RESEARCH COMMUNICATION

Epidermal detachment, desmosomal dissociation, and destabilization of cornodesmosin in Spink5<sup>−/−</sup> mice

Tao Yang,1,2,6 Dongcai Liang,1 Peter J. Koch,1,3 Daniel Hohl,5 Farrah Kheradmand,4 and Paul A. Overbeek1,2,7

1Department of Molecular and Cellular Biology, 2Department of Molecular and Human Genetics, 3Department of Dermatology, 4Biology of Inflammation Center and Department of Medicine, Baylor College of Medicine, Houston, Texas 77030, USA; 5Laboratory for Cutaneous Biology, Dermatology Unit, Beaumont Hospital, CHUV, Lausanne CH-1011, Switzerland

Netherton syndrome (NS) is a human autosomal recessive skin disease caused by mutations in the SPINK5 gene, which encodes the putative protease inhibitor LEKTI. We have generated a transgenic mouse line with an insertional mutation that inactivated the mouse SPINK5 ortholog. Mutant mice exhibit fragile stratum corneum and perinatal death due to dehydration. Our analysis suggests that the phenotype is a consequence of desmosomal fragility associated with premature prolylisis of cornodesmosin, an extracellular desmosomal component. Our mouse mutant provides a model system for molecular studies of desmosomal stability and keratinocyte adhesion, and for designing therapeutic strategies to treat NS.

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Netherton Syndrome [NS] [MIM 256500] is a severe, recessively inherited skin disease in humans with high neonatal lethality. It is characterized by ichthyosiform erythroderma, atopic dermatitis, bamboo hair, skin barrier defects, and elevated IgE levels in survivors (Krafchik et al. 1983; Judge et al. 1994). Mutations have been identified in the SPINK5 (serine proteinase inhibitor Kazal type 5) gene of NS patients (Chavanas et al. 2000; Sprecher et al. 2001; Walley et al. 2001; Bitoun et al. 2002; Komatsu et al. 2002). SPINK5 encodes LEKTI [lympho-epithelial Kazal-type-related inhibitor], which is a putative protease inhibitor that contains an N-terminal signal peptide and 15 domains with high internal homology [Magert et al. 1999]. Each domain has four conserved cysteines. Domains 2 and 15 possess two additional cysteines, which make them typical Kazal-type proteinase inhibitor domains [Magert et al. 1999]. LEKTI exhibits proteinase inhibitor activity in vitro [Magert et al. 1999; Komatsu et al. 2002; Walden et al. 2002; Mitsudo et al. 2003]. In NS patients, loss or reduction of LEKTI activity is presumed to result in elevated proteolytic activity in the suprabasal epidermis, leading to erythroderma and skin-barrier defects. However, the specific proteins that are targeted for degradation in these patients have not been identified. We describe here a Spink5 mutant mouse line that shows severe skin defects associated with desmosomal fragility, and thus, provides insights into the molecular pathogenesis of NS and a novel model system for studies of keratinocyte adhesion.

Results and Discussion

Transgenic mouse line OVE1498 was generated by coinjection of a tyrosinase-tagged [Yokoyama et al. 1990] Sleeping Beauty transposon [Ivics et al. 1997] [termed pT-Tybs-3’E] along with PGK2-SB10 [Ivics et al. 1997] [see Supplementary Fig. S1] into inbred FVB/N embryos. The transgenic founder and its transgenic F1 offspring were phenotypically normal and showed no evidence for transposition of the transgene[s] [data not shown]. When transgenic F1 mice were intercrossed, ∼25% of the newborn offspring developed severe skin blistering and water barrier defects, leading to death within several hours after birth [Fig. 1A]. This observation suggested that this transgenic line carried a recessive lethal insertion mutation.

To identify the transgenic integration site, we used an inverse PCR approach [Ochman et al. 1988] to amplify genomic sequences flanking the right arm of the tyrosinase-tagged transgene [see Supplementary Fig. S2]. Sequences linked to the transgene-matched mouse genomic sequences from contig NT_078853 on chromosome 18B3 [data not shown]. The junction between transgenic and genomic sequences was located 6 kb downstream from the stop codon of mouse gene 2310065D10Rik [NCBI accession no. XM_283487].

PCR walking was used to identify the other integration junction [see Supplementary Material]. The left junction was found to be 3.8 kb upstream of the start codon of the 2310065D10Rik gene. Integration of the transgenic DNA was accompanied by a deletion of 66.8 kb in mouse chromosome 18, including the entire coding region of 2310065D10Rik, but excluding any exons from the predicted adjacent genes [Fig. 1B]. The adjacent genes [Loc225443 and Ttid] are expressed at normal levels by RT–PCR analysis in the mutant newborns [Supplementary Fig. S3].

The human cDNA with the highest homology (68%) to 2310065D10Rik is the SPINK5 cDNA. SPINK5 maps to chromosome 5q32, which is syntenic to mouse chromosome 18B3. SPINK5 has 33 exons and 2310065D10Rik has 32 exons. The positions of the exon–intron junctions are well conserved between mouse and human [see Supplementary Fig. S4]. On the basis of the homology of the genes and the similarity of the mutant phenotypes, we propose that 2310065D10Rik and the protein encoded by this gene should be named Spink5 and Lekt1,
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evated expression of keratin 6 can indicate epidermal hyperplasia, BrdU incorporation was restricted to the basal keratinocytes and occurred at comparable levels in Spink5−/− embryos [E19] and in wild-type controls [data not shown].

Because newborn Spink5−/− mice were typically found as dehydrated corpses within 24 h after birth, we assayed for changes in skin barrier development by X-gal staining [Hardman et al. 1998]. This procedure uses incubation at an acidic pH to detect endogenous β-galactosidase activity. At E17.5 and E19, mutant skin was largely unstained, which showed evidence for skin-barrier formation. Nevertheless, focal lack of barrier function (i.e., blue staining) was observed in the articular regions such as the knees, elbows, cheeks, and neck [Fig. 3B]. After birth, Spink5−/− mice stained blue over their entire body surface, demonstrating complete loss of barrier function [Fig. 3B]. These results are reminiscent of clinical observations in neonatal patients with severe NS, where skin lesions occur preferentially in articular regions and aggravate soon after birth with generalized exfoliative erythroderma [Komatsu et al. 2002; Muller et al. 2002].

Transmission electron microscopy on skin samples from newborn Spink5−/− and wild-type littermates revealed abnormal cornification in the mutant mice [Fig. 4C]. Specifically, we observed loosely interconnected keratinocytes that contained abnormal low electron-dense vesicles in their cytoplasm [Fig. 4F], a phenomenon observed in human NS samples as well [Muller et al. 2002]. Furthermore, we found a distinctive architectural loss of cell–cell adhesion [acantholysis] in the granular layer of mutant epidermis [Fig. 4D]. Half desmosomes with attached keratin filaments were visible at the plasma membranes of most granular cells [Fig. 4E]. This desmosomal defect is reminiscent of the ultrastructural lesions observed in the skin of pemphigus foliaceus [PF] patients, who suffer from suprabasal intraepidermal blistering due to the presence of Dsg1 auto-antibodies [Stanley 2000; Stanley et al. 2001].
Histology and epidermal barrier assay on Spink5−/− mice. (A) Histology of P0 Spink5−/− (MT) and wild-type (WT) mouse skin. The stratum corneum has detached and is not seen in the mutant skin. In addition, granular cells are seen prematurely scaling off from the mutant skin (arrows). (SC) Stratum corneum; (GR) granular layer; (BL) basal layer; (hf) hair follicle. Bar, 50 µM. (B) Epidermal barrier assays by X-gal staining. Wild-type and Spink5−/− embryos and neonates were incubated with X-gal. Skin without a functional epidermal water barrier stains blue.

The similarities in the desmosomal defects of PF patients and the Spink5−/− mice suggested that the stability of desmosomal proteins might be altered in our mutant mice. Because the Spink5 gene encodes a putative secreted proteinase inhibitor, we tested for an increase in proteinase activity using an in situ zymogram (ISZ) assay. The mutant SC exhibited higher caseinolytic activity than wild-type SC, and increased proteinase activity was also detected in the upper spinous and granular layers of Spink5−/− skin [Fig. 5A]. Therefore, the desmosomal defect seen in the mutant epidermis might result from an abnormal increase in proteinase activity that leads to premature degradation of extracellular desmosomal component[s].

The major transmembrane desmosomal proteins in the upper spinous and granular layers are Dsg1 and Dsc1 (Garrod et al. 2002b). It is thought that heterophilic interactions between these two types of transmembrane glycoproteins establish desmosome-mediated cell–cell adhesion (Schwarz et al. 1990; Garrod et al. 2002a,b; Cheng and Koch 2004). Another extracellular component with a less well-defined function is corneodesmosin (CDSN) [Simon et al. 1997, 2001]. Human CDSN is a 52–56-kDa glycoprotein expressed in the upper spinous and granular layers of normal skin, and in the inner root sheath of hair follicles [Levy-Nissenbaum et al. 2003]. This protein is secreted into the intercellular space and becomes an extracellular component of desmosomes in the granular layer and of corneodesmosomes [cornified cell envelope (CE)] in the SC [Simon et al. 1997, 2001]. CDSN has been hypothesized to stabilize desmosomes and to become covalently associated with the cornified cell envelope [CE] [Simon et al. 1997]. Furthermore, expression of a chimeric protein consisting of the N-terminal domain of CDSN and the transmembrane domain of E-cadherin promotes cell–cell aggregation in transfection experiments, suggesting that CDSN might function as a homophilic adhesion molecule [Jonca et al. 2002]. Within normal skin, CDSN is progressively proteolysed during cornification and desquamation [Simon et al. 1997, 2001]. It has been hypothesized that the controlled degradation of CDSN leads to a destabilization of corneodesmosomes during physiological desquamation (Simon et al. 1997, 2001).

To determine whether Dsg1, Dsc1, or Cdsn [mouse ortholog of human CDSN, XP_111684], were affected in newborn Spink5−/− skin, we used Western blotting with Triton X-100 [TX 100]-soluble and TX 100-insoluble protein extracts [Fig. 5B]. The TX-100-insoluble fraction contains proteins integrated into mature desmosomes. Dsc1 and Dsg1 are mainly present in the TX 100-insoluble fraction [Pasdar and Nelson 1988]. Using Dsc1 and Dsg1/2 antibodies (Cheng et al. 2004), we did not detect a significant reduction in the steady-state level of Dsc1 or Dsg1/2 in either the TX 100-soluble or TX 100-insoluble fractions of Spink5−/− skin [Fig. 5B].

Next, we probed Western blots with an antibody [F28-F27] generated against human CDSN [Montezin et al. 1997]. In humans, this antibody recognizes three clusters of bands as follows: a full-length cluster [52–56 kDa], which is mainly found in the keratinosomes and present in the detergent-soluble pool, an intermediate-size cluster [40–48 kDa], which primarily exists in the detergent-insoluble fraction and is thought to contribute to desmosome stabilization, and a small-size cluster [33–36 kDa], which is normally present in the uppermost SC, and probably lacks adhesive properties [Montezin et al. 1997; Simon et al. 1997, 2001]. In mouse skin, F28-F27 also recognizes three Cdsn clusters, but the sizes are slightly different from those observed in humans, that is, 75 kDa,
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CDSN plays a role in hair-follicle growth and maintenance [Levy-Nissenaum et al. 2003]. So far, no homozygous CDSN/Cdsn mutants have been reported in humans or mice.

In summary, our results show a link between NS skin defects and impaired desmosome function in the suprabasal epidermis, and provide a possible molecular mechanism for the pathogenesis of NS. Spink5−/− mice mimic severe NS, and can be used to design and test perinatal therapies for treatment of the disease. In addition, our mouse can be used to study desmosomal maturation and stabilization in the suprabasal layers of the epidermis.

Materials and methods

Genotyping of mutant allele by PCR

Tail and yolksac DNA samples were used as PCR templates. Primers R3 (CCACCTGGGAAATGTGATGAAAGAAATAAAAGC), R-WT-s (GGCATCCATTAGTTTACACCG), and R-WT-as (GGGAGTGGATCACTTTCCCGTGT) were used to amplify a 434-bp Spink5 cDNA from wild-type samples. HPRT-s (ATGA CTAGATTTGTTTGTATACC) and HPRT-as (GTAGCTCTTCAGTCTGAAATATTTCT) primers were used to amplify hypoxanthine-guanine phosphoribosyltransferase (HPRT) cDNA as a positive control.

Epidermal permeability assay

Embryos or neonates were briefly washed with 0.9% NaCl and immediately immersed into acidic X-gal mix (100 mM phosphate buffer at pH 4.3, 3 mM K3Fe(CN)6, 3 mM K4Fe(CN)6, 2 mM MgCl2, 1 mg/mL X-gal), then incubated for 8 h at room temperature in the dark [Hardman et al. 1998]. To avoid damage to the neonatal epidermis, we separated pups from their mothers immediately after birth. In the absence of a mature epidermal barrier, X-gal penetrates into the cells of the epidermis where it is hydrolyzed to produce a blue color.

In situ hybridization

A Spink5 cDNA fragment generated by RT–PCR with Spink5-exon3-s and Spink5-exon7-as primers was subcloned into the pGEM-T Easy vector (Promega) and used as a template for generating an antisense RNA Probe. Digoxygenin-UTP, T7 RNA polymerase, and the Digoxygenin-UTP RNA polymerase were used to label the probe and to detect hybridization. [The nonradioactive in situ Protocol is described online at http://www.roche-applied-science.com/prod_inf/manuals/InSitu/pdf/ISH_149–163.pdf.]

CDSN degradation and dissolution of corneodesmosome-mediated adhesion are normal processes in the stratum corneum. In our mouse model, as well as human NS patients, absence of Lekti/LEKT1 leads to a premature activation of proteinases involved in this process. Serine proteinases SCCE (stratum corneum trypsin-like enzyme) [Hansson et al. 1994] and SCCTE (stratum corneum chymotrypsin-like enzyme) [Brattsand and Edgerud 1999], which are synthesized in granular cells and secreted into the intercellular space, can both digest human CDSN-producing proteolytic products of 30–33 kDa [Simon et al. 2001; Caulet et al. 2004]. They may cooperate with LEKT1 to regulate the CDSN stability in the suprabasal epidermis.

Mutations that produce truncated forms of CDSN have been described in humans [Levy-Nissenaum et al. 2003]. These mutations lead to a dominant hair disorder [hypoesthesia simplex of the scalp], implying that
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Triton X-100 with Complete Proteinase Inhibitors Cocktail from Roche). Soluble and insoluble fractions were separated by centrifugation. The insoluble pellets were dissolved with 5x Laemml buffer (0.05 M Tris-Cl, 4% SDS, 8% glycelyer, with 20% β-Mercaptoethanol) at 100°C for 20 min. Equal amounts of protein (determined using the Protein Assay Dye Reagent from Bio-Rad) were fractionated on 4%–15% gradient SDS-polyacrylamide gels (Bio-Rad), then transferred to PVDF membranes (Bio-Rad). The following primary antibodies were used: Dsg4/1 (1:800; from RDI), detects Dsg1/2; Dsc1 antibody (Cheng et al. 2004) (1:800), and corneodesmosin antibody F28-F27 (1:1000, from Dr. Guy Serre, University of Toulouse III, Toulouse, France).

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