Autosomal Ichthyosis with Hypotrichosis Syndrome Displays Low Matriptase Proteolytic Activity and Is Phenocopied in ST14 Hypomorphic Mice*

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Human autosomal recessive ichthyosis with hypotrichosis (ARIH) is an inherited disorder recently linked to homozygosity for a point mutation in the ST14 gene that causes a G827R mutation in the matriptase serine protease domain (G216 in chymotrypsin numbering). Here we show that human G827R matriptase has strongly reduced proteolytic activity toward small molecule substrates, as well as toward its candidate epidermal target, prostasin. To further investigate the possible contribution of low matriptase activity to ARIH, we generated an ST14 hypomorphic mouse strain that displays a 100-fold reduction in epidermal matriptase mRNA levels. Interestingly, unlike ST14 null mice, ST14 hypomorphic mice were viable and fertile but displayed a spectrum of abnormalities that strikingly resembled ARIH. Thus, ST14 hypomorphic mice developed hyperproliferative and retention ichthyosis with impaired desquamation, hypotrichosis with brittle, thin, uneven, and sparse hair, and tooth defects. Biochemical analysis of ST14 hypomorphic epidermis revealed reduced prostasin proteolytic activity and profilaggrin proteolytic processing, compatible with a primary role of matriptase in this process. This work strongly indicates that reduced activity of a matriptase-prostasin proteolytic cascade is the etiological origin of human ARIH and provides an important mouse model for the exploration of matriptase function in ARIH, as well as multiple other physiological and pathological processes.

Serine proteases are among the most abundant proteolytic enzymes in vertebrate genomes. This diverse family of extracellular proteases regulates a large number of biological processes associated with tissue development, tissue homeostasis, and tissue regeneration, and it is implicated in the genesis or progression of a wide spectrum of important human pathologies, including carcinogenesis (1–6).

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The epidermis of terrestrial vertebrates is formed by two interconnected compartments: the interfollicular epidermis and the follicular epidermis. The follicular epithelium provides a permeability barrier that prevents excessive water loss to the surrounding environment and noxious chemicals from entering the body. The follicular epidermis gives rise to epidermal appendages such as hair and feathers. The follicular epidermis is a stratified epithelium that is formed by proliferating basal cells that move outwards through a series of distinct differentiation events to form the stratum corneum: a two-compartment structure that consists of a lipid-enriched extracellular matrix in which an interlocking meshwork of dead corneocytes are embedded (7–10). Epidermal differentiation culminates with the regulated shedding (desquamation) of the outermost layer of corneocytes to control the thickness of the stratum corneum (11). The follicular epidermis is a complex, dynamic organ consisting of multiple keratinocyte populations. The outer root sheath of the hair follicle is continuous with the basal layer of the interfollicular epidermis. Within the follicular matrix, a zone located at the proximal end of the hair surrounding the dermal papilla, rapidly proliferating undifferentiated transient amplifying cells give rise to the cortex, medulla, cuticle of the hair shaft, and the inner root sheath cells, which continuously move upward. The matrix transient-amplifying cells periodically withdraw from the cell cycle and commit to terminal differentiation, giving rise to repeated cycles of active hair growth (anagen), hair follicle regression (catagen), and rest (telogen) (12–15).

A large number of serine proteases and serine protease inhibitors are expressed in the upper layers of the epidermis, suggesting a role of extracellular/pericellular proteolysis in terminal differentiation. Indeed, several recent studies have revealed that proper epidermal differentiation depends on a finely tuned interplay between specific serine proteases and their cognate serine protease inhibitors, and that perturbations of this proteolytic balance in either direction have serious pathophysiological consequences (15). Key steps of epidermal differentiation that are regulated by serine protease activity include profilaggrin proteolytic processing, lipid lamellar body extrusion, and desquamation of the interfollicular epidermis, as well as hair shaft eruption and hair growth (15–23). Generally speaking, the loss of serine protease proteolytic activity impairs...
terminal epidermal differentiation, whereas increased proteolytic activity accelerates the process. For example, genetic ablation of the membrane-associated serine proteases matriptase and prostatin impairs stratum corneum formation and causes follicular hypoplasia, whereas the genetic deficiency in the Kazal-type serine protease inhibitor lympho-epithelial Kazal-type inhibitor or targeted overexpression of stratum corneum chymotryptic enzyme is associated with accelerated stratum corneum formation (15–17, 20–24).

Matriptase (also known as MT-SP1, epithin, and TADG15) is a type II transmembrane serine protease encoded by the ST14 gene. The membrane protease is being extensively studied in the context of human carcinogenesis, due to its almost ubiquitous overexpression in human carcinomas and its potent tumor-promoting properties (25, 26). Matriptase is normally expressed in terminally differentiating cells of stratified epithelium but becomes expressed in undifferentiated epithelial cells that have the capacity to undergo malignant transformation after carcinogen exposure (27, 28). Null mutations in the ST14 gene cause neonatal death of mice due to the loss of epidermal and oral barrier function leading to fatal dehydration (24). The matriptase-deficient interfollicular epidermis is characterized by complete loss of proteolytically processed filaggrin, impaired epidermal lipid extrusion, and impaired desquamation of the stratum corneum (16). Recent evidence suggests that matriptase acts as part of a matriptase-prostasin proteolytic cascade during terminal epidermal differentiation (6). Loss of matriptase in mice also causes generalized follicular hypoplasia and impaired vibrissae eruption (16, 24).

Recently, a newly defined syndrome, autosomal recessive ichthyosis with hypotrichosis (ARIH),2 was linked to homozygosity for a c.2672G → A mutation located in exon 19 of the ST14 gene. This mutation results in a Gly → Arg substitution in residue 827 of the matriptase protein (henceforth G827R matriptase; G216 in chymotrypsin numbering) (29). ARIH patients present with thickened, scaling, and grayish skin and curly, sparse, fragile, brittle, dry, lusterless, and slow growing hair. Other manifestations linked to homozygosity for the c.2672G → A ST14 allele included corneal opacity, photophobia, and abnormal deciduous and permanent teeth. Some uncertainties remain, however, regarding the interpretation of the reported findings, because the effect of the G827R substitution on matriptase proteolytic activity was determined; the three homozygously affected individuals analyzed in the report were all siblings from one consanguineous marriage, and one individual also was diagnosed with Hirschsprung disease.

To further investigate the association between matriptase proteolytic activity and ARIH, we characterized the enzymatic properties of recombinant human G827R matriptase and report here that the mutant protease has highly reduced proteolytic activity toward low molecular weight peptide substrates, fluorophosphonate probes, and α2-macroglobulin, as well as toward its candidate epidermal substrate, the zymogen form of prostatin. Furthermore, we generated mice with a hypomorphic ST14 allele with ~100-fold reduced epidermal matriptase mRNA levels at birth and characterized the phenotypic consequences of reduced matriptase expression. Surprisingly, this very low epidermal matriptase mRNA level was compatible with postnatal development, long-term survival, and reproduction. Importantly, these ST14 hypomorphic mice were virtual phenocopies of ARIH patients, displaying near identical epidermis, hair, and tooth abnormalities. This study strongly implicates impaired matriptase proteolytic activity as the underlying cause of ARIH, and it provides a valuable animal model for the study of ARIH, as well as for the exploration of matriptase proteolytic function in other physiological and pathological processes.

**EXPERIMENTAL PROCEDURES**

**Expression of G827R, S805A Matriptase, and Wild-type Matriptase in *Pichia pastoris*—**The G → A and T → G mutations in nucleotides 2672 and 2606 of the human matriptase cDNA (GenBank™ NM021978), causing, respectively, G827R and S805A changes in the translated protein, were introduced using the QuikChange kit from Stratagene (La Jolla, CA). Nucleotides 2036–2762 of the wild-type and mutated matriptase serine protease domains were introduced into the *Pichia pastoris* expression vector pPIC9 (Invitrogen), immediately downstream from the coding region of the Leu-Glu-Lys-Arg KEX2 signal peptide, leading to the synthesis and secretion of active matriptase. The recombinant serine protease domains were affinity-purified on benzamidine-Sepharose columns as recommended by the manufacturer (Amersham Biosciences), dialyzed against 50 mM Tris-HCl, pH 8.0, and aliquots were snap-frozen in liquid nitrogen.

**Matriptase Proteolytic Activity Assays—**For prostatin zymogen activation, recombinant soluble prostatin was made as described previously (6). Briefly, HEK-293T cells were transfected with the pCMV-SPORT6 expression vector containing full-length human prostatin cDNA. Transfected cells were collected mechanically and incubated with 1 unit/ml PI-PLC (Sigma) in phosphate-buffered saline for 4 h at 4 °C, and the clarified supernatant containing the PI-PLC-released proteins was collected. The soluble prostatin was incubated with 1–10 nm wild-type or G827R matriptase serine protease domain for 2 h at 37 °C in 50 mM Tris-HCl, pH 8.5, 100 mM NaCl. For complex formation with prostatin nexin-1 (PN-1)/SERPINE2, 700 nm PN-1 (R&D Systems, Minneapolis, MN) was added to the activation reaction, and the incubation was continued for an additional 1 h at 37 °C. Proteins were analyzed by SDS-PAGE on 4–12% gradient gels under reducing conditions followed by Western blotting using a monoclonal anti-prostatin antibody (BD Biosciences Pharmingen).

**Fluorogenic Peptide Assays—**0.1–100 nm wild-type, G827R, and S805A matriptase serine protease domains were added to 200 μl of 50 mM Tris/HCl, pH 8.0, 2 mM CaCl2, reaction buffer containing 10 μM t-butyloxycarbonyl-Gln-Ala-Arg-7-amido-4-methylcoumarin HCl fluorogenic peptide substrate (R&D Systems). The assay was performed at 25 °C for 30 min, and fluorescence measurements with excitation at 380 nm and emission at 460 nm were obtained every minute using a

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2 The abbreviations used are: ARIH, autosomal recessive ichthyosis with hypotrichosis; PI-PLC, phosphatidylinositol-specific phospholipase C; PN-1, protease nexin-1.
**SAFIRE2TM Microplate Reader (Tecan, Durham, NC).** All measurements were performed in triplicates. Once measured, the reaction rate \( k_{\text{obs}} \) was determined as \( k_{\text{obs}} = -\ln[S]/\text{time} \). The \( k_{\text{cat}}/K_m \) ratio was then calculated from the Michaelis-Menten equation as \( k_{\text{cat}}/K_m = k_{\text{obs}}/E(\text{total}) \).

**Fluorophosphonate Labeling**—4–400 nm wild-type, G827R, and S805A matriptase serine protease domains in 50 mM Tris-HCl, pH 8.5, 100 mM NaCl, were incubated with 0.5 mM rhodamine-coupled fluorophosphonate probe for 1 h at room temperature (30). The resultant mixture was then separated by SDS-PAGE on 4–12% gels under reducing conditions, and the gels were photographed on a UV transilluminator 2040ev (Stratagene).

**α2-Macroglobulin Capture**—α2-Macroglobulin capture assays were performed by incubation of 200 nm wild-type and G827R matriptase with 200 nm α2-macroglobulin in 50 mM Tris-HCl, pH 8.5, 150 mM NaCl, 2 mM CaCl₂ for 1 h at room temperature as described previously (31). Complex formation was then analyzed by SDS-PAGE on 4–12% SDS-PAGE gradient gels under reducing conditions, followed by Western blotting using a rabbit anti-matriptase antibody (Calbiochem), and an alkaline phosphatase-labeled goat anti-rabbit secondary antibody (DakoCytomation, Glostrup, Denmark).

**Generation of ST14 Hypomorphic Mice**—ST14 hypomorphic mice were generated by interbreeding mice with an ST14 null allele (24) with mice carrying an ST14 allele in which the pGT0pfs gene trap (Bay Genomics, San Francisco, CA) is inserted into intron 1 (ST14 knockdown allele). ST14 hypomorphic mice were either bred for three generations into a C57BL/6j background or maintained in a 129/Black Swiss background. All experiments performed with ST14 hypomorphic mice were littermate and sibling controlled. Genotyping of ST14 hypomorphic mice was either bred for three generations into a C57BL/6j background or maintained in a 129/Black Swiss background. All experiments performed with ST14 hypomorphic mice were littermate and sibling controlled. Genotyping of ST14 null mice was performed as described (24, 28).

**Histology and Immunohistochemistry**—Histological analysis of ST14 hypomorphic skin was performed as described (24). Cell proliferation was visualized with Ki67 immunostaining as described (32). Bound antibodies were visualized with a Vectastain ABC peroxidase kit (Vector Laboratories, Burlingame, CA) using diaminobenzidine as the chromogenic substrate.

**Desquamation Assays**—Neonate ST14 hypmorphs and littermate controls were euthanized by CO₂ inhalation, and the dorsal skin area was sequentially stripped with 14-mm D-Squame disks (Cuderm Corp., Dallas, TX) as described before (33).

**Transepidermal Fluid Loss Assay**—Newborn pups were separated from their mother to prevent fluid intake and placed in a 37 °C incubator. The rate of epithelial fluid loss was estimated by measuring the reduction of body weight of the individual pups as a function of time over a period of 4 h.

**Scanning Electron Microscopy**—Tissues were fixed in 4% formaldehyde/4% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 3 days, rinsed in cacodylate buffer, and incubated overnight in 2% glycine, overnight in 2% tannic acid, pH 4.0, and 6 h in 2% OsO₄. The fixed tissues were dehydrated in graded ethanol solutions, put under vacuum for 2 h, mounted onto stubs with Leit-C adhesive (Electron Microscopy Sciences, Fort Washington, PA), and examined with a Philips XL-20 scanning electron microscope.

**Analysis of Epidermal Prostasin and Profilaggrin/Filaggrin**—Skins were removed from euthanized newborn mice, and dermis and epidermis were separated as described (24). For prostasin analysis, the epidermis was immediately ground into a fine powder in liquid nitrogen, and the powder was lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 2 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 0.5% Triton X-100, 0.1% SDS) supplemented with a protease inhibitor mixture (Sigma). Homogenized lysates were cleared by centrifugation at 13,000 × g for 20 min at 4 °C. Soluble protein lysates were quantified using a BCA Protein Assay (Pierce). Equal amounts of epidermal proteins were resolved by SDS-PAGE on a 10% gel under reducing conditions and transferred to a polyvinylidene difluoride membrane. Western blots were performed with the anti-prostasin antibody as described (6). For profilaggrin/filaggrin analysis, epidermal proteins were extracted as described (18) using 8 M urea, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0, with protease inhibitor mixture (Sigma). Equal amounts of epidermal proteins were separated by SDS-PAGE on 4–12% gels under reducing conditions and Coomassie stained or transferred to a polyvinylidene difluoride membrane. Profilaggrin/filaggrin was detected using a rabbit polyclonal antibody (Zymed Laboratories, San Francisco, CA) and an alkaline phosphatase-conjugated goat anti-rabbit IgG as secondary antibody (DakoCytomation).

**RESULTS**

**G827R Matriptase Has Reduced Proteolytic Activity**—G827R is part of the S12 β-sheet (34) (Fig. 1A). This glycine residue is highly conserved in trypsin-like serine proteases, although it is substituted by serine in the mouse hypothetical protease TESP2 and by aspartic acid in trypsin-α (2). To test if the Gly to Arg substitution reported in human ARHI affected substrate accessibility and/or hydrolysis, the wild-type and G827R matriptase serine protease domains were expressed in P. pastoris and purified by benzamidine affinity chromatography as described (34). Wild-type and G827R matriptase displayed identical absorption and elution profiles using this purification procedure, suggesting that the benzamidine binding pocket is retained in the mutant matriptase (data not shown). We first performed a peptide cleavage assay using a fluorogenic t-butyloxycarbonyl-Gln-Ala-Arg-7-amino-4-methylcoumarin peptide that is efficiently cleaved by matriptase. G827R matriptase hydrolyzed this model substrate poorly, displaying a catalytic activity that was ~1000-fold lower than that of wild-type matriptase, and was similar to a matriptase mutant (S805A) in which the active site serine was changed to alanine \( (k_{\text{cat}}/K_m; \text{wild-type matriptase}, 1.7 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}; \text{G827R matriptase}, 1.8 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}; \text{S805A matriptase}, 2.4 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}) \). We next incubated wild-type, G827R, and S805A matriptase with a rhodamine-labeled fluorophosphonate probe, which attaches covalently to the active site serine of serine proteases (and other serine hydrolases) after its cleavage (30, 35). This analysis showed efficient cleavage and covalent attachment of the fluorogenic probe to wild-type matriptase (Fig. 1B, lanes 1 and 2), and much lower, but measurable, cleavage and covalent attachment of the fluorogenic probe to G827R matriptase (Fig. 1B, lane 5), when analyzed by...
The above studies indicated that G827R matriptase would have impaired capacity to activate its candidate downstream epidermal substrate, the prostasinzymogen (6). To verify this, the wild-type and mutant enzymes were incubated with an excess of recombinant soluble prostasinzymogen. Active two-chain prostasin can be distinguished from the prostasinzymogen by a small increase in electrophoretic mobility in Western blots of high percentage SDS-PAGE gels after reduction of the single disulfide bridge that links the two chains, as well as by the capacity to form SDS-stable complexes with the cognate serpin, PN-1 (36). As described previously (6), exposure of soluble prostasinzymogen to either 10 or 1 nM active matriptase serine protease domain lead to the emergence of a new prostasin protein species with the mobility expected for active prostasin (Fig. 1D, compare lane 1 with lanes 3 and 5) that formed an SDS-stable complex with PN-1 (Fig. 1D, compare lane 2 with lanes 4 and 6). However, equivalent amounts of G827R matriptase were incapable of converting the prostasinzymogen into a faster migrating species that could form an SDS-stable complex with PN-1 (Fig. 1D, compare lane 1 with lanes 7 and 9, and lane 2 with lanes 8 and 10). Even very long exposures failed to detect any specific prostasin-PN-1 complexes (data not shown). Given the dynamic range of this assay of one logarithm or more, we conclude that the activity of G827R matriptase serine protease domain toward the prostasinzymogen is reduced by at least 10-fold.

**Very Low Epidermal Matriptase mRNA Level Is Compatible with Mouse Survival—De novo generation of a knock-in mouse strain that expresses mouse G827R matriptase to study the effects of low matriptase activity in mice is a lengthy process. However, we were able to mimic ARIH in mice by generating an ST14 hypomorphic mouse strain that combined a pre-existing ST14 null allele (28) in which an engrailed-2 splice acceptor site had been inserted between coding exons one and two (Fig. 2C, lane 3) or buffer (lanes 2 and 4). Matriptase-α2-macroglobulin complex formation was analyzed by SDS-PAGE followed by Western blotting with matriptase antibodies. Positions of matriptase-α2-macroglobulin complexes and matriptase are indicated at the left, and positions of molecular mass markers (kilodaltons) are indicated at the right. C, α2-macroglobulin capture assay. Vehicle (lane 1), 200 nM wild-type (lanes 2 and 3) or 200 nM G827R matriptase serine protease domain, 400 nM S805A matriptase serine protease domain (lane 4), or 400 nM G827R matriptase serine protease domain (lane 5). Fluorophosphonate probe attachment was examined by SDS-PAGE followed by photography of the gel under UV illumination. The position of matriptase is indicated at the left, and the positions of molecular mass markers (kilodaltons) are indicated at the right. C, α2-macroglobulin capture assay. Vehicle (lane 1), 200 nM wild-type (lanes 2 and 3) or 200 nM G827R matriptase serine protease domain, 400 nM S805A matriptase serine protease domain (lane 4), or 400 nM G827R matriptase serine protease domain (lane 5). Fluorophosphonate probe attachment was examined by SDS-PAGE followed by photography of the gel under UV illumination. The position of matriptase is indicated at the left, and the positions of molecular mass markers (kilodaltons) are indicated at the right. C, α2-macroglobulin capture assay. Vehicle (lane 1), 200 nM wild-type (lanes 2 and 3) or 200 nM G827R matriptase serine protease domain, 400 nM S805A matriptase serine protease domain (lane 4), or 400 nM G827R matriptase serine protease domain (lane 5). Fluorophosphonate probe attachment was examined by SDS-PAGE followed by photography of the gel under UV illumination. The position of matriptase is indicated at the left, and the positions of molecular mass markers (kilodaltons) are indicated at the right.

**FIGURE 1. G827R matriptase has reduced proteolytic activity. A, structure of the matriptase serine protease showing the positions of the glycine 827 residue that is changed in ARIH (corresponding to glycine 216 in chymotrypsin numbering) and the catalytic site amino acids His-656, Asp-711, and Ser-805 (His-57, Asp-102, and Ser-195 in chymotrypsin numbering). The matriptase structure (PDB code:1EAX (34)) is depicted in the ribbon-rendering mode (gray). Red, oxygen; cyan, carbon; and blue, nitrogen. The image was generated using the Visual Molecular Dynamics 1.8.6. program (47). B, 0.5 mM rhodamine-coupled fluorophosphonate probe was incubated with 400 nM (lane 1), 40 nM (lane 2), and 4 nM (lane 3) wild-type matriptase serine protease domain, 400 nM S805A matriptase serine protease domain (lane 4), or 400 nM G827R matriptase serine protease domain (lane 5). Fluorophosphonate probe attachment was examined by SDS-PAGE followed by photography of the gel under UV illumination. The position of matriptase is indicated at the left, and the positions of molecular mass markers (kilodaltons) are indicated at the right. C, α2-macroglobulin capture assay. Vehicle (lane 1), 200 nM wild-type (lanes 2 and 3) or 200 nM G827R matriptase serine protease domain, 400 nM S805A matriptase serine protease domain (lane 4), or 400 nM G827R matriptase serine protease domain (lane 5). Fluorophosphonate probe attachment was examined by SDS-PAGE followed by photography of the gel under UV illumination. The position of matriptase is indicated at the left, and the positions of molecular mass markers (kilodaltons) are indicated at the right. D, soluble human prostasinzymogen was incubated with buffer (lanes 1 and 2), 10 nM (lanes 3 and 4), or 1 nM (lanes 5 and 6) wild-type (lanes 3–6) or G827R (lanes 7–10) matriptase serine protease domains. At the end of the incubation, buffer (lanes 1, 3, 5, 7, and 9) or 700 nM PN-1 (lanes 2, 4, 6, 8, and 10) was added for 1 h. Proteins were separated by SDS-PAGE under reducing conditions, followed by Western blot with a monoclonal prostasin antibody. The positions of prostasin-PN-1 complexes, prostasinzymogen, and activated prostasin are indicated at the left. The positions of molecular mass markers (kilodaltons) are indicated at the right.
Matriptase Insufficiency in Epidermal Development

A

Wild type ST14 Allele

Null Allele

Knock-down Allele

B

Wild type matriptase mRNA

Null matriptase mRNA (1)

Null matriptase mRNA (2)

C

Matrisppase

Expression

Skin Newborn

Skin 3 weeks

Skin 7 months

Lung Newborn

Kidney

Real time PCR

FIGURE 2. Generation of ST14 hypomorphic mice. A, schematic structure of the wild-type mouse ST14 allele (top), ST14 null allele (middle), and ST14 knockdown allele (bottom). The ST14 null allele was generated as described (24), by replacing a 350-bp fragment of the ST14 allele containing the 3′ portion of intron 1 and most of exon 2 with a HPRT cassette, deleting the signal anchor-encoding sequences and introducing a frameshift mutation through the splicing of the exon 1 and exon 3 (red arrow in B). The ST14 knockdown allele was generated by insertion of a gene trap consisting of the engramed-2 (En2) splice acceptor site, β-galactosidase-neomycin fusion gene, and an SV-40 polyadenylation site that was inserted between exons 1 and 2. ST14 hypomorphic mice are compound heterozygous for one ST14 allele null and one ST14 knockdown allele. B, schematic depiction of the mRNAs transcripts generated from ST14 null and knock-down alleles and the position of the primers used for analysis of the abundance of wild-type matriptase mRNA by quantitative PCR. The null allele generates only "null matriptase mRNA (1)," whereas the knockdown allele generates both wild-type matriptase mRNA and "null matriptase mRNA (2)." C, quantitative PCR analysis of wild-type matriptase mRNA levels in newborn, weanling, and adult skin and in newborn lung and kidney of control and ST14 hypomorphic mice. The data are expressed as the average ± S.E. $p$ values were determined by the Student’s $t$ test, two-tailed.

the newborn epidermis. Thus, quantitative PCR analysis of total mRNA isolated from organs of newborn, weanling, and adult ST14 hypomorphic mice showed that the level of residual wild-type matriptase mRNA was ~1% in newborn epidermis, ~7% in weanling epidermis, and ~12% in adult epidermis (Fig. 2C). Wild-type matriptase mRNA levels in other organs of newborn ST14 hypomorphic mice, such as lungs and kidneys varied from 7 to 18% (Fig. 2C). ST14 hypomorphic mice were born in the expected Mendelian ratio, but unlike ST14 null mice, ST14 hypomorphic mice did not display neonatal lethality and had normal survival when observed for up to 1 year (data not shown). Furthermore, ST14 hypomorphic females and males were capable of producing and rearing offspring (data not shown). ST14 hypomorphic mice, however, were lighter than their siblings. This weight reduction persisted from the earliest time point measurable (days 1–2) throughout an observation period of 300 days, was gender-independent, and presented in two different backgrounds (129/Black Swiss and in C57BL6/J (N3)) (Fig. 3). Taken together, this shows that mice can tolerate a very dramatic reduction of epidermal matriptase mRNA levels.

ST14 Hypomorphic Mice Phenocopy ARIH-Ichthyosis and Hypotrichosis—ST14 hypomorphic mice were distinguishable from their siblings at birth by the absence of erupted whiskers (data not shown) but were otherwise unremarkable (Fig. 4A). However, the skin of ST14 hypomorphic mice gradually became wrinkled and scaly, strikingly resembling the external appearance of ARIH skin (Fig. 4, B and C). Pelage hair eruption in ST14 hypomorphic mice was delayed by more than 1 week (Fig. 4C), and ST14 hypomorphic mice displayed patchy, lusterless, and sparse fur at weaning resembling ARIH scalp hair (Fig. 4D), as well as curly and thin whiskers (Fig. 4, I and J). ST14 hypomorphic pelage hair appeared thin and uneven when examined by either scanning electron microscopy (Fig. 4, K–N) or light microscopy (Fig. 4, O and P). Like the hair of ARIH patients, the overall presentation of ST14 hypomorphic fur improved with age, and gradually became indistinguishable from that of normal mice at seven months of age (Fig. 4, E and F). However, the pinnae of the ears often presented with a persistently thickened and scaly epidermis (Fig. 4, E and F).

Histological analysis showed that the stratum corneum of newborn ST14 hypomorphic mice was abnormally compact, resembling the epidermis of ST14 null mice (Fig. 5, A–C). At days 10 and 21, ST14 hypomorphic epidermis displayed marked retention (Fig. 5, D–G) and hyperproliferative (Fig. 5H) ichthyosis. The histological appearance of ST14 hypomorphic epidermis generally normalized with age (Fig. 5, I and J). Exceptions to this included the skin of the pinnae of the ears of adult mice, which often presented with marked ichthyosis (Fig. 5, K and L) and fibrosis of the underlying dermis (Fig. 5M).

Tooth Abnormalities—The teeth of three individuals with ARIH have been described as conical (deciduous teeth) or notched and pitted (permanent teeth) (29). Moreover, we recently reported that matriptase is highly expressed in the enamel-producing ameloblasts of developing teeth (27). To examine if matriptase contributes to tooth formation, the morphology of 4-week-old control and ST14 hypomorphic teeth was examined by scanning electron microscopy. No gross morphological differences were observed between control and ST14 hypomorphic incisors (Fig. 6, A and D) or molars (Fig. 6, G and J). However, examination at high magnification revealed a rougher enamel surface of ST14 hypomorphic incisors (Fig. 6, compare B and E) and molars (Fig. 6, compare H and K) with markedly increased bacterial growth (Fig. 6, compare C with F, and I with L), suggesting a direct role of matriptase in enamelogenesis.

Barrier Function and Desquamation Are Impaired in ST14 Hypomorphic Epidermis—Having established the remarkable outward similarity between ST14 hypomorphic mice and ARIH individuals, we next performed functional and biochemical assays to gain mechanistic insights into the condition. The marked ichthyosis associated with ARIH is likely to be caused
Matriptase Insufficiency in Epidermal Development

by impaired barrier function (29) as a consequence of reduced matriptase activity. To investigate the barrier function of ST14 hypomorphic epidermis, the rate of fluid loss through transepidermal evaporation was determined in newborn ST14 hypomorphic and control mice. Newborn litters were separated from their mothers to prevent fluid intake, and the loss of fluids at 37 °C was recorded for each individual pup over a 4-h period by monitoring the loss of body weight. This experiment revealed that the barrier function of the ST14 hypomorphic epidermis was significantly compromised when compared with that of littermate control pups, showing that decreased matriptase compromises epidermal barrier function (Fig. 7). However, the rate of fluid loss of ST14 mice (−2-fold higher than control mice) was markedly lower than the previously recorded fluid loss rate of ST14 null mice (5-fold higher than control mice) (24), likely explaining the capacity of ST14 hypomorphic mice to survive the critical neonatal period.

Reduced degradation of corneodesmosomes is a prominent ultrastructural feature of ARIH epidermis and was proposed by Basel-Vanagaite et al. (29) to underlie the prominent retention ichthyosis. To test this hypothesis, newborn ST14 hypomorphic mice and littermate control mice were subjected to a sequential tape-stripping procedure routinely used to assess stratum corneum cohesiveness (33). Repeated tape stripping of dorsal epidermis of control mice resulted in complete removal of the stratum corneum and exposure of the granular/transitional layer (Fig. 8, A and B). In contrast, hypomorphic ST14 epidermis, even when subjected to 17 rounds of tape stripping, retained part of the stratum corneum, demonstrating an impaired desquamation compatible with reduced corneodesmosome degradation (Fig. 8, C and D).

Impaired Proteolytic Prostasin Zymogen Activation and Filaggrin Processing in ST14 Hypomorphic Epidermis—We have previously proposed that matriptase acts as part of a matriptase-prostasin proteolytic cascade to promote three key steps of terminal epidermal differentiation: profilaggrin proteolytic processing, epidermal lipid extrusion, and stratum corneum desquamation (6, 16). To query the effect of highly reduced epidermal matriptase mRNA on prostasin activation, protein lysates were prepared from the epidermis of newborn control, ST14 hypomorphic, and ST14 null mice. Prostasin processing was analyzed by the separation of the protein lysates by reducing SDS–PAGE on high percentage gradient gels, followed by Western blotting using prostasin antibodies (Fig. 9A). As described previously (6), control epidermis contained two forms of prostasin: a 39-kDa form, corresponding to the prostasin zymogen, and a slightly faster migrating species representing active prostasin (Fig. 9, lanes 1–3). As also reported earlier, in ST14 null epidermis, prostasin was exclusively found in the higher molecular mass 39-kDa zymogen form (Fig. 9A, lanes 7–9). Interestingly, although the amount of active prostasin was clearly reduced in ST14 hypomorphic epidermis, small amounts of the active prostasin, nevertheless, could be detected by Western blot, indicating that very low level matriptase

FIGURE 3. Persistent body weight reduction of ST14 hypomorphic mice. Prospective cohorts of male (A and C) and female (B and D) ST14 hypomorphic mice and littermate controls (carrying combinations of either two wild-type, wild-type and null, or wild-type and knockdown ST14 alleles) in mixed 129/Black Swiss (A and B) or C57BL/6J (N3) (C and D) were weighed daily until 35 days of age and every other week thereafter for up to 300 days. The number of mice of each genotype is indicated. Insets in each panel show weights on days 0–40. Weights are expressed as the average ± S.E. *p < 0.05; Student’s t test, two-tailed.
expression suffices to sustain some proteasin activation (Fig. 9A, lanes 4 – 6).

To determine the level of residual profilaggrin processing in ST14 hypomorphic mice, epidermal urea extracts enriched in profilaggrin and profilaggrin proteolytic products (18) were prepared from newborn control, ST14 hypomorphic, and ST14 null mice and analyzed by SDS-PAGE followed by either Coomassie staining or Western blot using a profilaggrin/ST14 antibody (Fig. 9B). As reported previously (16), proteolytically processed filaggrin monomer was readily detected in control epidermis by Coomassie staining and Western blot (Fig. 9B, lanes 1 and 5), whereas no filaggrin monomer was detected in ST14 null epidermis (Fig. 9B, lanes 4 and 8). The amount of proteolytically processed filaggrin monomer in newborn ST14 hypomorphic epidermis varied from pup to pup, from barely detectable to clearly detectable by Coomassie staining and Western blot, but was always much lower than in control epidermis (representative examples in Fig. 9B, lanes 2, 3, 6, and 7).

DISCUSSION

This study provides strong evidence that reduced matriptase proteolytic activity is responsible for most of the phenotypic manifestations of individuals with ARIH. First, our biochemical analysis revealed that G827R matriptase has highly reduced proteolytic activity. Second, we found that ST14 hypomorphic mice with very low matriptase mRNA levels faithfully phenocopied the key features of ARIH.

The Gly-827 residue that is mutated in ARIH (G216 using chymotrypsin numbering) is highly conserved in trypsin-like proteases. Pioneering mutagenesis work of trypsin, which displays 42% amino acid identity to matriptase, showed that an Ala
substitution in this residue increased the specificity of the mutant trypsin for small peptide substrates containing Arg in the P1 position (37). A charged residue in this position has been a characteristic of trypsin-like serine proteases (38). G216S substitutions have also been reported in human protein C deficiency and shown to cause the reduced activity of the enzyme (39). Consistent with these previous studies, we found that, although the capacity to bind benzamidine and cleave fluorophosphonate probes indicated some level of preservation of the integrity of the activity site, G827R matriptase serine protease domain displayed low proteolytic activity toward small peptide substrates as well as toward macromolecular substrates. In light of these kinetic data, it is tempting to conclude that the c.2672G → A mutation in ST14 gives rise to a null allele. However, this conclusion may well turn out to be premature. Thus, our ST14 hypomorphic mouse model presented here surprisingly revealed that mice can tolerate even a severe reduction in matriptase: mice with <1% epidermal matriptase mRNA at birth survive the neonatal period and display only a moderately impaired barrier function. Although methods for quantitatively measuring the residual matriptase proteolytic activity in ST14 hypomorphic epidermis are not at hand, the 100-fold reduction in epidermal mRNA levels most certainly is going to translate into strongly reduced matriptase proteolytic activity. However, the ability to tolerate drastic reductions in the activity of a serine protease cascade is not unprecedented in a physiological setting. For example, the activity of tissue factor, a membrane-associated receptor for coagulation factors VIIa and X that serves as the primary physiological initiator of blood coagulation, can be reduced by 99% with minimal adverse effects on mouse embryonic development, whereas complete tissue factor deficiency causes mid-gestational embryonic lethality (40).

In this respect, it is noteworthy that matriptase and tissue factor deficiency causes mid-gestational embryonic lethality (40). mouse embryonic development, whereas complete tissue factor deficiency causes mid-gestational embryonic lethality (40).

Matriptase Insufficiency in Epidermal Development

FIGURE 6. Abnormal enamel surface and increased bacterial colonization of ST14 hypomorphic teeth. Representative scanning electron microscopic images at low (A, D, G, and J) and high (B, C, E, F, H, I, K, and L) magnification of control (A–C and G–I) and ST14 hypomorphic (D–F and J–L) teeth from young adult mice reveal normal gross morphology, but roughened enamel surface and increased bacterial colonization of ST14 hypomorphic teeth. Magnifications: A, D, G, and J, 100×; B and E, 1000×; H and K, 2000×; and C, F, I, and L, 10,000×.

FIGURE 7. Compromised epidermal barrier function of ST14 hypomorphic mice. Litters of newborn pups were separated from their mother to prevent fluid intake, and the rate of epidermal fluid loss was estimated by measuring the reduction of body weight as a function of time. The data are expressed as the average body weight as a percent of initial body weight in hypomorphic (open circles, n = 4) and littermate control (dark squares, n = 4) pups. The data are expressed as the average ± S.E. *, p < 0.05; Student’s t test, two-tailed.

FIGURE 8. Impaired desquamation of ST14 hypomorphic epidermis. Microscopic appearance of control (top panels) and littermate ST14 hypomorphic (bottom panels) epidermis without (left panels) and after 17 rounds of tape stripping (right panels). Complete removal of the stratum corneum with exposed upper granular and transitional cell layers of control epidermis (B) and partial stratum corneum removal in ST14 hypomorphic epidermis (D) are shown. Hematoxylin and eosin staining was used. Size bars all frames, 20 μm.
Matriptase Insufficiency in Epidermal Development

FIGURE 9. Reduced proteolytic prostanin activation and profilaggrin processing in ST14 hypomorphic epidermis. A, Western blot using a mouse monoclonal anti-prostanin antibody of protein lysates (50 μg per lane) from the epidermis of three newborn control (lanes 1–3), three ST14 hypomorphic (lanes 4–6), and three ST14 null (lanes 7–9) mice separated by SDS-PAGE under reducing conditions showing reduced, but not abolished, active prostanin in ST14 hypomorphic epidermis. The positions of the prostanin zymogen and proteolytically activated prostanin are indicated at the left. The position of the mouse IgG heavy chain, which is recognized by the secondary antibody, is also indicated. The position of the molecular mass markers (kilodaltons) is indicated at the right. B, Cooomassie staining (left panel, lanes 1–4) or proflaggrin/filaggrin Western blot (right panel, lanes 5–8) of epidermal urea extracts from newborn control (lanes 1 and 5), ST14 hypomorph (lanes 2, 3, 6, and 7), and ST14 null (lanes 4 and 8) epidermis reveals diminished proflaggrin proteolytic processing in ST14 hypomorphic epidermis. The positions of proflaggrin (Pro-FG), proflaggrin-processing products, and proteolytically processed filaggrin monomer (FG) are indicated. The positions of the molecular mass markers (kilodaltons) are indicated at the right.

suggest that the physiological functions of the membrane serine protease are relatively conserved in the two species. These similarities included thickened, scaly epidermis, hypomorphic and dysmorphic hair, and tooth abnormalities. It is also noteworthy that the phenotypic manifestations of low matriptase improved with age in both humans and mice. Unlike ARIH patients, however, the eyes of ST14 hypomorphic mice appeared normal when observed for >300 days (data not shown), and the link between corneal opacity and matriptase-insufficiency proposed in Basel-Vanagaite et al. needs confirmation in future studies.

By using our ST14 hypomorphic mouse model, we were able to gain mechanistic insights into the physiological and molecular events underlying ARIH. Histological and functional analysis revealed that the ichthyosis of ST14 hypomorphic mouse epidermis was caused by a combination of hyperproliferation of basal keratinocytes (hyperproliferative ichthyosis) and increased retention of upper epidermal layers (retention ichthyosis), secondary to a markedly compromised epidermal barrier function. Biochemical analysis indicated that this compromised epidermal barrier function was again secondary to reduced prostanin proteolytic activation and proflaggrin pro-

teolytic processing, thus uncovering potential targets for treatment.

Matriptase is widely expressed in epithelial tissues and by some hematopoietic cells and has been proposed to play a critical role in multiple physiological processes. These include thy-

mic development (41), mammary gland development (42), turnover of intestinal epithelium (43), fibrinolytic surveil-

lance (44), and macrophage activation (45). Furthermore, matriptase proteolysis is linked to the progression of both early and late stages of epithelial carcinogenesis of multiple tissues, and the membrane serine protease constitutes an attractive target for cancer therapy (25, 26, 46). However, the exploration of matriptase physiological and pathological functions using the powerful approach of mouse genetics has been seriously impaired by the uniform and early lethality of ST14 null mice (16, 24). The ST14 hypomorphic mouse strain presented in this report combines strongly reduced matriptase mRNA levels with normal survival, within at least a 300-day period, in both mixed and partially inbred genetic backgrounds. This makes the ST14 hypomorphic mouse strain almost ideally suited for further exploration of the functions of matriptase in a wide spectrum of physiological and pathological processes.

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