Quasi-Normal Cornified Cell Envelopes in Loricrin Knockout Mice Imply the Existence of a Loricrin Backup System

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The cornified cell envelope, a lipoprotein layer that assembles at the surface of terminally differentiated keratinocytes, is a resilient structure on account of covalent crosslinking of its constituent proteins, principally loricrin, which accounts for up to 60%–80% of total protein. Despite the importance of the cell envelope as a protective barrier, knocking out the loricrin gene in mice results in only mild syndromes. We have investigated the epidermis and forestomach epithelium of these mice by electron microscopy. In both tissues, corneocytes have normal-looking cell envelopes, despite the absence of loricrin, which was confirmed by immunolabeling, and the absence of the distinctive loricrin-containing keratohyalin granules (L-granules). Isolated cell envelopes were normal in thickness (≈15 nm) and mass per unit area (≈7.3 kDa per nm^2); however, metal shadowing revealed an altered substructure on their cytoplasmic surface. Their amino acid compositions indicate altered protein compositions. Analysis of these data implies that the epidermal cell envelopes have elevated levels of the small proline-rich proteins, and cell envelopes of both kinds contain other protein(s) that, like loricrin, are rich in glycine and serine. These observations imply that, in the absence of loricrin, the mechanisms that govern cell envelope assembly function normally but employ different building-blocks. ***Key words: epidermal keratinocytes/immunoelectron microscopy/scanning transmission electron microscopy/small proline-rich proteins/terminal differentiation. J Invest Dermatol 118:102–109, 2002***

Late in terminal differentiation of keratinocytes in the epidermis and other squamous stratified epithelia, the cell membrane is replaced by the cornified cell envelope (CE) (**Fig 1**). The CE is a lamellar structure, approximately 15 nm thick (Jarnik *et al*, 1998), composed primarily of proteins cross-linked by covalent bonds formed by transglutaminase activity (Rice and Green, 1977; Thacher and Rice, 1985; Jeon *et al*, 1998). On its outer surface is a layer of covalently attached ceramide lipids (Swartzendruber *et al*, 1987; Wertz and Downing, 1987). This pattern of extensive cross-linking – in addition, perhaps, to intrinsic conformational properties of the proteins involved – make the CE a highly resilient structure, and it has been attributed a key role in conferring the toughness and impenetrability of cornified tissues (Reichert *et al*, 1993; Roop, 1995; Nemes and Steinert, 1999).

The major constituent of native epidermal CE is a protein called loricrin (Mehrel *et al*, 1990), which accounts for about 80% of their protein mass (Steven and Steinert, 1994). A number of other proteins have also been identified as CE components (reviewed by Hohl, 1990; Reichert *et al*, 1993; Simon, 1994), including the small proline-rich proteins (SPRRs; Kartasova and van den Putte, 1988; Kartasova *et al*, 1988; Marvin *et al*, 1992) and involucrin (Rice and Green, 1979; Eckert and Green, 1986). Loricrin has a molecular weight of 38 kDa in mouse and 26 kDa in human, and both proteins share the same domainal organization and unusual amino acid composition. They have extended tracts consisting almost exclusively of Gly and Ser residues, separating two terminal and one internal domain with more normal amino acid compositions, including Glu and Lys residues that are substrates for the cross-linking transglutaminases (Greenberg *et al*, 1991; Reichert *et al*, 1993). Interestingly, the terminal domains of loricrin are closely homologous to those of the SPRRs (Hohl *et al*, 1995), which fall into three subfamilies – SPRR1, SPRR2, and SPRR3 – according to their intervening sequences, which are multiple tandem repeats in which Pro is prominent (Kartasova *et al*, 1996; Tarcsa *et al*, 1998; Song *et al*, 1999).

Assembly of the CE from precursor proteins is thought to be a highly ordered event that proceeds in several stages: first, a thin layer composed of involucrin and other constituents is established, which subsequently serves as a scaffold for deposition of other components, including loricrin and the SPRRs (Eckert *et al*, 1993; Reichert *et al*, 1993; Steven and Steinert, 1994; Steinert and Marekov, 1997). This final phase of assembly establishes the main body of the CE, which has been proposed to consist either of multiple layers (Steinert and Marekov, 1995) or a monolayer (Jarnik *et al*, 1998) of cross-linked loricrin molecules. Moreover, barrier formation in the epidermis has been correlated with CE assembly (Marshall *et al*, 2000).

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Abbreviations: CE, cell envelope; DDW, doubly distilled water; EB, extraction buffer; LKO, loricrin knockout; SPRR, small proline-rich protein.

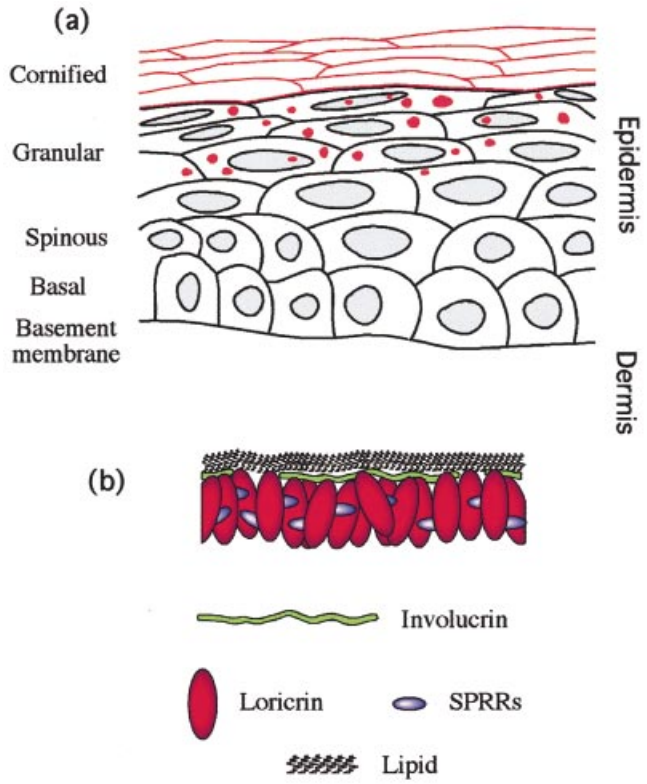

Figure 1. Assembly of the cornified CE in the outer layers of the epidermis. (*a*) The CE (Matoltsy, 1976) is assembled late in the terminal differentiation pathway of keratinocytes. Its major constituent is loricrin (Mehrel *et al*, 1990) – shown here in red – which first accumulates in L-granules in the cytoplasm and nucleus of granular layer cells before being redistributed into the assembling CE, where it is cross-linked with SPRRs and other proteins. (*b*) Molecular model of the CE, which is a uniformly 15 nm thick layer. Loricrin is on the cytoplasmic side where it forms either a monolayer – as shown here (Jarnik *et al*, 1998) – or several layers (Steinert and Marekov, 1997).

Thus the CE is a major structural element of terminally differentiated keratinocytes and has been assigned an important role as a protective barrier. As loricrin is its main constituent, one might expect that knocking out or interfering with loricrin expression would have severe repercussions. The production and characterization of loricrin knockout (LKO) mice have now been described (Koch *et al*, 2000): physiologically, the sequelae of loricrin elimination are surprisingly mild. In this study, we have used electron microscopy to examine the structure of CE *in situ* and as isolated from these animals. The absence of loricrin was confirmed by immunolabeling. Isolated LKO CE were found to be near-normal in thickness and mass per unit area, however, as measured from metal-shadowed specimens and from scanning transmission electron micrographs of unstained specimens, respectively. As the cross-linked character of CE precluded determining their protein composition by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, we measured the amino acid compositions of isolated CE and used mathematical modeling of these data (Steven and Steinert, 1994) to estimate the contents of proteins known to be present in wild-type CE. These observations indicate that, although loricrin is dispensable, the mechanisms for CE assembly remain in operation in LKO mice, but they incorporate different substrates.

MATERIALS AND METHODS

***In situ* electron microscopy of LKO mice** The LKO allele was generated in mice of a mixed (129/SvEv, C57D1/6) genetic background and crossed into the FvB strain as described by Koch *et al* (2000). For morphologic observation, small tissue samples (≈1 mm^3) were briefly washed in phosphate-buffered saline (PBS) and fixed for 3 × 1 h in 2% glutaraldehyde in 50 mM cacodylate buffer, pH 7.2, containing 2% sucrose; washed 3 × 1 h with the same buffer without sucrose; postfixed 2 × 1 h with 1% osmium tetroxide; washed overnight in water; dehydrated with gradually increasing concentrations of ethanol; infiltrated with EMbed-812 resin (EMS, Fort Washington, PA); and polymerized for 24 h at 65°C. Thin sections cut on an Ultracut E microtome (Leica, Deerfield, IL) were collected on collodion/carbon coated electron microscopic grids and stained with 2% uranyl acetate and lead citrate.

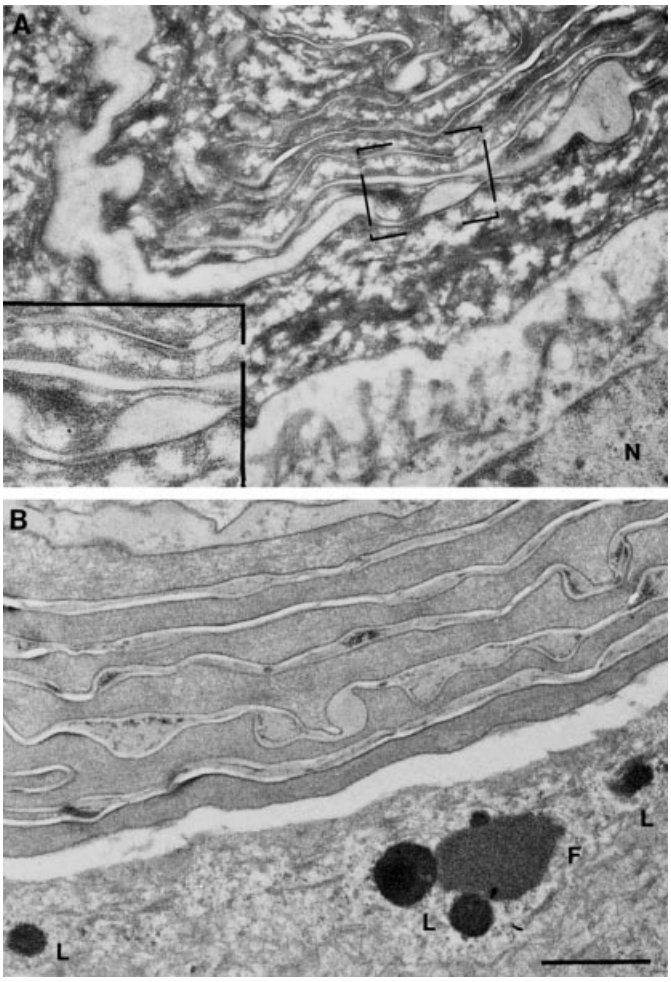

Figure 2. Ultrastructure of the upper granular and cornified layers of the forestomachs of an LKO mouse and a wild-type mouse. (*A*) LKO mouse; (*B*) wild-type mouse. The boxed area of the transverse thin section (*A*) is shown at double magnification at bottom left. The corneocytes are bounded by heavily staining cornified CE. N, nucleus. The granular layer cells underlying the cornified squames in the wild-type tissue contain abundant L-granules (L) (cf. Jarnik *et al*, 1996), which are completely absent in the knockout mouse. An F-granule (F) is also present. *Scale bar*: 1 μm.

Immunocytochemistry was performed according to Tokuyasu (1980). Briefly, the tissue was fixed in 6% formaldehyde in 0.1 M PIPES, pH 7.05; washed with that buffer; perfused with 2.1 M sucrose; and quenched in liquid nitrogen. Cryosections were cut on the same microtome equipped with a model FC4 cryochamber; transferred to a drop of 2.3 M sucrose; and mounted on formvar/carbon coated grids. After blocking nonspecific binding with 10% fetal bovine serum in PBS, the grids were incubated for 60–90 min with the primary antibody, washed three times with PBS, and incubated on a drop of protein A/10 nm gold (30 μg per ml of protein, BBInternational, U.K.) for 30 min. After washing in PBS, the grids were rinsed with doubly distilled water (DDW) and stained/embedded in 0.2% uranyl acetate/1% methylcellulose (Sigma, St. Louis, MO).

Specimens were viewed on an EM902 electron microscope (LEO, Thornwood, NY).

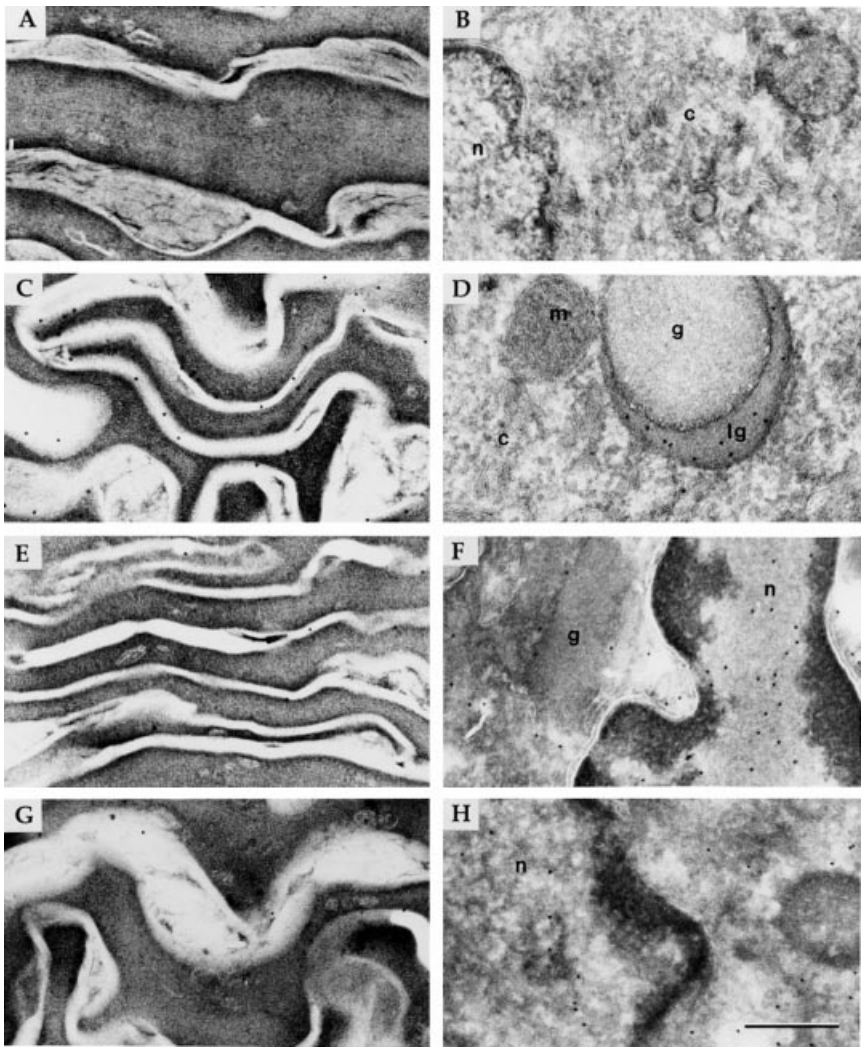

Figure 3. Immunolabeling of loricrin and SPRR1 in LKO mouse forestomach. Cryosections of formaldehyde-fixed tissues were labeled with antiloricrin (*A*, *B*) and anti-SPRR1 (*E*, *F*) antibodies, respectively. Bound antibodies were visualized with protein A complexed with 10 nm colloidal gold. Controls include normal mouse forestomach labeled with antiloricrin (*C*, *D*) and anti-SPRR1 (*G*, *H*). c, cytoplasm; n, nucleus; m, mitochondria; g, granule; lg, loricrin granule. *Scale bar*: 200 nm.

Isolation of CE To prepare CE from epidermis and forestomachs, the corresponding tissues were first obtained. Briefly, two to three newborn mice of the same genotype (LKO or wild-type) were sacrificed, and their skins were spread over Saran plastic foil. The epidermis was heat-separated from the dermis in PBS at 65°C for 30 s. Entire forestomachs were excised from two to three adult animals of the same genotype. Thereafter, CE were isolated essentially as described by Mehrel *et al* (1990). The tissues were rinsed in PBS, and extracted for 10 min in 2% sodium dodecyl sulfate (SDS) extraction buffer (EB) [100 mM Tris, pH 8.5, 2% SDS, 20 mM dithiothreitol (DTT), 5 mM ethylenediamine tetraacetic acid (EDTA)], 5 ml per epidermis or 2 ml per forestomach, on a boiling water bath. The resulting suspensions were centrifuged for 10 min at 12,000*g*, the supernatants were discarded, and the pellets were re-extracted under the same conditions. Then the pellets were washed twice with 0.2% SDS EB (100 mM Tris, pH 8.5, 0.2% SDS, 20 mM DTT, 5 mM EDTA), resuspended in 5 ml 0.2% SDS EB, and centrifuged for 5 min through 3% Ficoll (M_r 400 × 10^3; Sigma, St. Louis, MO) in 0.2% SDS EB at 5000*g*. The pellet containing tissue debris and disrupted cells was discarded and the supernatant retained. The preparations from LKO mice (both epidermis and forestomach) usually contained a larger fraction of partially disrupted corneocytes, in which case the centrifugation speed was reduced to 2500*g* to increase the yield. The suspension overlying the Ficoll cushion was checked by phase contrast microscopy to confirm the presence of intact corneocytes. The forestomach preparations were contaminated with bacteria, which were removed by repeated centrifugation over a Ficoll cushion. The resulting isolated corneocytes were washed twice with 0.2% SDS EB, disrupted by sonicating for 3 × 10 s at 70% of maximum power on a Model 911001 sonicator (Kontes, Vineland, NJ), and collected by centrifugation for 10 min at 12,000*g*. The pellet was resuspended and washed twice with 0.2% SDS EB. CE fragments were separated from the remaining intact corneocytes by centrifuging through 3% Ficoll. This pellet was washed four times with 0.2% SDS EB, resuspended to a final protein concentration of 0.5–5 mg per ml in 0.2% SDS EB, and stored at 4°C for up to several months.

Electron microscopy of isolated CE Replicas of freeze dried/metal shadowed samples (Henderson and Griffiths, 1972) were prepared as described previously (Jarnik *et al*, 1998). In brief, a small drop of a suspension of isolated CE was adsorbed for 5 min on a 5 × 5 mm square of freshly cleaved mica, washed with PBS and DDW, and, after blotting off excess water, quenched in liquid nitrogen. The specimen was then placed on a copper block precooled in liquid nitrogen, transferred to a BAF 060 freeze-fracturing apparatus (Bal-Tec AG, Liechtenstein), and freeze-dried for 3 h at –100°C, 1 h at –80°C, and 1 h at –60°C. Unidirectional shadowing with 0.7 nm of tantalum–tungsten was performed at –100°C at an elevation angle of 30°, and overlaid by ≈10 nm of carbon, applied at an elevation angle of 90°. Replicas were floated on DDW, cleaned with 25% sodium hypochlorite, washed 10 times with DDW, and picked up on SPI electron microscope grids (Athena, U.K.).

For antibody labeling, CE were adsorbed to freshly glow discharged, collodion/carbon coated, copper grids as described above for mica, and subjected to the immuno-gold labeling procedure described above. Instead of staining/embedding in uranyl acetate/methylcellulose, the samples were freeze-dried and shadowed with tantalum–tungsten as described above, except that only 3–5 nm of carbon was applied as a protective film.

Micrographs were recorded on a Zeiss EM902 at nominal magnifications of 12,000–30,000×. Thickness measurements were made from enlarged prints at ≈203,000×, with the magnification calibrated relative to negatively stained catalase crystals (Wrigley, 1968). As the thickness of the shadowing metal was very low (about 0.7 nm), no correction was necessary for the thickness of the metal layer (Moor, 1959).

Scanning transmission electron microscopy was performed with the Brookhaven Biotechnology Resource instrument (Wall *et al*, 1998). Freeze-dried specimens were prepared according to Wall and Hainfeld (1986). Briefly, 20 μl of isolated CE (see above) were pelleted, the supernatant was removed, and the CE were resuspended in 20 μl of DDW by brief sonication. A 3 μl drop of tobacco mosaic virus suspension in DDW (100 μg per ml) was placed on a thin carbon film supported on a holey carbon film mounted on a titanium grid, and adsorbed for 1 min. After four washes with DDW, a 3 μl drop of CE suspension was adsorbed for 1 min and washed 10 times with DDW. Excess water was blotted off, and the grid was frozen by plunging into liquid nitrogen and dried overnight by gradually warming to –80°C under vacuum. The grid was then transferred under vacuum into the microscope for viewing. Digital images of 512 × 512 pixels were recorded. In the images used for mass measurements, the pixel step was either 1 or 2 nm.

Mass measurements were performed by image analysis (Jarnik *et al*, 1998), using the PIC-III program (Trus *et al*, 1996), with each measurement representing an average over a circular area, 40 nm in diameter. Backgrounds were subtracted and the calculations were completed according to standard procedures (Steven *et al*, 1983; Thomas *et al*, 1994). These data were calibrated with reference to tobacco mosaic virus particles on the same grid.

Amino acid analysis and mathematical modeling of protein compositions Amino acid analysis was performed on a System 6300 analyzer (Beckman, Palo Alto, CA) using the ninhydrin detection method as described previously (Jarnik *et al*, 1996). The amino acid compositions of isolated CE were modeled as sums of the amino acid compositions of candidate proteins by regression analysis. This analysis was performed using a program called AACOMP written by P. Gryzenia, which incorporates a data base of amino acid compositions (Jarnik *et al*, 1996).

RESULTS

The morphology of the outer layers of LKO mouse forestomach is shown in transverse thin section in **Fig 2*A***. The overall organization of this stratified tissue corresponds closely with that of wild-type (cf. **Fig 2*B***). The loricrin-containing granules (L-granules) normally seen in the cytoplasm and nucleus of granulocytes (**Fig 2*B***; cf. Leapman *et al*, 1997), however, are absent (**Fig 2*A***). In particular, a normal-looking CE is unmistakably present in LKO corneocytes (**Fig 2*A***, *insert*). The epidermis of newborn LKO mice is also morphologically similar to wild-type, apart from the absence of L-granules (data not shown).

Absence of L-granules and loricrin-specific labeling from LKO epithelia Labeling of thin sections of LKO mouse forestomach with antiloricrin antibodies was negative, confirming the absence of the loricrin epitope (**Fig 3*A***, cornified layer; **Fig 3*B***, granular layer). In a positive control with wild-type tissue processed in parallel, we observed labeling of the CE at the cell margins of corneocytes (**Fig 3*C***) and of L-granules in the granular layer (**Fig 3*D***). Similar labeling patterns were observed for newborn skin (data not shown).

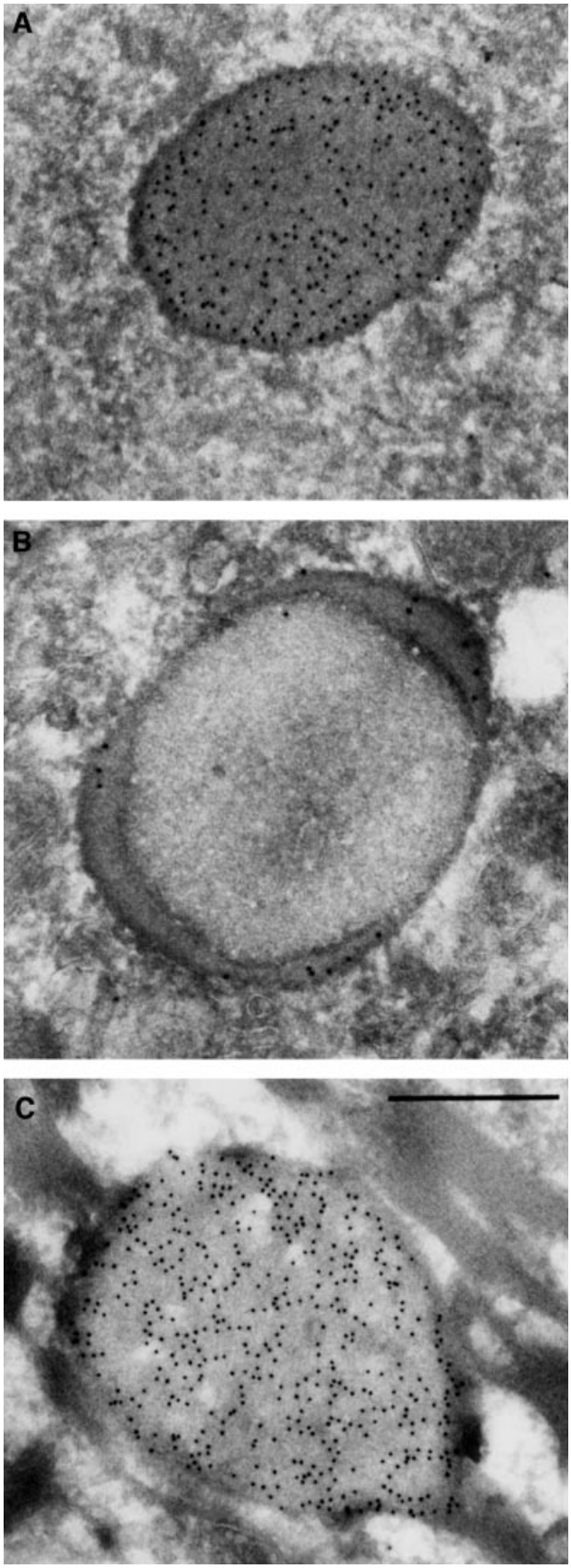

Figure 4. Immunolabeling of keratohyalin granules in newborn mouse skin. (*A*) A round L-granule in newborn epidermis of a Balb/c mouse, positively labeled for loricrin. (*B*) A composite granule in newborn epidermis for a mouse of the wild-type strain used to construct the LKO (129/SvEv-C57D1/6–FvB, wild-type for loricrin). The outer compartment labels positively for loricrin. In these granules, loricrin aggregates around another granule (see also **Fig 3*D***), instead of being unitary and round (cf. **Fig 4*A***). The nucleating granules may contain filaggrin, because granules with similar shapes and visual texture did label positively for filaggrin. (*C*) An oval F-granule in newborn epidermis (129/SvEv-C57D1/6–FvB, wild-type for loricrin), positively labeled for filaggrin. Previously described F-granules had stellate morphologies (Steven *et al*, 1990). Differences in granule morphology may represent a strain-specific property. Mixed 129/SvEv-C57D1/6–FvB mice were used in these experiments to facilitate genetic manipulation (Koch *et al*, 2000), whereas Balb/c mice were used in our earlier work (e.g., Jarnik *et al*, 1996). Composite granules have previously been reported in rat epidermis (Jessen *et al*, 1974), whose distinct compartments probably contained loricrin and filaggrin, respectively. In rat, however, they appeared to consist of small round L-granules embedded in larger, irregular, F-granules, unlike the present case (*B*). *Scale bar*: 0.5 μm.

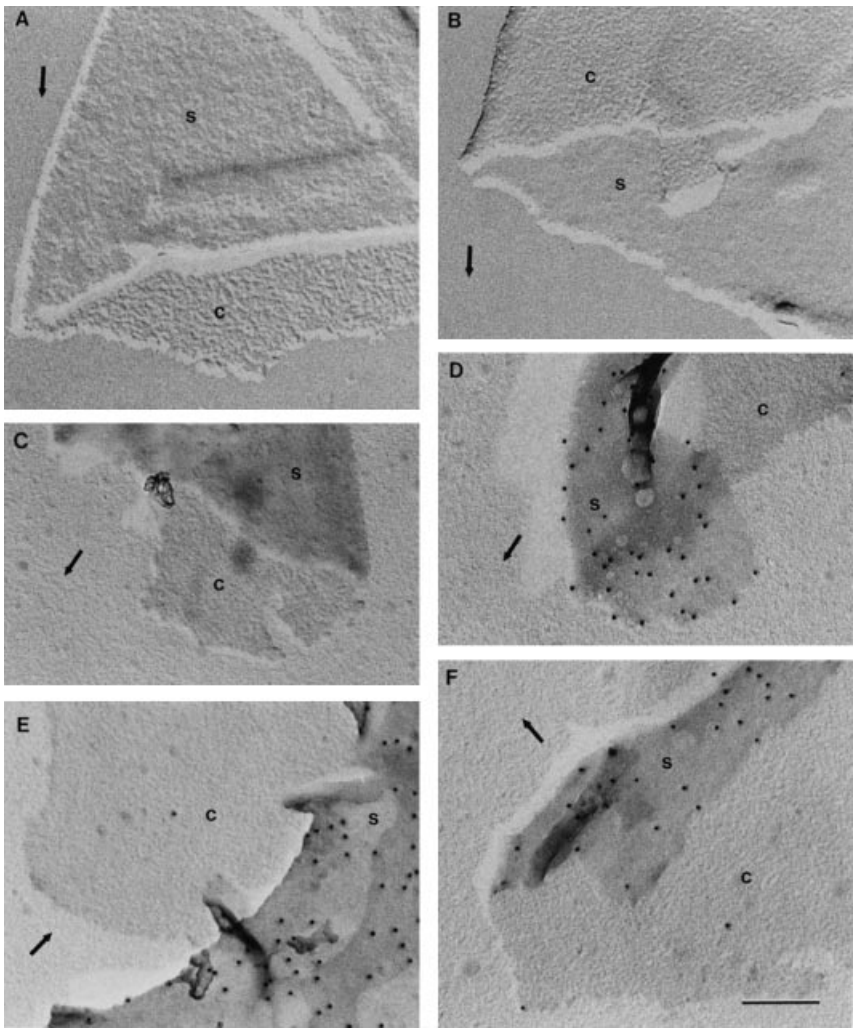

Figure 5. Electron micrographs of isolated cornified CE prepared by freeze-drying and metal shadowing. The samples are from the epidermis of a newborn LKO mouse (*A*) and a wild-type mouse (*B*), respectively. Note the two types of surface texture, coarse (c) and smooth (s). Similarly prepared samples of LKO mouse forestomach CE labeled with antiloricrin (*C*) and anti-SPRR1 (*E*) antibodies. Normal mouse forestomach CE labeled with antiloricrin (*D*)and anti-SPRR1 (*F*). Labeling was visualized by protein A complexed to 10 nm colloidal gold. c, cytoplasm. *Arrows* indicate the direction of the shadowing. *Scale bar*: 200 nm.

In contrast, the detection pattern for SPRR1, another marker for terminal differentiation in forestomach (Jarnik *et al*, 1996), appeared normal in LKO mice. In the granular layer, the anti-SPRR1 antibody labeled the cytoplasm diffusely, except for the granules; it also labeled the nuclei, except for regions occupied by condensed chromatin (**Fig 3*F*, *H***). In the cornified layer, sparse but very specific labeling of the cell periphery was observed (**Fig 3*E*, *G***).

F-granule and L-granule morphology As noted above, L-granules are absent in the epidermis of newborn LKO mice. They are present, however, in the parental strain from which these mice were derived, albeit with an altered morphology *vis-à-vis* L-granules previously reported in Balb/c mice (**Fig 4**). In this case, the L-granule appears to be the outer layer of a composite granule (**Fig 4*B***), not a unitary round granule as in Balb/c mice (**Fig 4*A***). The profilaggrin-containing F-granules also differ in shape in these mice, being smaller and rounder (**Fig 4*C***) than the less regular, stellate F-granules observed in Balb/c mice. Nevertheless, both proteins appear to be redistributed from granules and subsequently processed in the same way, as granulocytes mature into corneocytes (Steven *et al*, 1990).

Surface topography, physical thickness, and mass thickness of LKO CE Isolated CE were observed after freeze-drying and unidirectional shadowing (**Fig 5**). CE from normal epidermis present two distinct surfaces, corresponding to the smooth cytoplasmic side and the coarse extracellular side, respectively (Jarnik *et al*, 1998). On their coarse side, CE from LKO mice are essentially indistinguishable from wild-type, but their smooth side has a rougher texture than wild-type CE (cf. **Fig 5*A*, *B***). As expected, antiloricrin antibodies did not label isolated LKO CE (**Fig 5*C***), whereas in a positive control the smooth side of wild-type control CE was heavily labeled (**Fig 5*D***). Labeling with anti-SPRR1 antibody was confined to the smooth side of both LKO and wild-type CE, confirming the presence of this protein on the cytoplasmic side of both specimens (**Fig 5*E*, *F***).

The thickness of isolated CE was measured from the lengths of the shadows cast from the edges of specimens contrasted by unidirectional shadowing. The resulting data show that LKO CE are indistinguishable in thickness from wild-type CE, at 14.9 ± 1.0 nm and 15.6 ± 1.1 nm, respectively. The uncertainties quoted are standard deviations and more than 50 measurements were made in each case.

To determine the mass per unit area of LKO CE, we recorded and analyzed dark-field scanning transmission electron micrographs of unstained freeze-dried specimens (Wall and Hainfeld, 1986) of LKO and normal CE (**Fig 6*A*, *C***). Their values of mass per unit area are very similar at 7.3 ± 0.9 kDa per nm^2 (with more than 150 measurements) and 7.8 ± 0.8 kDa per nm^2 (current data, cf. Jarnik *et al*, 1998), respectively (**Fig 6*B*, *D***).

Amino acid composition of LKO CE In CE isolated from newborn skin and adult forestomach of LKO mice, the two amino acids that predominate in wild-type CE – Gly and Ser – are less prominent but still relatively high (**Table I**). These reductions are more pronounced for epidermal CE, which also have markedly higher contents of Pro and GlX (Glu or Gln) residues.

The implications of these data for the protein compositions of the respective CE were explored by mathematical modeling (Steven and Steinert, 1994). For this approach to be valid, the

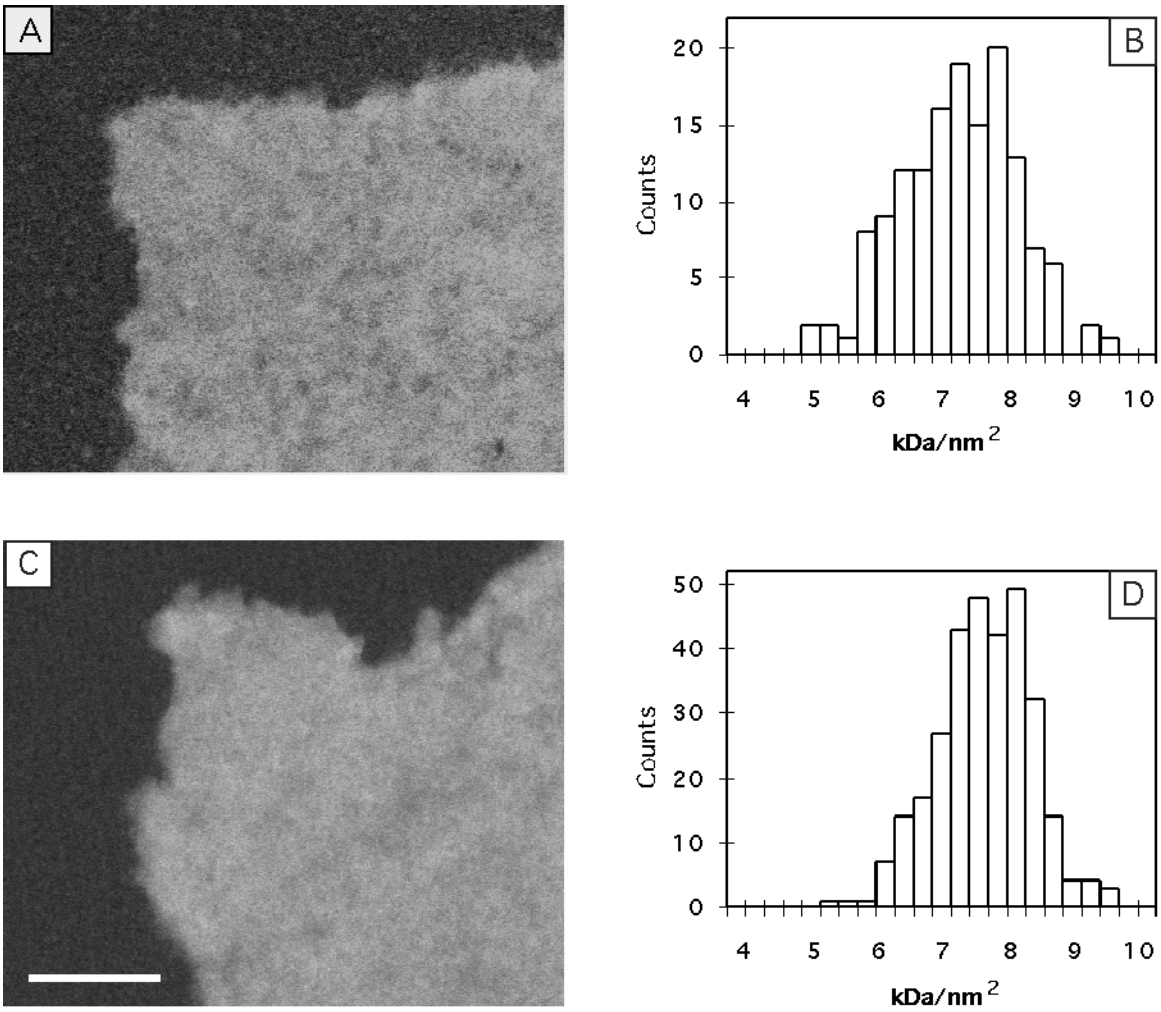

Figure 6. Dark-field scanning transmission electron microscopy of cornified CE isolated from LKO and wild-type mice. The samples are from newborn epidermis (*A*, LKO; *C*, wild-type) and were prepared by freeze-drying without shadowing or staining. *Scale bar*: 100 nm. Using tobacco mosaic virus as an internal mass standard, these images were used to make measurements of mass per unit area. The respective histograms are shown in (*B*) and (*D*).

components should be limited in number, and the CE preparations must have low levels of contaminants. The main conclusion to emerge from these calculations was that the predicted SPRR content of epidermal CE was found to increase approximately 3-fold from ≈9% to 25%–30% in LKO mice. (The three subclasses of SPRRs, having rather similar amino acid compositions, were not distinguished in this analysis.) In contrast, the SPRR content of forestomach CE, already at this level (Jarnik *et al*, 1996), did not change significantly. These numbers were robust, being essentially unaffected by the inclusion of various other components in the fits.

The second conclusion was that LKO CE from both tissues – and particularly epidermis – contain large amounts of proteins that are rich in Gly and Ser. When keratins K1 and K10, which have Gly- and Ser-rich domains, were included in the fits, elevated levels of these proteins were registered. We view these figures with suspicion, however, because, on attempting to detect keratins on isolated CE by immuno-gold electron microscopy, we observed essentially no labeling (data not shown). A second cause for concern was that the residuals for fits obtained with loricrin omitted but keratin included are many-fold higher than we obtained for wild-type CE (with loricrin included). Thus these keratin estimates are probably exaggerated, representing the attempt of the regression analysis to satisfy the requirement for a high content of Gly and Ser from the only source on offer. It appears that these CE contain some other Gly- and Ser-rich protein(s) substituting for loricrin. The inferred substitutions probably differ between epidermal CE and forestomach CE, as their reduced amino acid compositions (i.e., with the SPRR contribution subtracted) differ significantly: epidermis, 25% Gly, 21% Ser, 15% GlX; forestomach, 42% Gly, 20% Ser, 8% GlX.

None of the above conclusions was affected by including repetin, a recently discovered epidermal protein (Krieg *et al*, 1997) in the fits; hence we infer that repetin appears not to be a major CE component.

DISCUSSION

The cornified CE, a late product of terminally differentiating keratinocytes in squamous epithelia, is thought to play a vital role in protecting the underlying tissues against challenges posed by their environment (Hohl, 1990; Roop, 1995). Its formation proceeds in a highly organized manner and genetic disorders that affect this process (review: Ishida-Yamamoto and Iizuka, 1998) can have severe phenotypic consequences, as in lamellar ichthyosis, Vohwinkel syndrome (Maestrini *et al*, 1996; Korge *et al*, 1997; Ishida-Yamamoto *et al*, 1997; Suga *et al*, 2000). At 60%–80% mass fraction, loricrin is the major component of all native CE (as distinct from CE from cultured cells) characterized to date (Hohl *et al*, 1993; Steven and Steinert, 1994; Steinert and Marekov, 1995; Jarnik *et al*, 1996, 1998). In these circumstances, we expected that elimination of loricrin might be potentially disastrous for the

Table I. Amino acid compositions of cornified cell envelopes and some potential components (%, wt/wt)

	Newborn epidermis CE		Forestomach CE									
	LKO	wild-type	LKO	wild-type	Loricrin	Involucrin	Filaggrin[a]	Keratin[b]	Cystatin A	Elafin	SPRR[c]	Repetin
G	18.8 ± 1.1	50.3	34.1 ± 1.1	40.4	59.4	5.0	15.7	24.5	7.9	12.7	2.3	11.6
S	17.1 ± 1.7	21.8	16.6 ± 1.1	18.8	24.1	3.2	19.8	14.1	4.9	4.6	6.0	12.8
Y	2.3 ± 0.1	5.0	3.4 ± 0.1	4.0	5.6	0.9	0.0	3.7	2.9	0.0	2.0	2.8
Q/E	14.6 ± 0.9	5.9	9.6 ± 0.4	7.9	3.4	41.3	21.4	12.0	16.7	9.2	17.2	26.4
T	3.5 ± 0.3	1.3	3.1 ± 0.4	2.7	1.6	1.9	0.0	3.5	5.9	4.6	6.3	3.8
P	11.4 ± 2.3	5.1	11.7 ± 0.2	10.2	1.8	9.1	2.8	0.9	2.9	12.7	37.2	2.6
R	4.7 ± 1.3	1.4	2.4 ± 0.0	1.8	0.0	1.9	12.5	6.0	2.9	3.5	0.5	7.5
K	4.9 ± 0.4	3.4	4.4 ± 0.2	3.8	2.0	10.0	0.0	3.7	11.8	12.6	10.4	4.9
V	3.4 ± 0.5	1.5	3.0 ± 0.1	2.6	1.1	2.1	3.6	3.6	5.9	13.7	5.9	1.5
A	3.5 ± 0.3	1.1	2.4 ± 0.1	1.8	0.4	1.3	8.9	3.7	4.9	4.6	2.0	1.1
H	2.8 ± 0.6	1.1	2.6 ± 1.1	1.3	0.4	5.5	8.9	0.4	1.0	0.0	2.3	9.0
L	4.8 ± 0.8	0.8	1.8 ± 0.1	1.2	0.0	12.2	0.4	7.7	9.8	3.5	0.4	3.0
I	1.9 ± 0.4	0.2	1.3 ± 0.1	0.8	0.0	0.9	0.0	3.6	4.9	4.6	1.7	0.4
M	0.4 ± 0.4	0.1	0.5 ± 0.6	0.1	0.2	1.1	0.0	1.2	3.9	2.3	1.2	0.3
F	2.4 ± 1.3	0.3	0.4 ± 0.5	0.4	0.0	0.4	0.8	3.3	3.9	2.3	0.3	2.0
D/N	3.6 ± 1.9	0.7	3.1 ± 0.1	2.0	0.0	3.2	5.2	7.9	9.9	9.3	4.0	10.2

[a]Human protein: if not stated otherwise, mouse proteins were used in these calculations.
[b]Mean of keratins K1 and K10.
[c]Mean of SPRR1, SPRR2, and SPRR3. The LKO data represent the averages ± standard deviations for three and two independent determinations, for epidermis and forestomach, respectively.

organism on account of profound changes in CE structure. In contrast, loricrin-lacking mice survive and show only a mild phenotype during a brief neonatal period: the formation of skin barrier function is delayed during embryonic development, but an impaired barrier function is not detected in neonates (Koch *et al*, 2000). We have now found that LKO corneocytes from both epidermis and forestomach have CE that are close to wild-type in many of their properties.

LKO cell envelopes have conserved gross structure but altered substructure In LKO mice, the epidermis and forestomach epithelium are both morphologically normal apart from the absence of L-granules. In particular, a normal-looking CE was observed at the periphery of corneocytes (**Fig 2*A***). Moreover, the distribution of SPRR1 – a marker for terminal differentiation (Hohl *et al*, 1995) and an integral component of forestomach CE (Jarnik *et al*, 1996) – resembled that of wild-type. In thickness and projected density, isolated LKO CE are essentially the same as wild-type CE.

Nevertheless, there are differences at a substructural level. The cytoplasmic surface of the CE, which is coated with loricrin and is extremely smooth in the case of wild-type mice (Jarnik *et al*, 1998), is visibly rougher in texture in LKO CE (cf. **Fig 5*A***, ***B***). The unchanged thickness and projected density of the CE imply that the amount of protein per unit area and its packing density are conserved, to a good approximation. The observed changes in amino acid composition (**Table I**), however, confirm that the protein constituents have changed markedly.

In light of these observations, the conserved thickness of the CE (15 nm) is noteworthy. Two mechanisms have been proposed for the uniform thickness of loricrin-containing CE: that it reflects either the dimensions of the loricrin molecule or the limited reach of a membrane-bound transglutaminase (Jarnik *et al*, 1998). The latter mechanism readily applies to LKO CE, with the enzyme simply incorporating different substrates into the assembling CE. The former mechanism requires that the molecule(s) that substitute for loricrin as building-blocks should have similar dimensions to loricrin.

Altered composition of LKO CE Compositional analysis by mathematical modeling indicated a 3-fold increase in net content of SPRRs in LKO mice (the three subtypes of SPRRs could not be distinguished by this technique). Consistent with this observation, it has been determined that two members of the SPRR2 subfamily are upregulated by factors of 5 and 2, respectively, in newborn mouse skin (Koch *et al*, 2000). As noted above, LKO CE labeled positively with anti-SPRR1 antibodies both *in situ* and *in vitro*.

Which proteins contribute the remaining 70% or so mass fraction of LKO CE? Repetin is upregulated by a factor of 2 in newborn LKO skin (Koch *et al*, 2000) but our compositional analysis suggests that it is not a major component. Major components should be rich in Gly and Ser and, in the case of newborn epidermal CE, in Glu/Gln. Keratins are likely contributors (see Steinert and Marekov, 1995; 1997; Candi *et al*, 1998) but we consider them unlikely to predominate, for reasons given above. After the compositional analysis was completed, we began to search for Gly/Ser-rich proteins that were upregulated in LKO epidermis. This has resulted in the discovery of a new family of Gly/Ser-rich proteins that are putative CE components (D.R.R., unpublished results).

Cell envelope assembly in the absence of loricrin Taken together, these results indicate that the organism is able to mobilize a back-up system in the event of failure to synthesize loricrin in the epidermis or other epithelia in which it is normally expressed. We note that elimination of another CE component, involucrin, has no effect on mice (Djian *et al*, 2000), indicating that this protein is either dispensible or substituted when unavailable, as in the case of loricrin. In consequence of the loricrin substitution, a CE with native-like properties but altered composition is assembled. Our data imply that it should include, as a major component, protein(s) that are similar in amino acid composition to loricrin (see above). As such, they might be the products of other as yet unknown genes stemming from the same ancestor as loricrin, as occurs in the SPRR family (Hohl *et al*, 1995; Steinert *et al*, 1998). Genes clustering in the epidermal differentiation complex (Mischke *et al*, 1996) would be likely candidates.

In this context, we note that loricrin is not expressed in some cornifying tissues, including some internal epithelia (Hohl *et al*, 1993). Although CE from such sources have not been characterized in depth, it may be that the genes coding for their major proteins are upregulated in the epidermis and forestomach of LKO mice. Another situation in which loricrin would normally enter the CE but does not do so is represented by transgenic mice expressing a loricrin variant with an altered C-terminal portion, simulating mutants that have been observed in Vohwinkel's syndrome (Maestrini *et al*, 1996). When the transgene is expressed in mice, the mutant protein is transported into the nucleus (Suga *et al*, 2000).

Nevertheless, the CE of transgenic mice with a −/− background appear normal in transverse thin section electron micrographs (Suga *et al*, 2000; MJ, unpublished observations), suggesting that loricrin is again replaced by other components. A similar mutant loricrin expressed in the skin of human patients with the genetic condition progressive symmetric erythrokeratoderma (PSEK) also enters the nucleus (Ishida-Yamamoto *et al*, 2000): in this case, it has been reported that involved epidermis of PSEK patients has abnormally thin CE (Ishida-Yamamoto *et al*, 1997).

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