Identification of Human Hornerin and Its Expression in Regenerating and Psoriatic Skin

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We previously isolated a new member of the fused-type S100 protein family (hornerin) from the mouse (Makino, T., Takaishi, M., Morohashi, M., and Huh, N.-h. (2001) J. Biol. Chem. 276, 47445–47452). Mouse hornerin shares structural features, expression profiles, and intracellular localization with profilaggrin, indicating possible involvement of hornerin in cornification. In this study, we identified and partially characterized a human ortholog of mouse hornerin. The human hornerin gene was mapped between trichohyalin and filaggrin on chromosome 1q21.3, the region being completely syntenic with the counterpart of the mouse. The deduced amino acid sequence of 2850 residues shows typical structural features of “fused-type” S100 protein family members. Mature transcripts and protein from human hornerin were not detected in normal stratified epithelium, including the trunk epidermis, tongue, and esophagus. After screening of various normal and pathological human tissues, we found that human hornerin was expressed in psoriatic skin. Hornerin protein was present in the keratinizing region, although at a lower level and in fewer cells compared with filaggrin. Mature transcripts and protein from hornerin were also detected in regenerating human skin after wounding. Hornerin mRNA was induced 5 days after wounding. The mRNA level remained almost constant until 15 days and declined at 30 days after wounding. Hornerin protein was detected in the proximal epidermis (but not in the distal epidermis) at 15 days after wounding. These results indicate that hornerin has a function similar to but distinct from that of filaggrin in cornification.

Cornification is an essential process to confer upon skin in terrestrial mammals a barrier function to resist noxious biological and physicochemical insults from the environment and to prevent loss of body fluid (1, 2). Formation of a cornified envelope, an insoluble structure beneath the plasma membrane, is a hallmark of cornification (3). The cornified envelope is composed of many different proteins (4, 5), including relatively small proteins with short tandem repeats (involutin, loricrin, and small proline-rich proteins) (6–10), fused-type S100 proteins (profilaggrin, retin, and trichohyalin) (11–13), cytokeratins (14), and other miscellaneous proteins (elafin, cystatin A, envoplakin, and desmosomal components) (15–19). Some of them are essential for triggering and promoting the cornification process, and the others are probably bystanders engulfed in the cornified envelope (20). These proteins are cross-linked through ε-(γ-glutamyl)–lysine bonds catalyzed by epidermal transglutaminases (21). Abnormality in the cornification process results in various human diseases (22, 23), including absent granular layer-type ichthyosis vulgaris (24, 25), lamellar ichthyosis (26–28), and Vohwinkel syndrome with ichthyosis (29).

Among the proteins found in the cornified envelope, filaggrin plays a unique pivotal role in cornification. Filaggrin produced by post-translational proteolysis of the precursor protein, profilaggrin, promotes the aggregation of keratin filaments, resulting in the formation of disulfide bonds among them (30–33). At a later stage, filaggrin is further processed by modification of some amino acid residues and degradation to free amino acids, this being instrumental in keeping epidermal hydration and flexibility (34) and in absorbing UV (35).

We previously identified a protein (hornerin) in the mouse by RNA differential display (36). It shares structural features with fused-type S100 proteins, particularly with profilaggrin, i.e. having Ca$$^{2+}$$-binding EF-hand domains at the N terminus, followed by a spacer sequence and a large repetitive domain. Hornerin was found to be expressed in cornifying stratified epithelium, including the epidermis, tongue, esophagus, and forestomach. In the epidermis, hornerin was found to be colocalized with profilaggrin in keratohyalin granules in cells in the granular layer (37). These findings indicate that hornerin has a function that is similar to or that mutually complements profilaggrin in the cornifying epithelium.

In this study, we identified and characterized a human homolog of mouse hornerin. Unexpectedly, human hornerin was not detected in normal trunk skin, but it was expressed in regenerating and psoriatic skin. Thus, human hornerin may have a function similar to but distinct from that of profilaggrin.

EXPERIMENTAL PROCEDURES

Identification of Human Hornerin cDNA—A cDNA library of normal human skin was prepared using aZAPII (Stratagene, La Jolla, CA) and screened using a 674-bp segment covering the 3′-end of the coding region as a probe under conventional conditions. 5′-RACE$$^1$$ was performed using a 5′-Full RACE Core Set (Takara Bio, Shiga, Japan) under the conditions recommended by the manufacturer. Template

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank$$^{TM}$$/EBI Data Bank with accession number(s) AB180729 and AB104446.

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$ The abbreviations used are: RACE, rapid amplification of cDNA ends; RT, reverse transcription; TRITC, tetramethylrhodamine isothiocyanate; contigs, groups of overlapping clones.
RNA was derived from psoriatic skin tissue, and the primers used were as follows: RT-PCR, 5-TGAAATCTGTCACGCTCTG-3; first PCR sense, 5-CATATTGCACGGCTTC-3; first PCR antisense, 5-GTATAGCTCG-3; second PCR sense, 5-CGAAGTTTCAGGGTCA-3; and second PCR antisense, 5-TATCCACAGTATCCTGATCG-3. Altogether, five clones were isolated and sequenced.

**Preparation of Antiserum**—To prepare antiserum, an oligopeptide (CS-RGPYESGSSHS) corresponding to part of the repetitive units of human hornerin was synthesized, conjugated to keyhole limpet hemocyanin, and injected with adjuvant (TyterMax Gold, CytRx Corp.) into rabbits. The resulting antibody was affinity-purified using a HiTrap N-hydroxysuccinimide-activated column (Amersham Biosciences) conjugated to the peptides.

**Western Blot Analysis**—Normal human skin and psoriatic skin were obtained from the trunk and thigh, respectively, after acquiring written informed consents. After thoroughly removing dermal tissues, protein extracts were prepared and analyzed by Western blotting as described previously (37). Briefly, proteins were extracted in 0.1 M Tris-HCl (pH 7.5) and 5 mM EDTA containing a protease inhibitor mixture after homogenization. The proteins were precipitated with cold 10% trichloroacetic acid and redissolved in 9M urea, 2% Triton X-100, and 5% dithiothreitol. The pH of the solution was adjusted to 7.0 with 1M Tris-HCl (pH 8.0). 10- or 20-μg eq of protein were applied to 5–20% gradient SDS-polyacrylamide gels (PAGE, Atto, Tokyo, Japan), electrophoresed, and transferred to PolyScreen transfer membranes (PerkinElmer Life Sciences). The membranes were treated with an anti-hornerin antibody or an antihuman filaggrin monoclonal antibody (Biomedical Technologies, Inc., Stoughton, MA), and positive signals were visualized by ECL Plus Western blotting detection reagents (Amersham Biosciences). The following antibodies were also used as primary antibodies: anti-human cytokeratin-6 monoclonal antibody (Zymed Laboratories Inc., South San Francisco, CA), anti-human cytokeratin-14 monoclonal antibody (Biomed, Foster City, CA), and anti-bovine desmoplakin-1/2 monoclonal antibody (Progen Biotechnik, Heidelberg, Germany). Preimmune

**Detection of mRNA**—Human multiple tissue expression MTA™ Array 2 (Clontech) was hybridized with a 32P-labeled probe of the 3′-untranslated region of human hornerin cDNA under the conditions recommended by the manufacturer. For RT-PCR, total RNA was prepared from psoriatic skin tissue, and the primers used were

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antisera and the antibodies absorbed by the peptide were used for negative controls.

**Immunohistochemistry**—Psoriatic human skin tissues were fixed with 10% formalin solution. 5-μm thick sections were treated with heat at 105 °C for 5 min in 1 mM EDTA and cooled down spontaneously. The sections were blocked with serum-free protein block (Dako Corp., Carpinteria, CA) for 30 min and incubated with primary antibodies (anti-human hornerin or anti-human filaggrin described above) and then with TRITC-labeled anti-rabbit IgG or Alexa Fluor 488-labeled anti-mouse IgG (Molecular Probes, Inc., Eugene, OR).

Regenerating skin tissues were fixed with 4% paraformaldehyde in phosphate-buffered saline and processed and immunostained under the same conditions as those described for the psoriatic skin tissues. The antibody used for cytokeratin-6 was the same as that used in the Western blot analysis. Preimmune serum or anti-human hornerin antibody pre-absorbed by the immunogen peptide gave consistently negative results. The tissue sections were observed under an Olympus fluorescence microscope or a Zeiss LSM510 confocal laser microscope.

**RESULTS**

**Identification of Human Hornerin cDNA**—By a homology search using a sequence covering the EF-hand domains (EF) at the N terminus, followed by a spacer (S) and repetitive segments 1A–6B. Each repetitive segment is composed of four homologous units 39 ± 2 amino acids in length. B, homology among the EF-hand domains of representative human (hu) and mouse (mu) fused-type S100 proteins. An unrooted dendrogram made by ClustalW is shown. C, alignment of EF-hand domains of human hornerin (huHorn), mouse hornerin (muHorn), and human filaggrin (huFilg). Asterisks indicate identical residues, and dots indicate similar residues.

![Image](http://www.jbc.org/)

**Fig. 2. Structural and homology analysis of human hornerin protein.** A, structural features of the human hornerin protein. EF-hand domains (EF) are located at the N terminus, followed by a spacer (S) and repetitive segments 1A–6B. Each repetitive segment is composed of four homologous units 39 ± 2 amino acids in length. B, homology among the EF-hand domains of representative human (hu) and mouse (mu) fused-type S100 proteins. An unrooted dendrogram made by ClustalW is shown. C, alignment of EF-hand domains of human hornerin (huHorn), mouse hornerin (muHorn), and human filaggrin (huFilg). Asterisks indicate identical residues, and dots indicate similar residues.

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**Structural Features of Human Hornerin Protein**—The deduced amino acid sequence comprises EF-hand domains at the N terminus, followed by a spacer and repetitive segments 1A–6B. Each repetitive segment is composed of four homologous units 39 ± 2 amino acids in length. B, homology among the EF-hand domains of representative human (hu) and mouse (mu) fused-type S100 proteins. An unrooted dendrogram made by ClustalW is shown. C, alignment of EF-hand domains of human hornerin (huHorn), mouse hornerin (muHorn), and human filaggrin (huFilg). Asterisks indicate identical residues, and dots indicate similar residues.

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**Identification of Human Hornerin cDNA**—By a homology search using a sequence covering the EF-hand domain of mouse hornerin, we identified a human homolog of mouse hornerin at a chromosomal locus between those of filaggrin and trichohyalin on chromosome 1q21.3 (Fig. 1A). The region including hornerin and neighboring genes on human chromosome 1q21.3 was completely syntenic with a region on mouse chromosome 3F2.1 (Fig. 1B). Based on this sequence information, we obtained a 5’-terminal cDNA clone by library screening and 5’-terminal fragments by 5’-RACE. The base sequence of the isolated cDNA fragments matched well with that registered in the human genome data base. Altogether, we identified a 9621-bp cDNA, followed by a poly(A) tail. An open reading frame encoding a protein of 2850 amino acids was found. The human hornerin gene is composed of three exons 51, 161, and 9409 bp in length (Fig. 1C). The coding region occupies the 3’-half of the second exon and most of the third exon. The nucleotide sequence of the 5’-end (240 bp) is shown in Fig. 1D with precise insertion sites of the introns. A putative TATA box (TATAAA) was found in the genomic DNA sequence from −26 to −31 upstream of the 5’-end of the present cDNA. The sequences of the 5’- and 3’-terminal regions of human hornerin were registered in the GenBank™/EBI Data Bank under accession numbers AB180729 and AR104446, respectively. The putative full-length cDNA sequence was assigned as BR000036.

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glycine (24.4%), glutamine/glutamate (12.1%), and histidine (9.5%) residues, a common characteristic of mouse hornerin and mouse and human filaggrins.

Clustering of EF-hand domains of the representative fused-type S100 proteins revealed that the EF-hand domain of human hornerin is most similar to that of mouse hornerin among the members (Fig. 2B). This finding and the syntenic chromosomal loci of the human and mouse hornerin genes (Fig. 1A) indicate that the gene we identified is a genuine ortholog of mouse hornerin. Sequence similarity between human and mouse hornerin is high in the EF-hand domains (>75%) (Fig. 2C) and the spacer region (>60%), but limited in the repetitive region (<50%).

The repetitive region was classified at first into six segments, 1–6 (Fig. 2A), which were each further divided into three segments (*A, *B, and *C; asterisks indicate wild-card figures of segments 1–6) 153–158 amino acids in length. Segment 6B is truncated, having only 128 amino acids. Each of segments *A, *B, and *C is further composed of four repeats 39 ± 2 amino acids in length (*AI–IV, *BI–IV, and *CI–IV). As shown in Fig. 3A, the difference in amino acid sequence within segments A, B, or C is smaller than that among the segments with different first numeral designations but the same alphabetical designations. Within each of segments *A, *B, and *C, the smallest units of ~39 amino acids showed moderate homology (54–69%) to each other. When the smallest units were analyzed by an algorithm of ClustalW (available at clustalw.genome.jp/), segment *AI, for example, showed higher homology to other *AI segments than to segments *AI–IV, *BI–IV, or *CI–IV (data not shown). This was also the case for other units. Alignment of amino acid sequences of representative segments, i.e. 1A–6A and 4A–4C, is shown in Fig. 3 (B and C, respectively). These results are compatible with the notion that a progenitor unit of ~39 amino acids was at first amplified 4-fold and then triplicated during evolution to form segments A, B, and C in tandem, these being further amplified 6-fold.

Expression of Human Hornerin in Psoriatic Skin—We first examined the expression of hornerin in various human tissues using a poly(A)+ RNA array. The array covers most of the human tissues (except for skin), including those of the central
nervous system, digestive system (including the esophagus), respiratory system, and urogenital tract, but none of them were positive for expression of hornerin. We then examined various human skin tissues for hornerin expression by RT-PCR. Mature processed mRNA from hornerin was detected in psoriatic skin and vulva as evidenced by a 198-bp band amplified with the 5′/H11032-terminal primers, but not in normal human fetal and adult skin and tongue (Fig. 4A). Intriguingly, unprocessed transcripts of hornerin containing the second intron, giving rise to a DNA fragment 1823 bp in length, were present even in normal skin and tongue. In accordance with the results of RT-PCR, hornerin protein was detected by Western blot analysis in psoriatic skin, but not in normal skin, whereas filaggrin was expressed in both tissues (Fig. 4B). The ladder-like bands of hornerin are probably partially processed proteins, and the arrow points to fully processed filaggrin.

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psoriatic lesion. Hornerin was detected in the area positive for profilaggrin. However, hornerin protein was present in the keratinizing region, although at a lower level and in fewer cells.
compared with filaggrin. In an image at a higher magnification, it is clear that only some of the filaggrin-positive cells synthesized hornerin protein (Fig. 5B). Both hornerin and filaggrin proteins were present in a granular pattern in the cytoplasm. Hornerin was expressed in the lesional epidermis but not in the non-lesional epidermis of psoriatic patients (Fig. 5A).

Expression of Human Hornerin in Regenerating Epidermis—Hornerin protein was detected not in the entire area of the psoriatic lesion, but mainly in the periphery of the lesion, where the skin was in a process of healing. This prompted us to examine the expression of hornerin in regenerating skin after wounding. Full-thickness skin lesions 3 mm in diameter were made with a skin punch biopsy instrument in the medial side of the upper arm of a healthy volunteer. 2, 5, 15, and 30 days after wounding, 5-mm punch biopsies were taken and analyzed. Processed transcripts of hornerin lacking the second intron were induced at 5 days, and the level remained almost constant until 15 days and declined at 30 days after wounding (Fig. 6A). Unprocessed hornerin transcripts were persistently present.

Cytokeratin-16 was induced 2 days after wounding. Skin biopsies obtained 15 days after wounding were used for immunohistochemistry. Hornerin protein was detected in the proximal epidermis (−2 mm from the center of the wound), but not in the distal epidermis (−5 mm from the center of the wound) (Fig. 6B). Cytokeratin-6, which is known to be expressed in hyperproliferative epidermal keratinocytes (41, 42), was induced in the proximal region from the wound (Fig. 6C). Cytokeratin-6-positive cells were restricted to the proximal epidermis from the center of the wound, whereas hornerin was detected in a larger area, but only in limited layers.

DISCUSSION

**Gene Structure**—The synteny of the chromosomal region surrounding the hornerin gene is perfectly conserved between humans and mice (Fig. 1A). The hornerin gene is composed of three exons and two introns. The initiation codon is located in the second exon, and the long repetitive region is devoid of introns. This exon/intron composition is shared by the mouse hornerin gene (36), the human and mouse profilaggrin genes, and some other fused-type S100 protein genes (32, 33, 38, 39).

The amino acid sequences of the EF-hand domains of fused-type S100 proteins are well conserved in comparison with the remaining parts of the genes. The EF-hand domain of human hornerin shows 76% identity and 97% similarity to that of mouse hornerin and 50%–60% identity and 85%–90% similarity to those of human profilaggrin, repetin, and trichohyalin (Fig. 2, B and C) (data not shown). In contrast, the amino acid sequences of the repetitive regions of human and mouse hornerin are only ~40% identical.

**Repetitive Segments**—The repetitive region of human hornerin is composed of six triplet segments (A, B, and C) 153–158 amino acids in length (Fig. 2A). The amino acid sequences of the repetitive segments are well conserved. For example, the amino acid sequence of segment 3A shows ~97% identity to those of segments 4A and 5A (Fig. 3B). Each segment is further composed of four units 39 amino acids in length. Homology analysis indicated that a progenitor unit of ~39 amino acids was at first amplified 4-fold and then triplicated during evolution to form segments A, B, and C in tandem, these being further amplified 6-fold in tandem (see “Results”).
In the case of mouse hornerin, repetitive units are ~170 amino acids in length, and no smaller unit structure is noted (36). Thus, the structural features of the repetitive regions of human and mouse hornerin differ greatly at least at the present stage of evolution. Similarly, human profilaggrin has 10–12 repeats 324 amino acids in length, whereas mouse profilaggrin has >20 repeats of 250 or 255 amino acids (32, 33). In contrast to the structures of the repetitive regions, the general structures of the gene, the amino acid sequences of the EF-hand domains, and the amino acid composition of the repetitive region are well conserved not only in human and mouse hornerin, but also among other fused-type S100 proteins, including profilaggrin. Intriguing questions of how the repetitive structures were diversified and why such diverse structures are tolerated remain unanswered.

Expression of Hornerin—In the mouse, the expression profile of hornerin is similar to that of profilaggrin, and both proteins are co-localized in keratohyalin granules (37). Hornerin and filaggrin proteins were detected in the epidermis, tongue, esophagus, and forestomach. Unexpectedly, human hornerin was not expressed in the normal trunk epidermis, tongue, and esophagus. By screening various normal and pathological human tissues, we found that hornerin was expressed in psoriatic and regenerating epidermis (Figs. 4 and 5). Trichohyalin is a fused-type S100 protein that was originally found to be expressed in the inner root sheath in hair follicles (43, 44). It was later shown to be expressed in a variety of pathological human epidermal tissues as well as in the tongue, hard palate, and nail bed (45, 46). In these tissues, the expression of trichohyalin was found to be co-localized with the expression of cytokeratin-6 and cytokeratin-16. Human hornerin was also detected in some cytokeratin-6-positive epidermal keratinocytes in regenerating skin after wounding, but hornerin-positive cells only partially overlapped with cytokeratin-6-positive cells in regenerating skin (Fig. 6C).

RNA Processing—Neither mature mRNA nor protein from hornerin was detected in normal human skin. However, RT-PCR showed the presence of unprocessed transcripts of hornerin with intron 2 in normal human skin (Fig. 4A). Mature mRNA was induced in regenerating epidermis 5 days after wounding, and the level remained constant until 15 days and declined at 30 days after wounding (Fig. 6A). The consistent presence of unprocessed hornerin transcripts indicates that the expression level of human hornerin in the epidermis is at least partly regulated post-transcriptionally through modulation of the efficiency of RNA processing. Jarrous and Kaempfer (47) reported that super-induction of retinoic acid-induced interleukin-1β mRNA by cycloheximide is dependent on enhanced processing of the precursor transcripts. Regulation of gene expression at the stage of RNA processing probably facilitates a rapid response to an inductive signal. It is not clear, however, why human hornerin needs to be regulated in such a way and whether other fused-type S100 protein genes encoded in the epidermal differentiation complex are regulated similarly. Our present observation could be a step toward exploring such questions.

Functions of Human Hornerin in Normal and Pathological Skin—Evidence obtained in our previous studies on mouse hornerin indicates that hornerin is coexpressed with profilaggrin and plays roles similar to those of profilaggrin in the cornifying epithelium (36, 37). In humans, however, hornerin is not expressed in normal adult and fetal skin, as revealed in this study. Expression of hornerin appears not to be related to hair since human hair follicles and scalp epidermis are devoid of hornerin expression (data not shown). Instead, human hornerin protein was detected in regenerat-
Identification of Human Hornerin

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