Comment

The Complexity and Redundancy of Epithelial Barrier Function

Peter M. Steinert

Laboratory of Skin Biology, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, Maryland 20892-2752

Recent analyses of mummified or long-frozen human specimens have revealed that the outermost layer of our skin, the epidermis, is one of the most durable soft tissues of the body. The major function of the epidermis is to provide a protective barrier, and recent work now suggests that evolution has devoted an enormous amount of energy to providing this critical function. Several recent publications and three papers in this issue have shed new light on the complexity and redundancy of barrier function in the epidermis.

During terminal differentiation, stratified squamous epithelia, including internal wet and external dry epithelia, such as the epidermis, make a specialized structure termed the cell envelope (CE)[1] (Reichert et al., 1993; Nemes and Steinert, 1999). This is a key aspect of barrier function, as it provides a flexible physical protection against trauma and wear-and-tear, and a platform for organized layers of lipids, which in turn afford water barrier function. The CE is an insoluble proteinaceous layer 10-nm thick and of uniform density (Jarnik et al., 1998) deposited subjacent to the plasma membrane. The constituent proteins become cross-linked together by transglutaminases. As the isopeptide bond formed by these enzymes cannot be cleaved in vertebrate cells, this affords a clever method of forming a permanent, stable, insoluble macromolecular protein complex.

To date, cell biological, biochemical, and protein sequencing studies have shown that at least 20 proteins are used to assemble CEs. How CE assembly proceeds during differentiation in epithelia is still speculative, but extant data have now provided the following working model (Fig. 1). An early event upon initiation of terminal differentiation is the expression of envoplakin and periplakin (Ruhrberg et al., 1996, 1997) that become associated together at, and in between, desmosomes. A short time later, involucrin is expressed, which was the first described CE precursor (Rice and Green, 1979). Data from this laboratory have suggested that it binds spontaneously to membranes in a Ca^{2+} dependent manner (Nemes et al., 1999a). Another early expression product is the transglutaminase 1 enzyme (Kim et al., 1995), which self-assembles onto membranes by way of its acyl lipid adducts. As localized Ca^{2+} concentrations rise, the enzyme cross-links involucrin to form a two-dimensional head-to-head and head-to-tail oligomeric mesh, and involucrin to envoplakin (and perhaps periplakin; LaCelle et al., 1998; Steinert and Marekov, 1999). Shortly later, members of the small proline rich (SPR) family of proteins become cross-linked to both envoplakin and involucrin. Eventually, this amalgam spreads across the inner surface of the plasma membrane, including the desmosomes, so that many cell junctional proteins, including desmoplakin, annexin I, and keratin intermediate filaments become cross-linked too. Together, these form a uniform layer that serves as a template or scaffold for subsequent maturation or reinforcement stages of CE assembly (Yaffe et al., 1992; Ruhrberg et al., 1997; Steinert and Marekov, 1999). This process varies between epithelia, presumably in concert with tissue-specific requirements. For example, in the epidermis, the major CE reinforcement proteins are loricrin cross-linked together with lesser amounts of SPRs; in oral epithelia, the CEs are ~70% SPRs and ~10% loricrin; and in the hair cuticle, cysteine-rich proteins are used. In addition, in the epidermis, transglutaminase 1 attaches ceramide lipids by ester linkages to involucrin, envoplakin, and periplakin for water barrier function (Marekov and Steinert, 1998; Nemes et al., 1999b).

Based on this hypothetical model of CE structure and assembly, which predicts a key role for involucrin, what would be the expected phenotype of an involucrin knockout mouse? The answer, presented by Djian et al. (2000, this issue) is: none. Surprisingly, by several tests, the mice were phenotypically normal. Two tested CE precursor proteins were expressed in normal amounts. However, it would be of interest to also examine the levels of envoplakin, periplakin, and multiple members of the SPR family, which are typically expressed in stratified squamous epithelia, or repetin or trichohyalin, commonly expressed in the CEs of toughened epithelia, including the epidermis. Some of these are known to be upregulated in response to epidermal injury. Previous studies have shown that overexpression of involucrin in transgenic mice leads to an abnormal epithelial phenotype (Crish et al., 1993). Thus, the observation of Djian et al. (2000) implies that compensatory mechanisms exist in epithelia that can overcome the absence of involucrin.

Address correspondence to Peter M. Steinert, Building 6, Room 425, NIAMS, NIH, Bethesda, MD 20892-2752. Tel.: 301-496-1578. Fax: 301-402-2886. E-mail: pemast@helix.nih.gov

[1]*Abbreviations used in this paper:* CE, cell envelope; SPR, small proline rich.

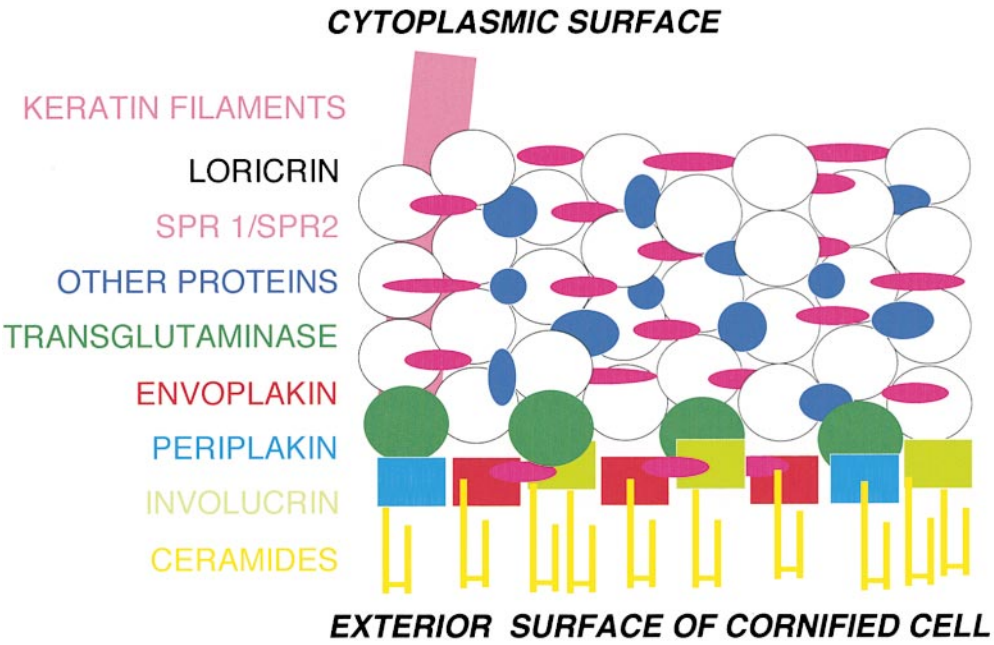

Figure 1. A model of the epidermal CE. Loricrin (white circles) is the major CE protein, admixed with small amounts of SPRs 1 and 2 (pink ellipsoids). Together, these constitute ~85% of the CE and represent the final reinforcement stage of CE assembly. However, the number of layers of loricrin remains unresolved. It may be one (Jarnik et al., 1998), in which case the axial ratio of each loricrin molecule should be ~1:4, or at least two and up to four, if the loricrin molecules are spherical. In the case of the loricrin$^{-/-}$ mice (Koch et al., 2000), it is anticipated that SPRs and some other proteins (blue circles), such as repetin and trichohyalin, compensate for absent loricrin. It is speculated that the loricrin–SPR complex is cross-linked onto a scaffold composed of several proteins cross-linked together at or near the plasma membrane, including keratin filaments (long red rod), envoplakin (red box), periplakin (blue box), and involucrin (green box), perhaps by transglutaminase 1 (green circles). If these do form a redundant scaffold, this could explain the individual phenotypes of the involucrin$^{-/-}$ (Djian et al., 2000) and envoplakin$^{-/-}$ (Määttä, A., and F. Watt, personal communication) mice, as one may compensate for the absence of the other. The yellow rods denote ceramide lipids that become ester-linked by transglutaminase 1 onto involucrin, envoplakin, and periplakin. These replace the plasma membrane of the cornified cell and are important for interdigitation with extracellular lipids that together confer water barrier function. Modified from Nemes and Steinert (1999).

Further, young envoplakin$^{-/-}$ mice also have no discernable phenotype (Määttä, A., and F. Watt, personal communication). Does this mean that envoplakin and involucrin are redundantly used for CE scaffold formation, and that one can substitute for the other? The obvious experiment of breeding the two knockout mouse lines is in progress.

Loricrin and SPRs have the dubious distinctions of having the highest contents of glycine or proline residues, respectively, of any other proteins. Whereas the amount of SPRs in CEs varies from <1% in human trunk epidermis, to >10% in palmaplantar and lip epidermis, the sum of loricrin plus SPRs seems to remain constant at ~85% (Steinert et al., 1998). Together, these two are thought to form a flexible, tough, cross-linked layer that forms the bulk of the CE barrier in human epidermis. Loricrin thus constitutes ~10% of the mass of the epidermis.

What would be the expected phenotype of loricrin knockout mouse? The answer, presented in a paper by Koch et al. (2000, this issue), reveals that there is almost no phenotype. At birth, there is mild epidermal erythema and the CEs are fragile as demonstrated by experimental fragmentation. However, the CEs and skin condition improve within a few days after birth, apparently coincident with upregulation of certain members of the SPR family (mouse *Sprr*2D and *Sprr*2H), and repetin. Thus, these and perhaps other proteins seem to have compensated for the absence of loricrin. One interesting aspect of this study is that the content of glycine in CEs in the loricrin$^{-/-}$ mice was similar to that of wild-type (Steven, A., personal communication). Does this mean that other loricrin-like CE proteins exist which, while normally silent, are upregulated in loricrin$^{-/-}$ mice? Two ways to explore these possibilities are: examine mRNAs from the loricrin$^{-/-}$ mice for novel expression products; and undertake the somewhat laborious sequencing of CEs from the loricrin$^{-/-}$ mice, as has been done for normal epidermal CEs.

In addition, mutations in the human loricrin gene occur in the rare ichthyosis disorders, Vohwinkel's Syndrome (Meastrini et al., 1996), and progressive symmetric erythrokeratoderma (Ishida-Yamamoto et al., 1997). They arise from similar nucleotide deletions in the latter half of the coding sequences, causing a frameshift that converts a glycine-rich segment into an arginine-rich one, and loss of residues involved in cross-linking. These disorders cause phenotypes with diminished barrier function, generalized scaling, and epidermal constrictions on the digits. In an adjoining article, Suga et al. (2000) have successfully recreated this phenotype in transgenic mice. However, they note the mutant protein relocates to the nucleus instead of the CE because it possesses a nuclear localization signal. Mating with the above loricrin$^{-/-}$ mice revealed that the phenotype observed in the mutant mice is probably caused by general interference with transcriptional function, that is, a gain of function rather than a dominant negative effect per se. Further studies will be needed to address more general questions pertaining to the role of loricrin in barrier function. How is this incredibly insoluble protein dispatched to the cell periphery for CE assembly? What is the function of the extraordinarily long runs of glycines that are also present on the keratins 1, 2e, and 10, which form the bulk of the mass of the epidermal keratinocyte?

Several hundred million years ago, our early vertebrate ancestors crawled out of swamps to begin a new era of life on land. One of their earliest adaptations to this new envi-

ronment was improved barrier function of their skin. These three new papers, together with a number of other recent studies, suggest that the subsequent evolution of barrier function has not only been bewilderingly complex, but compensatory backup systems have been built in to substitute in the absence of one or other of the major players.

Submitted: 8 September 2000
Accepted: 13 September 2000

References

Crish, J.F., J.M. Howard, T.M. Zaim, S. Murthy, and R.L. Eckert. 1993. Tissue-specific and differentiation-appropriate expression of the human involucrin gene in transgenic mice: an abnormal epidermal phenotype. *Differentiation* 53:91–100.

Djian, P., K. Easley, and H. Green. 2000. Targeted ablation of the murine involucrin gene. *J. Cell Biol*. 151:381–387.

Ishida-Yamamoto, A., J.A. McGrath, H. Lam, H. Iizuka, R.A. Friedman, and A.M. Christiano. 1997. The molecular pathology of progressive symmetric erythrokeratoderma: a frameshift mutation in the loricrin gene and perturbation in the cornified cell envelope. *Am. J. Hum. Genet*. 61:581–589.

Jarnik, M., M.N. Simon, and A.C. Steven. 1998. Cornified cell envelope assembly: a model based on electron microscopic determinations of thickness and projected density. *J. Cell Sci*. 111:1051–1060.

Kim, S.Y., S.-I. Chung, K. Yoneda, and P.M. Steinert. 1995. Expression of transglutaminase 1 in human epidermis. *J. Invest. Dermatol.* 104:211–217.

Koch, P.J., P.A. de Viragh, E. Sharer, D. Bundman, M.A. Longley, J. Bickenbach, Y. Kawachi, Y. Suga, Z. Zhou, M. Huber et al. 2000. Lessons learned from loricrin-deficient mice: compensatory mechanisms maintaining skin barrier function in the absence of a major cornified envelope precursor. *J. Cell Biol*. 151:389–400.

LaCelle, P.T., A. Lambert, M.C. Ekambaram, N.A. Robinson, and R.L. Eckert. 1998. In vitro cross-linking of recombinant human involucrin. *Skin Pharmacol. Appl. Skin Physiol*. 11:14–226.

Meastrini, E., A.P. Monaco, J.A. McGrath, A. Ishida-Yamamoto, C. Camisa, A. Hovnanian, D.E. Weeks, M. Lathrop, J. Uitto, and A.M. Christiano. 1996. A molecular defect in loricrin, the major component of the cornified cell envelope, underlies Vohwinkel's syndrome. *Nat. Genetics.* 13:70–77.

Marekov, L.N., and P.M. Steinert. 1998. Ceramides are bound to structural proteins of the human foreskin epidermal cornified cell envelope. *J. Biol. Chem.* 273: 17763–17770.

Nemes, Z., and P.M. Steinert. 1999. Bricks and mortar of the epidermal barrier. *Exp. Mol. Med*. 31:5–19.

Nemes, Z., L.N. Marekov, and P.M. Steinert. 1999a. Involucrin cross-linking by transglutaminase 1: binding to anionic membranes directs residue specificity. *J. Biol. Chem.* 274:11013–11021.

Nemes, Z., L.N. Marekov, L. Fesus, and P.M. Steinert. 1999b. A novel function for the transglutaminase 1 enzyme: attachment of long chain ω-hydroxyceramides to involucrin by ester bond formation. *Proc. Natl. Acad. Sci. USA*. 96:8402–8407.

Reichert, U., S. Michel, and R. Schmidt. 1993. The cornified envelope. *In* Molecular Biology of the Skin. Darmon, M., and M. Blumenberg, editors. Academic Press Inc., New York, pp. 107–150.

Rice, R.H., and H. Green. 1979. Presence in epidermal cells of a soluble protein precursor of the crosslinked envelope: activation of the crosslinking by calcium ions. *Cell.* 18:681–694.

Ruhrberg, C., M.A. Hajibagheri, M. Simon, T.P. Dooley, and F.M. Watt. 1996. Envoplakin, a novel precursor of the cornified envelope that has homology to desmoplakin. *J. Cell Biol*. 134:715–729.

Ruhrberg, C., M.A. Hajibagheri, D.A. Parry, and F.M. Watt. 1997. Periplakin, a novel component of cornified envelopes and desmosomes that belongs to the plakin family and forms complexes with envoplakin. *J. Cell Biol*. 139: 1835–1849.

Steinert, P.M., and L.N. Marekov. 1999. Initiation of assembly of the cell envelope barrier structure of stratified squamous epithelia. *Mol. Biol. Cell* 10: 4247–4261.

Steinert, P.M., T. Kartasova, and L.N. Marekov. 1998. Biochemical evidence that small proline rich proteins and trichohyalin function in epithelia to modulate the biomechanical properties of their cornified cell envelopes. *J. Biol. Chem*. 273:11758–11769.

Suga, Y., M. Jarnik, P.S. Attar, M.A. Longley, D. Bundman, A.C. Steven, P.J. Koch, and D.R. Roop. 2000. Transgenic mice expressing a mutant form of loricrin reveal the molecular basis of the skin diseases Vohwinkel syndrome and progressive symmetric erythrokeratoderma. *J. Cell Biol*. 151:401–412.

Yaffe, M.B., H. Beegen, and R.L. Eckert. 1992. Biophysical characterization of involucrin reveals a molecule ideally suited to function as an intermolecular cross-bridge of the keratinocyte cornified envelope. *J. Biol. Chem*. 267: 12233–12238.