposits of membraneous structures clearly resembling lamellar bodies. They potentially consisted of irregularly processed contents of lamellar bodies (Figure 1e–f). Lamellar bodies in the granular cells were of normal morphology. The ultrastructural findings were very similar in patients from both families.

Identification of mutations in *ST14*

First we have analyzed the larger, Arab pedigree in a genome-wide scan with microsatellites. Linkage analysis identified two regions with lod scores over 3, with maximum two-point lod scores of 3.6 at $\Theta = 0.0$ to D11S910 and 3.4 at $\Theta = 0.0$ to D2S1363. The Turkish pedigree was only compatible with linkage to the region on chromosome 11. Refined mapping confirmed the locus with a combined lod score of 4.0 at D11S4131 and D11S910 (Table S1). Haplotype analysis defined the candidate region on chromosome 11q24 between markers D11S4150 and M11TA01 (Figure 2). This region is ~10.9 cM in length, corresponding to 4.1 Mb.

The candidate region contained 29 genes and transcripts, including a number of promising candidate genes. The most propitious candidate was *ST14*. We identified a homozygous splice-site mutation of exon 17, c.2269 + 1G > A, in the Arab family, and a homozygous frame-shift mutation c.2034delG in the Turkish family (Figure 2). Although the frame shift was predicted to result in a premature termination codon after 84 residues (p.Leu678PhefsX84), the outcome of the splice-site mutation could not be tested directly as we were unable to obtain fresh tissue samples from the family. Therefore, we cloned a genomic fragment comprising exons 16–18 around the mutant splice site and analyzed splicing after transfection of the construct into HEK-293 cells. This mini-gene assay revealed several splice products: one predominant product activating a cryptic splice site in exon 17, and minor products with retention of intron 17, usage of a cryptic splice site in intron 17, and complete skipping of exon 17. All of these were predicted to result in premature stop codons, p.Val727AlafsX5 being the major mutant peptide.

Functional characterization of matriptase deficiency

The gene product of *ST14* is matriptase, a type II transmembrane serine protease expressed in most human epithelia. Both mutations were expected to result in complete loss of matriptase. To demonstrate this, we analyzed the presence of matriptase in differentiated primary keratinocytes from the Turkish patient. Whereas the presence of terminal differentiation markers keratin 1 and desmoglein 1 could be confirmed, matriptase was completely missing in the patient cells (Figure 3).

Prostasin is a GPI-anchored serine protease encoded by *PRSS8*, which is present in terminally differentiating keratinocytes (Leyvraz *et al.*, 2005; List *et al.*, 2006b). Proprostasin is activated by proteolytic cleavage between residues Arg-12 and Ile-13 (Yu *et al.*, 1995). Mouse models for matriptase and prostasin deficiencies showed similar phenotypes and suggested that matriptase acts upstream of prostasin (List *et al.*, 2003, 2007a; Leyvraz *et al.*, 2005; Netzel-Arnett *et al.*, 2006). Therefore, we analyzed the activation of prostasin in the patient. Expression of *PRSS8* was normal, as expected, however, only prostasin zymogen was detected in lysates from differentiated patient keratinocytes and the active form was completely missing (Figure 4a). As filaggrin is a major player in terminal epidermal differentiation and loss of profilaggrin processing was seen in matriptase-deficient mice (List *et al.*, 2003, 2007a), we then analyzed profilaggrin and filaggrin units in the IFAH patient. Although profilaggrin was almost undetectable in differentiated keratinocytes from control persons, we observed a strong signal of profilaggrin in patient keratinocytes (Figure 4b). In contrast, there was a clearly reduced amount of filaggrin monomers as compared to normal keratinocytes. An intermediate product, probably consisting of four filaggrin units, was slightly increased, and an additional peptide of ~48 kDa could be seen (Figure 4c).

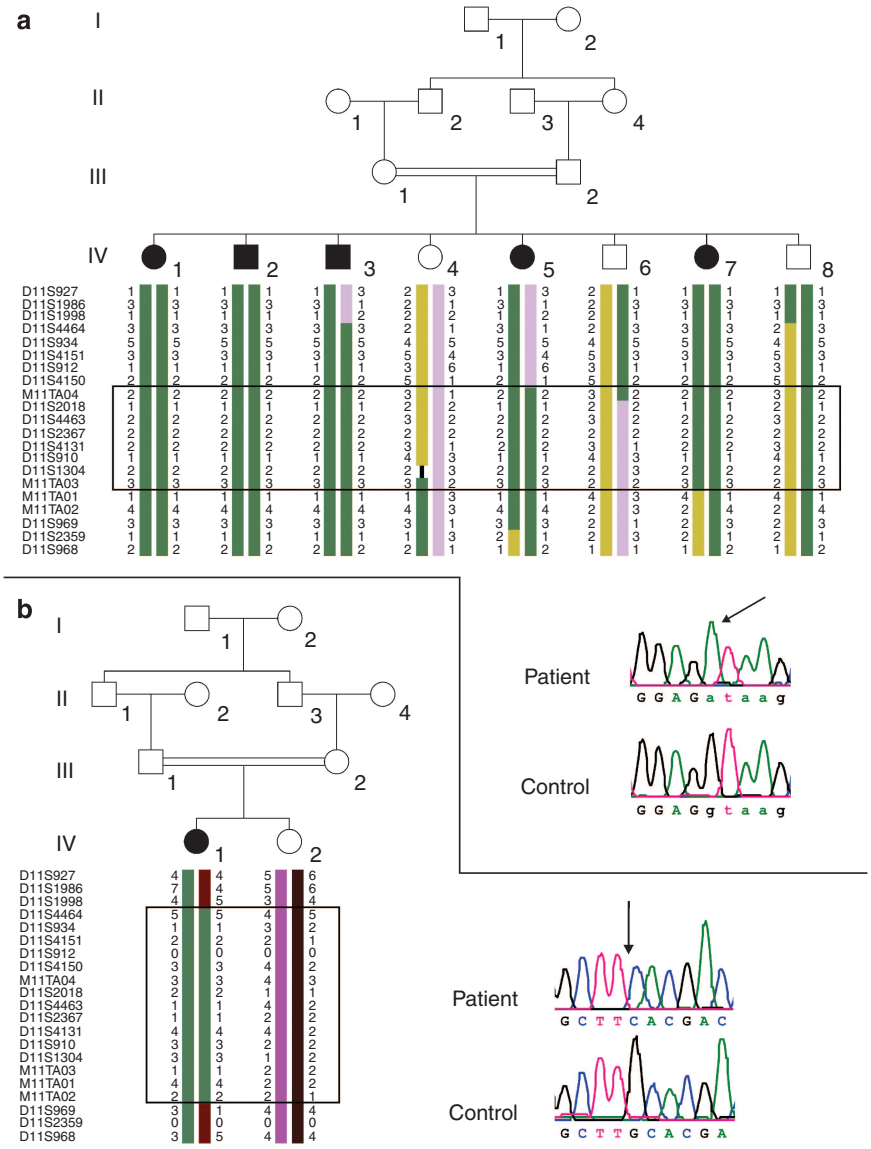

Figure 2. Pedigrees, haplotype analysis, and mutations in *ST14*. (**a**) Pedigree of the Arab family with five affected children. The parents were first cousins. Homozygosity defined the critical interval (marked by the box) between D11S4150 and M11TA01. Sequence analysis identified the homozygous mutation c.2269 + 1G > A in all affected siblings. Exon nucleotides are shown as uppercase, intron nucleotides as lowercase letters. (**b**) Pedigree of the Turkish family with one affected child. The parents and the paternal grandparents were first cousins. Homozygosity defined the critical interval (marked by the box) between D11S1998 and D11S969. Sequence analysis identified the homozygous mutation c.2034delG.

DISCUSSION

Congenital ichthyosis is a feature of several cornification disorders characterized by epidermal barrier impairment, which leads to severe transepidermal water loss. Here we have demonstrated that IFAH, an autosomal recessive syndrome of congenital ichthyosis and follicular atrophoderma, can be caused by loss-of-function mutations in *ST14*, the gene for matriptase. We have shown that the synthesis of matriptase is completely abolished. An *ST14* mutation has been described recently in an Israeli-Arab family with ARIH; those patients, however, had a homozygous missense mutation, p.Gly827Arg (Basel-Vanagaite *et al.*, 2007). A second patient with ARIH from Turkey also showed a homozygous missense mutation, though affecting the initial methionine codon, p.Met1Ile (Avrahami *et al.*, 2008). Interestingly, there are a number of remarkable differences in the clinical appearance between the ARIH family and our patients with IFAH. The scaling of the skin is similar in extent and color, the hair phenotype is milder in our patients. The hair is lighter than expected, at least in the Turkish patient, but hypotrichosis is less pronounced and particularly characterized by the high forehead; the eyebrows, however, are almost completely missing. In contrast to ARIH, we have not seen eye abnormalities besides pingueculum and late-onset photophobia and corneal opacities in one patient. Moreover, there were no tooth abnormalities and no itching. Follicular atrophoderma is an important characteristic of IFAH, which is not present in ARIH and generally a rare sign

of skin disorders; it is found in chondrodysplasia punctata (Conradi-Hünermann-Happle syndrome, OMIM 302960) and in Bazex syndrome (OMIM 301845). Although the proteolytic activity of Gly827Arg matriptase identified in ARIH is strongly reduced (List *et al.*, 2007a; Désilets *et al.*, 2008), these findings point out that the presence of mutant matriptase is still important.

Phenotypic differences caused by residual amounts of matriptase have been shown in mouse models. Mice with an *St14* knockout died within 48 hours after birth; they showed malformations of the stratum corneum, a defect of the epidermal barrier, and generalized follicular hypoplasia (List *et al.*, 2002, 2003). Their skin developed a hyperproliferative ichthyosis 3 weeks after transplantation. In contrast, *St14* hypomorphic animals survived, they were viable and fertile and developed ichthyosis, hypotrichosis with brittle hair, and tooth defects resembling the phenoytpe of ARIH, although only minimal amounts of epidermal matriptase mRNA were detected (List *et al.*, 2007a). Proteolytic activation of prostasin was undectable in *St14* null mice and reduced in *St14* hypomorphic mice, suggesting that matriptase acts upstream of prostasin in an epidermal protease cascade. Here we have demonstrated that the activation of prostasin is abolished in IFAH patients, in accordance with the complete loss of matriptase in our patients, which is in contrast to the finding in *St14* hypomoprohic mice. Profilaggrin is a huge protein of 400–450 kDa, composed of 10–12 filaggrin units, 2 incomplete filaggrin units, and N- and C-terminal domains. Profilaggrin is accumulated in keratohyalin granules in the granular layer and proteolytically cleaved during terminal differentiation to release filaggrin monomers. Filaggrin molecules play a role in stabilizing and packing intermediate filaments during formation of the cell envelope. Moreover, free amino acids formed by degradation of filaggrin are needed for the hydration and osmolarity of the stratum corneum. In our patient, we have found a drastic reduction of filaggrin monomers in differentiated keratinocytes. There is an additional product of ~48 kDa, which may represent an uncleaved complete and an incomplete filaggrin unit. Though the final steps of profilaggrin processing are not known in detail, these findings and the disturbance of profilaggrin processing in *Prss8*-deficient mouse epidermis (Leyvraz *et al.*, 2005) indicate that matriptase/prostasin are involved in controlled filaggrin formation. The retention of unprocessed profilaggrin in primary keratinocytes demonstrates the disturbance of terminal differentiation caused by loss of matriptase, which is further underscored by the presence of unprocessed content of lamellar bodies in the stratum corneum, in contrast to the finding of rare and disorganized lamellar bodies already in the uppermost granular layer of matriptase-deficient mouse epidermis (List *et al.*, 2003). These deposits can be found in other

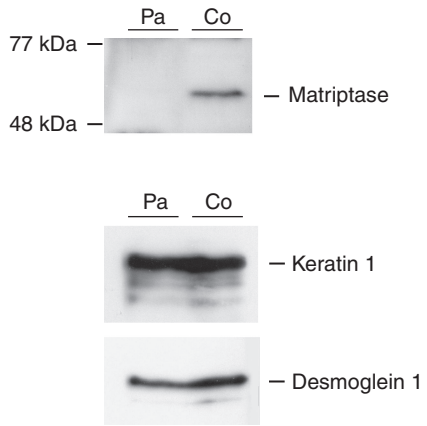

Figure 3. Western blot analysis of matriptase. Primary keratinocytes from the Turkish IFAH patient and control persons were cultivated and differentiated. Differentiation markers keratin 1 and desmoglein 1 were detected in equal amounts in patient and control. Matriptase was completely missing in patient cells as shown with an anti-matriptase antibody recognizing the N-terminal domain of the mature protein.

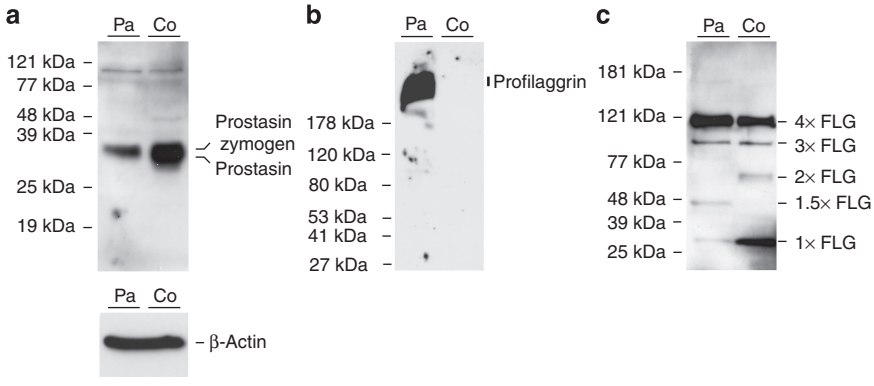

Figure 4. Western blot analysis of prostasin and filaggrin. (**a**) Proprostasin is activated by proteolytic cleavage after residue Arg-12. Keratinocytes from control persons showed a double band of prostasin zymogen and mature prostasin. Only unprocessed prostasin zymogen was detected in patient samples. (**b**) Profilaggrin is a large protein of 400–450 kDa, composed of 10–12 filaggrin units. Although profilaggrin was almost undetectable in control cells, we observed a massive signal of profilaggrin in patient keratinocytes. (**c**) Profilaggrin is proteolytically cleaved in terminally differentiating keratinocytes. Only a faint signal is present in patient cells; a significantly larger amount of filaggrin monomers was found in control lysates than in samples from the patient. Probable intermediate products are indicated. Equal protein loading was confirmed by detecting β-actin in patient and control samples. The positions of molecular weight marker signals are shown on the left of each panel. Pa, patient; Co, control.

keratinization disorders as well, more generally pointing to a disturbance of terminal differentiation of keratinocytes.

Netherton syndrome is an autosomal recessive genodermatosis characterized by congenital ichthyosiform erythroderma, so-called bamboo hair, and atopic disease. Netherton syndrome is caused by mutations in *SPINK5*, which encodes LEKTI, a serine protease inhibitor that specifically interacts with kallikreins 5 and 7 and probably other proteases (Chavanas *et al.*, 2000; Egelrud *et al.*, 2005; Deraison *et al.*, 2007). The inactivation of LEKTI leads to an extended processing of profilaggrin and an increased formation of filaggrin monomers (Hewett *et al.*, 2005). This is in accordance with our finding of reduced filaggrin formation and may point to a role of matriptase in desquamation. Correspondingly, a *Casp14*$^{-/-}$ mouse line showed a serious barrier impairment and disturbed filaggrin processing (Denecker *et al.*, 2007). Moreover, ichthyosis vulgaris and atopic dermatitis can be associated with loss-of-function mutations in the filaggrin gene (*FLG*) (Presland *et al.*, 2000; Palmer *et al.*, 2006; Smith *et al.*, 2006). A number of serine proteases, including matriptase, prostasin, kallikrein 5, kallikrein 7, and furin, and their respective inhibitors are synthesized in epidermal keratinocytes and involved in terminal differentiation (List *et al.*, 2003, 2007b; Zeeuwen, 2004; Hachem *et al.*, 2006; Netzel-Arnett *et al.*, 2006). It is not yet clear whether there is a common pathway and a coordinated regulation of their activity; matriptase, for instance, is able to utilize autoactivation whereas prostasin needs an activator. It becomes clear, however, that the regulation of serine protease activity is a key event for epidermal homeostasis and therefore also for terminal differentiation of epidermal keratinocytes.

In summary, we have shown that IFAH and ARIH can be allelic but clinical heterogeneity is caused by different types of mutations. Our results suggest that impairment of profilaggrin processing, abnormal interlamellar lipid extrusion, and defective corneocyte maturation may all result from the loss of matriptase and prostasin zymogen activation. Furthermore, our findings and the identification of missense mutations in ichthyosis and hypotrichosis indicate that matriptase plays a role in diverse processes in interfollicular epidermis and hair follicle formation, which is in line with detailed expression studies in mice (List *et al.*, 2006b) and recent findings in zebrafish epidermis (Carney *et al.*, 2007; Mathias *et al.*, 2007), which pointed out that the balance between matriptase and hepatocyte growth factor activator inhibitor-1 is important in various mechanisms to regulate skin homeostasis.

MATERIALS AND METHODS

Subjects

The study has been approved by the local Institutional Review Board and followed the Declaration of Helsinki protocols. Blood samples were drawn from 14 members of two consanguineous families from the United Arab Emirates and from Turkey, including six affected individuals, after obtaining written informed consent. Biopsies were taken from ichthyotic areas of the forearm of one affected individual from each family and from forearms or thighs of control persons without skin abnormalities. Genomic DNA was isolated according to standard protocols. A total of 381 microsatellite markers were selected for the whole-genome scan of the Emirati family.

Linkage and mutation analysis

Genotyping was performed by PCR and subsequent electrophoresis on ABI3730 genetic analyzers (Applied Biosystems, Foster City, CA). Data were checked using the programs Graphical Representation of Relationship Errors (Abecasis *et al.*, 2001) and PEDCHECK (O'Connell and Weeks, 1998). Parametric linkage analysis was performed with the program package LINKAGE v5.2 (Lathrop and Lalouel, 1984), assuming autosomal recessive inheritance and full penetrance. Multipoint lod score analysis was done with Simwalk2 (Sobel and Lange, 1996) using the Marshfield genetic map (Broman *et al.*, 1998). For refined mapping, additional markers were generated using dinucleotide microsatellites found in the genomic sequence NT_033899.7. Most likely haplotypes were constructed either manually or with Simwalk2. The 19 exons of *ST14* were amplified from genomic DNA with primers designed from the genomic sequence (NT_033899.7). The primer sequences will be provided upon request. PCR products were enzymatically purified and bidirectionally sequenced with the BigDye Terminator Kit (Applied Biosystems).

Splice assay

To confirm the splice-site mutation c.2269+1G>A found in the Emirati patients, we performed PCR from genomic DNA to amplify a fragment of ~10 kb including exons 16, 17, and 18 using the primer pair 5′-CACCCTTCCCCACACAGGGTCTC-3′ and 5′-GCCCCTATA CCCCAGTGTTC-3′. After purification, the product was cloned into pENTR/TEV/D-TOPO (Invitrogen, Carlsbad, CA). Expression clones were generated by cloning into the pcDNA-DEST40 eukaryotic expression vector. Human HEK-293 cells were transfected with plasmid DNA using the CalPhos mammalian transfection protocol (Clontech, Mountain View, CA). Transiently transfected cells were obtained after 48 hours. RNA was purified from the transfected HEK-293 cells using the RNeasy Kit (Qiagen, Hilden, Germany) and cDNA was prepared with M-MLV reverse transcriptase (Promega, Madison, WI) using oligo(dT) primers. After amplification by PCR, products were separated by electrophoresis and sequenced.

Western blot analysis

Primary human keratinocytes were isolated from biopsy specimens, grown in KGM and differentiated by 1.3 mM Ca^{2+}. For Western blotting analysis, protein was prepared by the lysis of keratinocytes using RIPA buffer containing 0.1% Nonidet-P40, 0.5% sodium deoxycholate, 0.1% SDS. Debris was removed and the protein concentration measured using the Bradford assay. Equal amounts of protein were electrophoresed, transferred to Immobilon-P polyvinylidene fluoride membranes (Millipore, Billerica, MA), and probed with polyclonal antibodies against human matriptase (Abcam, Cambridge, UK), human prostasin (BD Biosciences, San Jose, CA), and human filaggrin (Abcam). Primary antibodies were recognized by donkey anti-rabbit (Abcam) and goat anti-mouse (Zymed Laboratories, South San Francisco, CA) horseradish peroxidase-conjugated secondary antibodies, respectively. Detection was

performed with Immobilon Western chemiluminescent horseradish peroxidase substrate.

Electron microscopy

For electron microscopy, biopsy specimens from ichthyotic areas of the forearms were fixed for at least 2 hours at room temperature in 3% glutaraldehyde solution in 0.1 M cacodylate buffer (pH 7.4), cut into pieces of $\sim 1\,mm^3$, washed in buffer, post-fixed for 1 hour at 4 °C in 1% osmium tetroxide, dehydrated through graded ethanol solutions, transferred into propylene oxide, and embedded in epoxy resin. Semithin and ultrathin sections were cut with an ultramicrotome (Ultracut E, Reichert, Nußloch, Germany). Ultrathin sections were treated with uranyl acetate and lead citrate and examined with an EM 400 electron microscope (Philips, Eindhoven, The Netherlands).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Table S1. Combined two-point lod scores for both IFAH families in the candidate region on chromosome 11q24–q25.

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