Lessons from Loricrin-deficient Mice: Compensatory Mechanisms Maintaining Skin Barrier Function in the Absence of a Major Cornified Envelope Protein

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Abstract. The epidermal cornified cell envelope (CE) is a complex protein–lipid composite that replaces the plasma membrane of terminally differentiated keratinocytes. This lamellar structure is essential for the barrier function of the skin and has the ability to prevent the loss of water and ions and to protect from environmental hazards. The major protein of the epidermal CE is loricrin, contributing ~70% by mass. We have generated mice that are deficient for this protein. These mice showed a delay in the formation of the skin barrier in embryonic development. At birth, homozygous mutant mice weighed less than control littermates and showed skin abnormalities, such as congenital erythroderma with a shiny, translucent skin. Tape stripping experiments suggested that the stratum corneum stability was reduced in newborn Lor−/− mice compared with wild-type controls. Isolated mutant CEs were more easily fragmented by sonication in vitro, indicating a greater susceptibility to mechanical stress. Nevertheless, we did not detect impaired epidermal barrier function in these mice. Surprisingly, the skin phenotype disappeared 4–5 d after birth. At least one of the compensatory mechanisms preventing a more severe skin phenotype in newborn Lor−/− mice is an increase in the expression of other CE components, such as SPRRP2D and SPRRP2H, members of the family of “small proline rich proteins”, and repetin, a member of the “fused gene” subgroup of the S100 gene family.

Key words: loricrin • knockout mice • cornified envelope • small proline-rich protein • repetin

Introduction

The cornified cell envelope (CE)1 is a protein–lipid layer that replaces the plasma membrane of corneocytes and is crucial for epidermal barrier function (Roop, 1995). The CE consists of a complex mixture of covalently cross-linked proteins and a layer of characteristic lipids attached to the extracellular surface of the protein layer (Wertz et al., 1989; Steinert and Marekov, 1995; Ishida-Yamamoto and Iizuka, 1998; Marekov and Steinert, 1998; Steinert et al., 1998a,b). On the cytoplasmic side, keratin filaments and their attachment sites at the plasma membrane, the desmosomes, are embedded in the CE (Koch and Franke, 1994). The CE components are cross-linked mainly via e-(γ-glutamyl)lysine isopeptide bonds, which are catalyzed by calcium-dependent transglutaminases (TGases). This cross-linking leads to a protein complex that is highly insoluble and resistant to conventional biochemical extraction procedures. The significance of the TGase-mediated cross-linking for skin function has been demonstrated by the discovery that mutations in one of these enzymes (TGase 1) can lead to lamellar ichthyosis, an autosomal recessive skin

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1Abbreviations used in this paper: CE, cornified cell envelope; E, embryonic day; ES, embryonic stem; HSV-tk, herpes simplex virus type 1 thymidine-kinase minigene; RPA, RNase protection assay; SPRRP, small proline-rich protein; TEWL, transdermal water loss; TGase, transglutaminase; VS, Vohwinkel’s syndrome.
Several protein components of the CE have been identified, among them are involucrin, members of a family of small proline-rich proteins (SPRRPs), loricrin as well as cystatin α, desmoplakin, envoplakin, elafin, filaggrin, and several keratins (Ishida-Yamamoto and Iizuka, 1998). Furthermore, repetin has been identified as a putative CE precursor protein (Krieg et al., 1997). Some of these proteins (involucrin, SPRRPs, loricrin, filaggrin, and repetin) are encoded by genes clustered in the so-called “epidermal differentiation complex” on human chromosome 1q21 (Volz et al., 1993; Marenholz et al., 1996; Mischke et al., 1996) and mouse chromosome 3 (Rothnagel et al., 1994; Song et al., 1999), respectively. Involutcin, SPRRPs, repetin, and loricrin also share amino acid sequence homologies in their COOH-terminal domains, indicating a common origin of these genes (Backendorf and Hohl, 1992; Krieg et al., 1997).

Interestingly, the protein composition of the CE varies between different tissues and body sites. Loricrin is the major component of the CE in the interfollicular epidermis, in which as much as 70% of the CE mass is derived from this protein (Hohl et al., 1991; Steven and Steinert, 1994; Jarnik et al., 1996; Steinert et al., 1998a). For example, in human foreskin epidermis, the inner (cytoplasmic) two thirds of the CE consists of >85% of loricrin (Steinert and Marekov, 1995). On the other hand, loricrin is absent in most internal epithelia, though one notable exception is the mouse forestomach (Hohl et al., 1993). Furthermore, the molar ratio of the CE components may vary between different tissues. A comparison of mouse epidermal and forestomach CE showed that the ratio of loricrin to SPRRPs was 100:1 in the newborn epidermis and 3:1 in the forestomach (Jarnik et al., 1996; Steinert et al., 1998a). SPRRP proteins are coexpressed with loricrin and serve as molecular cross-linkers (Steinert et al., 1998a). It has been speculated that the relative molar ratio of loricrin and SPRRP proteins may determine the biomechanical properties of the CE. For example, CE that are subject to mechanical stress may have a higher relative amount of SPRRP proteins (Jarnik et al., 1996; Steinert et al., 1998a).

It has been proposed that the first step in epidermal CE formation in the stratum granulosum is the deposition of involucrin at the inner surface of the plasma membrane (Steinert and Marekov, 1997). Involucrin is thought to serve as a scaffold for the addition of other CE components (Steinert and Marekov, 1997). Loricrin (Mehrel et al., 1990) is expressed in the granular layer of the epidermis where it accumulates in granules (termed L-granules to differentiate them from profilaggrin-containing F-granules) before it is integrated into the developing CE (Steven et al., 1990; Bickenbach et al., 1995).

Mouse loricrin is a glycine- (55.1% of total amino acids), serine- (22.3%), and cysteine-rich (7.1%) basic protein. It consists of 481 amino acids and has a predicted molecular weight of 37,828 (Mehrel et al., 1990). The protein contains tandem repeats that have been predicted to form “glycine loops” (Hohl et al., 1991; Steinert et al., 1991). It is thought that these structures confer flexibility to the protein and the CE. Interestingly, these biochemical features of loricrin, glycine-rich sequences and high flexibility, are also characteristic for the NH2-terminal and COOH-terminal domains of keratins 1 and 10 (Jorcano et al., 1984; Zhou et al., 1988). This pair of intermediate filament proteins is coexpressed with loricrin in embryonic development of the epidermis (Bickenbach et al., 1995). The glycine loops of loricrin are flanked by glutamine- or glutamine/lysine-rich sequences, which are targets for TGase-mediated intramolecular and intermolecular cross-linking through isopeptide bonds (Steinert and Marekov, 1995). The abundance of cysteine residues in the loricrin sequence suggests that this protein also forms extensive disulfide bridges (Hohl et al., 1991). This prediction is supported by the observation that loricrin can only be solubilized with high concentrations of reducing agents (Mehrel et al., 1990).

The unique structural features of loricrin and its abundance in orthokeratinizing epithelia, in particular tissues that are subject to considerable mechanical stress, such as the epidermis and forestomach (in mouse), have led to the assumption that expression of this protein is essential for the function of these tissues. To test this hypothesis, we have generated loricrin knockout mice. Unexpectedly, loss of loricrin led to a mild transient phenotype in mice. We present evidence suggesting that Lor−/− mice compensate for the loss of the main epidermal CE component by up-regulating the expression of other CE components, in particular SPRRPs and repetin.

Materials and Methods

Construction of Targeting Vector

We have described previously the isolation and characterization of the loricrin gene of the BALB/c mouse strain (Rothnagel et al., 1994). To achieve a high frequency of recombination with the genome of mouse embryonic stem (ES) cells, gene targeting/replacement vectors were constructed using gene sequences from the 129/Sv mouse strain (Capecchi, 1989; Deng and Capcelle, 1992; Ramirez-Solis et al., 1993; Horie et al., 1994). We have isolated the loricrin gene from this mouse strain. In our targeting vector (Fig. 1 A), a 2.457-kb DNA fragment encompassing the entire loricrin coding sequence is deleted and replaced by a neomycin-resistance minigene (see Fig. 1 A, PGK neo pA) (provided by Allan Bradley, Baylor College of Medicine). The minigene is flanked by DNA sequences derived from the loricrin gene locus (5.8-kb 5′ flanking sequence; 1.5-kb 3′ flanking sequence). To facilitate the isolation of targeted ES cell clones, we inserted a herpes simplex virus type 1 thymidine-kinase minigene (HSV tk; Fig. 1 A) (Manoury et al., 1988) into the targeting vector (provided by John Lydon, Baylor College of Medicine). Both minigenes are under the transcriptional control of the phosphoglycerate kinase I promoter.

Targeting of ES Cells and Generation of Loricrin-deficient Mice

The ES cell line AB2.2 (provided by Allan Bradley, Baylor College of Medicine) was cultured as described previously (Ramirez-Solis et al., 1993). Linearized targeting vector was introduced into ES cells by electroporation with a Bio-Rad Gene Pulser II (Bio-Rad Laboratories). Electroporated ES cells were grown on gamma-irradiated neomycin-resistant SNL feeder cells (Hogan et al., 1994), provided by Allan Bradley (Baylor College of Medicine). Both minigenes are under the transcriptional control of the phosphoglycerate kinase I promoter.
fragment (5’ probe) and a SacI-BamHI fragment (3’ probe) as probes in this Southern blot analysis. Recombinant ES cell clones that showed the expected DNA bands in Southern blots, indicating a deletion of the loricrin coding sequence in one allele, were also tested with a probe derived from the neomycin-resistance minigene. This probe indicated the presence of a single copy of the neomycin-resistance minigene in the recombinant ES cell clones tested (data not shown). The recombinant ES cells were injected into C57Bl/6j blastocysts. Chimeric offspring were crossed with C57Bl/6j mice. DNA from agouti offspring was prepared from the tail (Hogan et al., 1994) and tested for the presence of the targeted allele by Southern blot hybridization using the 5’ and/or 3’ probes. Mice heterozygous for the targeted mutation were intercrossed to obtain homozygous loricin-null mutants. To minimize phenotype variations due to the founder effect, each line was backcrossed into the FvB mouse strain. The experiments described in this paper were performed using mice from the fifth back cross.

Southern Blot Analysis

Genomic DNA from mouse tails (Hogan et al., 1994) was digested overnight with the appropriate restriction enzymes. DNA fragments were separated by agarose gel electrophoresis, transferred to GeneScreen Plus membranes (NEN Life Science Products), and then UV cross-linked (UV Stratalinker 1800; Stratagene). Radioactive DNA probes were synthesized using the DecaprimeII kit (Ambion) with [α-32P]dCTP (NEN Life Science Product). Blots were hybridized for 2 h at 65°C with probes in “rapid hybridization buffer” (Amersham Pharmacia Biotech), washed once for 10 min at room temperature in 2× SSC/0.1% SDS, followed by two washes (20 min each) at 65°C in 0.2× SSC/0.1% SDS. The membranes were then exposed to Kodak BIOMAX MR film (Eastman Kodak).

RNase Protection Assay

The following mouse DNA clones were used to synthesize antisense cRNA probes: loricrin (200-bp 3’ coding sequence), SPRRP2D (position 309–640, sequence data available from EMBL/GenBank/DDBJ under accession number AJ005562), SPRRP2H (position 469–642, sequence data available from EMBL/GenBank/DDBJ under accession number AJ005562), repetin (position 8651–8854, sequence data available from EMBL/GenBank/DDBJ under accession number AJ005562), and SPRRP2A (position 309–640, sequence data available from EMBL/GenBank/DDBJ under accession number AJ005562), repetin (position 8651–8854, sequence data available from EMBL/GenBank/DDBJ under accession number AJ005562). RNAs were determined by scanning RPA autoradiograms followed by quantification of the digitalized images using the BrightStar Biodetect system (Ambion). RPAII products were separated in 6% polyacrylamide/urea gels, UV cross-linked (UV Stratalinker 1800; Stratagene), and then biotinylated and hybridized to separate nitrocellulose membranes (S&S NC; Schleicher & Schuell) with a Mini-Trans-Blot Cell (Bio-Rad Laboratories). Membranes were blocked for 1 h in blocking buffer (5% nonfat milk/TBS) and then incubated with the primary antibody, which was dissolved in blocking buffer, for 1 h. After three washes (10 min each) in TBST buffer (0.05% Tween 20 in TBS), membranes were incubated with the secondary antibody (alkaline phosphatase–conjugated anti–rabbit or anti–mouse antibodies; Zymed Laboratories) for 30 min and then washed as described above. Antibody binding was visualized with the NBT/BCIP system (Boehringer).

Immunofluorescence Microscopy

Cryosections (5-μm thick) were fixed in acetone for 10 min at −20°C and then washed in PBS. The tissues samples were incubated with the first antibody at the appropriate dilution in 1% BSA/PBS over night at 4°C. After washing three times for 10 min each in PBS, samples were incubated with a fluorescent dye–coupled antibody diluted in 1% BSA/PBS for 30 min and washed as described above. Stained sections were examined and photographed with a Nikon OPTIPHOT photomicroscope (Nikon).

Preparation of CEs and Sonication Experiments

Epidermis from newborn and 4- to 5-old mice was isolated essentially as described previously (Jarnik et al., 1998). CEs were prepared by boiling epidermis for 20–40 min in a buffer consisting of 20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 10 mM DTT, and 2% SDS. After centrifugation (5,000 g), CEs were washed twice with a buffer consisting of 20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 10 mM DTT, and 0.2% SDS. For sonication, CEs were suspended in a 2% SDS solution. The concentration of CE was determined with a hemacytometer. The CE suspension was sonicated in a Branson 2210 cup sonicator (Branson Ultrasonics) at 4°C, 20%, 30%, and 60°C. At various time points, CE aliquots were removed from the sonicator, counted with the hemacytometer, and photographed with a Nikon OPTIPHOT photomicroscope.

Tape Stripping Experiments

Newborn mice were tape stripped 10 times. Backskin from treated and untreated areas were excised, fixed, and processed for electron microscopy as described previously (Bickenbach et al., 1996).

Toluidine Blue Staining of Mouse Embryos

The developmental stage of mouse embryos was determined based on the assumption that fertilization occurred in the middle of the dark cycle the day before plugs were identified. The embryos were stained essentially as described (Hardman et al., 1998). In brief, embryos were dehydrated by incubations (1 min each) in 25%, 50%, and 75% methanol/PBS followed by 1 min in 100% methanol. The embryos were then rehydrated with the same series of methanol solutions (1 min incubations), washed in PBS, and stained for 1 min in 0.0125% toluidine blue O (Fisher Scientific)/PBS. After destaining in PBS, embryos were photographed with a Nikon SMZ-2T microscope.

Measurement of Transepidermal Water Loss

Transepidermal water loss (TEWL) from the ventral skin of newborn mice was determined using a Tewameter TM210 (Courage and Khazaka), as described previously (Matsuki et al., 1998). In addition, we used a simple dye penetration assay to analyze potential barrier defects (Matsuki et al., 1998). In brief, the backs of mice were immersed in 1 mM Lucifer yellow dye (Fluka Chemical Co.). Mice were killed after 1 h incubation and frozen sections were prepared from the back skin. Penetration of the dye was analyzed using a fluorescence microscope.

Results

Cloning of the Mouse Loricrin Gene and Generation of Loricin-deficient Mice

We have described previously the structure and chromosomal localization of the BALB/c loricin gene (Rothnagel et al., 1994). To construct an efficient gene targeting vec-
tor, we isolated the loricrin gene from the 129Sv/J mouse strain. It has been shown that the use of isogenic DNA can significantly increase the efficiency of gene targeting vectors in ES cells (Deng and Capecchi, 1992; Zhang et al., 1994; Simpson, 1997).

The loricrin protein sequence is encoded by a single exon (Yoneda et al., 1992; Rothnagel et al., 1994). In the targeting vector pDV140, a 2.4-kb PstI fragment encompassing the entire loricrin coding sequence was deleted and replaced by a neomycin-resistance minigene (Fig. 1 A). To allow homologous recombination between targeting vector and the loricrin gene in ES cells, the neomycin minigene was flanked by 5.8-kb 5' and 1.5-kb 3' loricrin sequences, respectively. Furthermore, a herpes simplex virus thymidine kinase (Fig. 1 A, HSV-tk) expression cassette was introduced into the targeting construct to allow a “positive/negative” selection procedure (Mansour et al., 1988). As shown in Fig. 1 A, successful recombination between the targeting vector and the loricrin gene was predicted to eliminate a BamHI site and introduce a new SacI site into the loricrin locus (129-mut, bottom) and the recombinant loricrin gene of 129Sv mice (129-wt, middle) and the wild-type loricrin gene locus deleted a BamHI site (middle, solid box) and introduced a new SacI site (in PGKneoAPa cassette, bottom). (B) Example of a Southern blot analysis. Tail DNA from the offspring of a loricrin knockout mouse was cut with BamHI and hybridized to the 3' probe. The genotype of the mice analyzed is indicated above the lanes. Arrows indicate the position of mutant (mut) and wild-type (wt) DNA fragments.

Effects of Loricrin Ablation on Prenatal Development

Hardman and colleagues recently developed a method to follow epidermal barrier formation in mouse embryonic development (Hardman et al., 1998). This method is based on the staining of embryos with a histological dye (see Materials and Methods). Impermeability of the skin for the dye indicates maturation of the skin barrier and is accompanied by the development of the cornified cell layers. At the molecular level, deposition of loricrin at the plasma membrane coincides with dye impermeability.

Focal expression of loricrin in the skin is first detected at embryonic day (E) 16 (Bickenbach et al., 1995; Hardman et al., 1998). We wondered whether the ablation of loricrin affected skin barrier formation at that stage of develop-
ment. Therefore, we examined Lor\(^{-/-}\) and control embryos at E16.5, E17.5, and E18.5 (Fig. 3). Lor\(^{1/-}\) and \(^{+/+}\) embryos showed a similar staining pattern at E16.5, with a large unstained area on the backskin indicating barrier formation. Lor\(^{-/-}\) embryos showed a different staining pattern. Their back skin was either completely stained or showed smaller areas of unstained skin compared with control animals, clearly indicating impaired barrier formation. However, at E17.5, Lor\(^{-/-}\) and control animals did not show any skin staining. These results suggest that the development of skin barrier function, at least as defined by the staining method, is delayed in Lor\(^{-/-}\) mice, but reaches wild-type level at E17.5. Nevertheless, given that Lor\(^{-/-}\) mice are born with a skin phenotype (see below), it is quite likely that skin barrier defects persist beyond E17.5.

**Loricin Knockout Mice Develop a Transient Congenital Erythroderma with a Shiny, Translucent Skin**

Mice heterozygous for the loricrin mutation (Lor\(^{1/-}\)) were phenotypically indistinguishable from wild-type littermates. However, Lor\(^{-/-}\) mice were clearly abnormal at birth. They were runted and had a shiny, translucent skin with signs of erythroderma (Fig. 4). To determine whether runted Lor\(^{-/-}\) mice grew more slowly than control littermates, we monitored their body weight. The postpartum growth rate of Lor\(^{-/-}\) mice was similar to control animals (data not shown). This suggested that food intake and digestion were normal in these mice, that is the loricrin-expressing tissues of the gastrointestinal tract were functioning properly. Consequently, histological examination did not reveal abnormalities in the tissues that are known to express loricrin, oral cavity, esophagus, and forestomach, of Lor\(^{-/-}\) mice (Hohl et al., 1993). The skin phenotype persisted for \(~4–5\) d and then gradually disappeared. Histological examination of newborn Lor\(^{-/-}\) skin did not reveal abnormalities with respect to thickness and proper development and differentiation (data not shown).

**Mechanical Properties of Lor\(^{-/-}\) CE**

We next prepared CEs from wild-type and Lor\(^{-/-}\) newborn epidermis. Microscopic examination revealed structural abnormalities in Lor\(^{-/-}\) CE (Fig. 5). It has been proposed that loricrin confers flexibility to the epidermal CE. To test whether Lor\(^{-/-}\) CEs were more susceptible to mechanical stress, we exposed purified mutant and wild-type CE to ultrasound (Table I). The percentage of intact CE from Lor\(^{-/-}\) epidermis decreased dramatically after 10

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CEs were isolated from four Lor\(^{-/-}\) mice and two wild-type controls. Each sample was sonicated as indicated at four different temperatures. Numbers indicate percentage of intact CE.

ND, not done.
min of ultrasound treatment (50% mutant CE versus 100% wild-type CE) (Fig. 5 and Table I). After 15 min, essentially all mutant CEs were destroyed. In contrast, no damage was evident in wild-type CE after 15 min of ultrasound treatment. Interestingly, the susceptibility of mutant CE to ultrasound was temperature dependent. Initially, CEs were treated with ultrasound at 4°C. At higher temperature, mutant CE were partially protected from ultrasound damage (Table I). For example, whereas essentially all mutant CEs were destroyed after 15 min treatment at 4°C, only 10% were damaged at 20°C (Table I).

Adult Lor+/2 CE also showed the same susceptibility to sonication (data not shown).

To determine whether the abnormal CE affected the biomechanical properties of the mutant epidermis, we performed tape stripping experiments and determined the loss of cell layers from the stratum corneum by electron microscopy. As summarized in Fig. 6, the stratum corneum of Lor−/− backskin was less resistant to mechanical stress than wild-type control tissue. Mutant mice lost approximately three times more cell layers than control mice. Nevertheless, the fact that tissues that are naturally exposed to mechanical stress (paws, legs, and axilla) do not show gross or microscopic lesions, suggests that the mechanical stability of Lor−/− epidermis is not significantly impaired.

The Lor−/− Mutation Does Not Increase TEWL

We speculated that the shiny skin phenotype of Lor−/− mice resulted from dehydration, that is an increased TEWL due to a skin barrier defect. To investigate this possibility, we measured water loss in newborn mice with a Tewameter (Barel and Clarys, 1995). We also determined whether the passive diffusion of the dye Lucifer yellow (Matsuki et al., 1998) through the stratum corneum of newborn Lor−/− mice was increased compared with wild-type controls. We have shown previously that the disruption of the epidermal barrier in transgenic mice expressing a mutant form of loricrin leads to an increase in the permeability of the skin for this dye, as well as an increase in TEWL (Suga et al., 2000). However, neither of these methods revealed an apparent increase in TEWL of Lor−/− epidermis (data not shown).

Upregulation of Other CE Components in Lor−/− Epidermis

The fast regeneration of the Lor−/− epidermis with normal TEWL shortly after birth and the disappearance of the phenotype by day 4 or 5, suggested a compensatory mechanism that was induced either in utero or immediately after birth. The fact that the CE of newborn Lor−/− mice was of normal thickness (Jarnik, M., P.A. de Viragh, D. Bundman, M. Simon, D.R. Roop, and A.C. Stevens, manuscript submitted for publication), but abnormal structure, further supported the idea that another CE component was compensating for the loss of loricrin. Therefore, we compared the expression levels of several known CE components in E16.5 and E17.5 embryos, newborn, and 4-d-old Lor−/− and Lor+/+ mice. We found an increase in the expression of repetin and two members of the SPRRPs protein family in Lor−/− epidermis at all time points examined (Fig. 7, A and B), except for E16.5. At E16.5, we did not obtain de-
tectable signals with the repetin and SPRRP2D probe, respectively. The SPRRP2H probe, however, gave reproducible signals. The expression levels of this gene were identical in Lor^{2/2} and wild-type mice at E16.5 (Fig. 7 C).

SPRRPs are components of the innermost cytoplasmic layer of the CE and serve as cross-linkers between loricrin molecules in orthokeratinizing epithelia (see Introduction). Mouse SPRRPs constitute a family of proteins that have been subdivided into three groups based on their amino acid sequences: SPRRP1 (2 genes), SPRRP2 (11 genes), and SPRRP3 (1 gene) (Ishida-Yamamoto and Iizuka, 1998; Song et al., 1999). Preliminary RT-PCR experiments indicated that two members of the SPRRP2 subfamily, SPRRP2D and SPRRP2H, were upregulated in Lor^{-/-} epidermis (data not shown). To confirm these results, we used RPAs to determine the mRNA levels of SPRRP2D and SPRRP2H in Lor^{-/-} and control (Lor^{+/+} and Lor^{2/2}) back skin epidermis.

As shown in Fig. 7 A, the two SPRRP2 mRNAs were already upregulated in E17.5 embryos, ~1 d after loricrin expression is initiated in normal mice (see Introduction). The expression of both SPRRP2D and SPRRP2H increased by approximately fivefold at E17.5. The expression in newborn mice increased by fivefold (SPRRP2D) and twofold (SPRRP2H), respectively. At day 4 of postnatal development, SPRRP2D and SPRRP2H expression decreased, coinciding with the disappearance of the shiny skin phenotype. Nevertheless, whereas both mRNAs were easily detectable in RPA analysis of Lor^{-/-} skin, no (SPRRP2D) or only faint signals (SPRRP2H) were obtained using wild-type skin mRNA (Fig. 7 A).

We also examined the expression of repetin by RPA. Repetin is a relatively new member of the “fused gene” S100 subgroup consisting of profilaggrin, trichohyalin, and repetin (Krieg et al., 1997). Repetin also shares homology with CE components and was recently shown to be upreg-
ulated along with SPRRP2A in another knockout mouse exhibiting a barrier defect (Segre et al., 1999). Repetin transcripts were increased approximately threefold at E17.5, twofold in newborns, and fourfold in 4-d-old Lor-/- back skin when compared with controls (Fig. 7 B). We were also able to demonstrate an increase in repetin in Lor-/- epidermis at the protein level by immunofluorescence microscopy. In wild-type epidermis, repetin is expressed focally, and it appears to be localized in granules. There is no obvious accumulation in the stratum corneum (Fig. 8 A). However, in Lor-/- epidermis, repetin is expressed more uniformly, and it appears to localize at the periphery of CE in the stratum corneum (Fig. 8 B).

Other biochemical markers of keratinocyte differentiation, such as profilaggrin, keratin 10, involucrin, scielin, and trichohyalin (which is a loricrin cross-linker in mouse forestomach) (Steinert et al., 1998a), were expressed at similar levels in newborn Lor-/- and control backskin (data not shown). However, in the case of profilaggrin, we noticed an additional high molecular weight band in Western blots from newborn Lor-/- back skin extracts which was not present in Lor+/- extracts (Fig. 7 D). Profilaggrin is a polyprotein consisting of multiple filaggrin units joined by linker sequences and flanked by unique NH2- and COOH-terminal domains (Rothenagel et al., 1987). The polyprotein is processed to yield filaggrin in a complex biochemical cascade involving phosphatases and proteases. Filaggrin has a high affinity for keratins and provides a matrix in which keratin filaments are embedded. In the stratum corneum, filaggrin is eventually completely hydrolyzed. Free amino acids derived from this protein are thought to contribute to water retention in the stratum corneum.

Interestingly, one of the filaggrin antibodies used in this study (AF133, Fig. 7 D) indicated a reduction of the amount of mature filaggrin in Lor-/- epidermis extracts.

Figure 6. The stratum corneum of Lor-/- mice shows increased susceptibility to mechanical stress. The back skin of newborn Lor-/- (Null) and wild-type pups was tape stripped ten times. Animals were killed and the number of stratum corneum cell layers was determined by electron microscopy. Open bars (untreated skin); closed bars (tape stripped skin). Note that mutant mice lost approximately three times more cell layers.

Figure 7. Alterations in the expression of SPRRPs (A and C), repetin (B), and of filaggrin (D) in Lor-/- mice. RPA and Western blot analysis were done with littermates from lor-/+ intercrosses. (A) RPA analysis of SPRRP expression in the back skin epidermis of E17.5 embryos, newborn, and 4-d-old pups. The genotypes of the animals are indicated above the lanes. b-Actin, cyclophilin, and desmoglein 3 (Dsg3; Koch et al., 1997) probes were used as internal standards. Note that the expression of SPRRP2D and SPRRP2H was increased at all time points examined (for details see text). (B) RPA analysis of repetin expression in the back skin of E17.5 embryos, newborn, and 4-d-old mice. Cyclophilin was used as an internal standard. The expression of repetin was increased in Lor-/- mice at all time points (see text for details). Note that the repetin probe produces two bands, most likely due to a polymorphism in the repetin gene. (C) RPA analysis of SPRRP2H expression in the back skin epidermis of E16.5 embryos. Note similar expression levels in mutant and wild-type mice. (D) Western blot analysis of protein extracts from newborn back skin with antibodies f111 and f133. The genotypes of the animals are indicated above the lanes. An additional profilaggrin band was detected in extracts from Lor-/- mice (arrows). Note that f133 indicates a reduction in the amount of mature filaggrin (Fil.) in Lor-/- mice.
compared with Lor$^{+/+}$ control extracts. However, the other antibody (AF111, Fig. 7 D) showed equal amounts of filaggrin in Lor$^{-/-}$ and Lor$^{+/+}$ extracts. Nevertheless, both antibodies detected the additional high molecular band. AF133 was raised against a peptide sequence from the linker region that connects the filaggrin units in the profilaggrin precursor polypeptide (GYYYEQEHSEEESD). On the other hand, AF111 was raised against a peptide found in the filaggrin repeat (DSQVHSGVQVEGRRGH).

Therefore, the Western blot results suggest abnormal proteolytic processing of profilaggrin. Immunofluorescence microscopy with AF133 showed a normal distribution of the protein in Lor$^{-/-}$ epidermis (data not shown). Furthermore, electron microscopy on newborn epidermis showed that Lor$^{-/-}$ mice form normal profilaggrin–containing F-granules. It remains to be seen whether the observed aberrant processing of profilaggrin contributes to the phenotype of Lor$^{-/-}$ mice.

### Discussion

**Delayed Development of Skin Barrier Function in Embryonic Lor$^{-/-}$ Mice**

Loricrin expression is first detected at day E16 in mouse embryonic development. The protein initially accumulates in L-granules (Bickenbach et al., 1995) where it is subsequently dispersed and incorporated into the nascent CE. At this stage, a rudimentary barrier function is established. By staining methanol-extracted E16.5 embryos with a histological dye, a process that presumably extracts polar lipids from the epidermis, unstained patches of skin become apparent in normal mouse embryos, indicating the initiation of barrier formation (Hardman et al., 1998). At this stage, Lor$^{-/-}$ embryos showed a different staining pattern than control embryos, indicating impaired skin barrier function. Their back skin was either completely stained or

![Figure 8. Increased repetin expression in Lor$^{-/-}$ mice. (A) Staining of back skin from a newborn wild-type mouse with antibodies against repetin (green) and K14 (red). Overlapping expression of both proteins results in yellow fluorescence. Note the expression pattern and lack of accumulation in the stratum corneum. (B) Staining of Lor$^{-/-}$ neonatal back skin. Note the more uniform expression pattern and the presence of repetin at the periphery of corneocytes in the stratum corneum. Also, note the apparent accumulation of repetin in granules in the granular layer of both wild-type and Lor$^{-/-}$ epidermis.](image-url)
showed only small patches of epidermis that were impermeable to the dye. In contrast, Lor+/− and Lor+/+ embryos showed large unstained areas on their backs (Fig. 3), and in most cases, the entire back was unstained. However, E17.5 mutant and control back skins were equally impermeable to the dye. Thus, the development of skin barrier function was delayed, but not abolished, in Lor−/− mutant embryos. Coincident with establishment of the barrier function in Lor−/− embryos, we observed increased expression of certain CE components in utero (see below).

**Lor−/− Epidermis Is More Prone To Desquamation but Not To TEWL**

Although the mutant epidermis eventually became impermeable to the dye, it was not completely normal. The skin of Lor−/− mice was red and shiny at birth, and the mutants weighed less than +/− and +/+ littermates. Interestingly, E18.5 embryos derived by cesarean section did not show gross skin abnormalities (data not shown), indicating that the skin phenotype developed shortly after birth, most likely within minutes.

Based on the gross appearance of newborn Lor−/− mice, which was very similar to another transgenic mouse model we characterized that exhibited dehydration due to a defect in epidermal barrier function (Imakado et al., 1995), we expected to see an increase in TEWL. However, at the time of measurement (~1 h after birth), we were not able to measure significant differences in TEWL in Lor−/− neonates.

The physiological basis for the red and shiny skin remains unclear. Staining tissue sections of Lor−/− and control mice with antibodies against endothelial markers did not reveal an increase in the number or size of blood vessels in Lor−/− mice (data not shown). Nevertheless, it is tempting to speculate that changes in the molecular composition of mutant CE might affect the optical properties of the skin.

A significant difference between Lor−/− and control epidermis was revealed by tape striping experiments. Mutant mice lost significantly more layers of back skin stratum corneum cells than control mice.

**Lor−/− Cell Envelopes Show Enhanced Susceptibility to Mechanical Stress**

The above observation suggested that the loss of loricrin affects the biomechanical properties of the stratum corneum, making it more susceptible to physical stress. This hypothesis is supported by our sonication experiments, which showed that purified Lor−/− CEs were reduced to small fragments under conditions in which wild-type CEs remained largely intact (Table I). Although CEs isolated from Lor−/− mice had less regular shapes than wild-type CEs (Fig. 5), their thickness, as determined by electron microscopy, was essentially normal, and was measured to be ~15 nm (a detailed analysis is reported in Jarnik, M., P.A. de Viragh, D. Bundman, M. Simon, D.R. Roop, and A.C. Stevens, manuscript submitted for publication). Since the procedure used to isolate CEs is harsh, it may be that the altered overall shapes of Lor−/− CEs reflects their enhanced susceptibility to mechanical stress (in situ, the CE of an individual corneocyte should be a continuous closed bag that is ruptured and perhaps fragmented during isolation).

**Upregulation of SPRRP2 and Repetin in Lor−/− Epidermis**

From the conserved thickness of the mutant CE, we suspected that another CE component(s) substitutes for loricrin in Lor−/− mice. Of several known CE components examined, increased transcript levels were detected only for SPRRP2D, SPRRP2H, and repetin. SPRRPs mainly serve as loricrin cross-linkers in orthokeratinizing epithelia. Interestingly, although the total amount of loricrin and SPRRP proteins is approximately constant at 80–85% of CE protein mass, their relative molar ratio varies markedly between different epithelia and even between different body sites (Jarnik et al., 1996; Steinert et al., 1998a). The loricrin/SPRRP ratio is >100:1 in the newborn epidermis, 4.5–7.5:1 in the footpad, and 3:1 in the forestomach (Steinert et al., 1998a). In general, tissues that have to withstand higher mechanical stress seem to have more SPRRP protein. Although the elevated levels of SPRRP2 in Lor−/− mice would imply a more robust CE, their effect appears to be offset by other components of the mutant cell envelope.

Increased expression in Lor−/− mice was also observed for repetin, a recently identified member of the “fused gene” subgroup of S100 proteins (Krieg et al., 1997). Repetin is expressed in terminally differentiated keratinocytes of mouse epidermis, tongue, and forestomach (Krieg et al., 1997). This protein has a high content of glutamine residues (20.2%) that might serve as a substrate for TGases. Repetin also shares sequence homologies in its COOH terminus with the corresponding domains of loricrin, involucrin, and SPRRP proteins (Krieg et al., 1997). These properties, together with the observation that repetin accumulates at the periphery of corneocytes in Lor−/− mice (Fig. 8 b), suggests that repetin is a component of the CE.

Analysis of the amino acid composition of Lor−/− CEs from newborn back skin indicated an approximately threefold increase in total SPRRP proteins compared with wild-type (Jarnik, M., P.A. de Viragh, D. Bundman, M. Simon, D.R. Roop, and A.C. Stevens, manuscript submitted for publication), which is consistent with the observed increase in SPRRP2 transcripts (this study). According to this analysis, repetin is unlikely to be a major component of either mutant or wild-type CEs, because it is detected at levels less than the estimated detection threshold of a few percent. This method was not sensitive enough to detect the increased repetin content of the CE implied by its elevated transcript level. More generally, mutant CEs have high contents of Gly and Ser, like wild-type CEs, in which these amino acids are contributed mainly by loricrin. The high Gly/Ser contents of the Lor−/− mutant may be contributed in part by keratins 1 and 10, whose end domains are rich in these residues, but other components are likely to be involved (Jarnik, M., P.A. de Viragh, D. Bundman, M. Simon, D.R. Roop, and A.C. Stevens, manuscript submitted for publication). Moreover, our Western blot analysis and RPA of Lor−/− epidermis did not indicate increased expression of the keratins. Further studies will be necessary to determine if a novel loricrin-like protein is indeed integrated into the CE of loricrin-deficient mice.

The expression of other CE components, such as involucrin, scinllin, filaggrin, trichohylin, and K10, were similar in Lor−/− and control animals. However, in the case of profil-
agrin, we noted an additional band in Western blots of total epidermal lysates, indicating abnormal processing of the precursor protein. Both immunofluorescence and immunoelectron microscopy of Lor⁻/⁻ epidermis showed a normal distribution of profilaggrin and F-granules (data not shown). Therefore, it is unclear whether the abnormal processing of profilaggrin contributes to the skin phenotype of Lor⁻/⁻ mice.

**Loricrin Mutations in Vohwinkel’s Syndrome and Progressive Symmetric Erythrokeratoderma**

Although loricrin-null mutations have not been reported in a human skin disease, a subset of patients with Vohwinkel’s syndrome (VS) (Maestrini et al., 1996; Korge et al., 1997; Armstrong et al., 1998; Ishida-Yamamoto et al., 1998; Takahashi et al., 1999) and one pedigree with “progressive symmetric erythrokeratoderma” (Ishida-Yamamoto et al., 1997) were shown to have mutations in the loricin gene. These diseases are rare autosomal dominant disorders that are clinically characterized by diffuse palmoplantar keratoderma (thickening of the stratum corneum of palms and soles) with a honeycomb appearance and the development of constricting bands around digits that can lead to amputation. In pedigrees analyzed at the molecular level, mutations in gene sequences coding for the COOH terminus of loricin have been found (Maestrini et al., 1996; Ishida-Yamamoto et al., 1997; Korge et al., 1997; Armstrong et al., 1998; Takahashi et al., 1999). All of the mutations reported to date are single base pair insertions that lead to the synthesis of an aberrant protein in which COOH-terminal amino acid sequences are replaced by nonsense amino acids. These mutations also eliminate several glutamine and lysine residues that normally form isopeptide bonds (compare mutations reported in Maestrini et al., 1996; Armstrong et al., 1998; Takahashi et al., 1999; with cross-linking sites in Candi et al., 1995; Steinert et al., 1998a).

We have recently generated a transgenic mouse model for this disease that reproduces most of the characteristics of VS (Suga et al., 2000). The severe phenotype resulting from expression of the loricin-VS mutant, as compared with the mild Lor⁻/⁻ phenotype, suggests that it is not functioning in a classic dominant negative fashion; it does not produce a functional knockout of loricin. To test this prediction, we mated the loricin-VS mutation into the Lor⁻/⁻ background. The severe phenotype resulting from this cross confirmed that the VS mutation was interfering with late stages of epidermal differentiation in a more global manner and did not require a direct interaction with wild-type loricin (Suga et al., 2000).

**Implications of the Mild Transient Phenotype Resulting from Loss of Loricin Expression**

In summary, we have shown that in newborn mice, the Lor⁻/⁻ phenotype includes erythroderma and a shiny, translucent skin. The absence of a more severe phenotype is, at least in part, due to a compensatory increase in the expression of SPRRPs and repetin. Although CEs of mutant mice show diminished stability when subjected to extreme mechanical stress in vitro, we did not notice skin abnormalities in adult mice. This might be due to the fact that the dense coat of mice provides protection against mechanical stress, since the CE isolated from adult Lor⁻/⁻ mice exhibited the same susceptibility to sonication as newborn Lor⁺/⁺ CE. This leaves one to speculate about the potential phenotypic consequences of loricrin deficiency in man. Patients born with a self-healing skin disorder similar to lamellar ichthyosis have been reported (Frenk and de Techtermann, 1992), however preliminary screening of a few of these patients did not reveal a deficiency in loricrin (Hohl, D., and P. de Viragh, unpublished data). The longer gestation time and the earlier onset of epidermal differentiation in human embryonic development may result in a subclinical presentation of loricrin deficiency at birth. If human Lor⁻/⁻ CE exhibit the same brittle characteristics as mouse Lor⁻/⁻ CE, would the absence of a dense coat of hair make these individuals more susceptible to harsh environmental conditions such as moisture and cold? Alternatively, are the requirements for fully functional CE in the maintenance of epidermal barrier function so critical that this need was met with the evolution of a series of replacement components that almost completely compensate for each other?

In this respect, it is interesting to note that mice deficient for involucrin do not develop an obvious phenotype (Djian et al., 2000). Regardless of the answers, an understanding of the molecular mechanisms that allow an embryo to compensate for a defect in epidermal barrier function in utero could have practical implications for the treatment of premature infants in the future. The Lor⁻/⁻ mouse provides us with an excellent model to discover these mechanisms.

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