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Bricks and mortar of the epidermal barrier

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Abbreviations: CE, cornified cell envelope; KIF, keratin intermediate filaments; SPR, small proline rich protein; TGase, transglutaminase

Abstract

A specialized tissue type, the keratinizing epithelium, protects terrestrial mammals from water loss and noxious physical, chemical and mechanical insults. This barrier between the body and the environment is constantly maintained by reproduction of inner living epidermal keratinocytes which undergo a process of terminal differentiation and then migrate to the surface as interlocking layers of dead stratum corneum cells. These cells provide the bulwark of mechanical and chemical protection, and together with their intercellular lipid surroundings, confer water-impermeability. Much of this barrier function is provided by the cornified cell envelope (CE), an extremely tough protein/lipid polymer structure formed just below the cytoplasmic membrane and subsequently resides on the exterior of the dead cornified cells. It consists of two parts: a protein envelope and a lipid envelope. The protein envelope is thought to contribute to the biomechanical properties of the CE as a result of cross-linking of specialized CE structural proteins by both disulfide bonds and N^{ε}-(γ-glutamyl)lysine isopeptide bonds formed by transglutaminases. Some of the structural proteins involved include involucrin, loricrin, small proline rich proteins, keratin intermediate filaments, elafin, cystatin A, and desmosomal proteins. The lipid envelope is located on the exterior of and covalently attached by ester bonds to the protein envelope and consists of a monomolecular layer of ω-hydroxy-ceramides. These not only serve of provide a Teflon-like coating to the cell, but also interdigitate with the intercellular lipid lamellae perhaps in a Velcro-like fashion. In fact the CE is a common feature of all stratified squamous epithelia, although its precise composition, structure and barrier function require-ments vary widely between epithelia. Recent work has shown that a number of diseases which display defective epidermal barrier function, generically known as ichthyoses, are the result of genetic defects of the synthesis of either CE proteins, the transglutaminase 1 cross-linking enzyme, or defective metabolism of skin lipids.

Introduction

Protection of the body from dehydration and noxious physical, mechanical and chemical insults from the environment is essential for terrestrial life. All beings from bacteria to plants and humans protect themselves by some form of barrier. For mammals the outermost bulwark of this defense-line are layers of terminally differentiated dead cornified cells on the surface of the epidermis of the skin.

Akin to a wall built from bricks and mortar, the cornified layer also consists of hard building blocks (the individual corneocytes) stuck together with space-filling mortar (intercorneocyte lipids). Barrier function of normal epidermis depends on the quality of its bricks and mortar. The building blocks of the epidermal barrier are formed during the complex terminal differentiation program from inner living dividing basal keratinocytes, culminating in the formation flattened cornified cells (corneocytes) which, as they are moved toward the surface, are eventually sloughed by abrasion. Each individual corneocyte consists largely of tightly bundled keratin filaments aligned roughly parallel to the skin surface (80-90% of total mass) encased in a sturdy bag termed the cornified cell envelope (CE) (about 10% of total mass). The CE is extremely insoluble, ~15 nm thick, and is composed of two major parts. The protein envelope (~10 nm thick) is formed by covalent cross-linking of specific structural proteins by sulfhydryl oxidases and transglutaminases (TGases) (Hohl, 1990; Polakowska and Goldsmith, 1991; Reichert *et al*., 1993; Simon, 1994; Eckert *et al*., 1997). This is coated by the lipid envelope which is a ~5 nm thick layer of lipids that are covalently attached to exterior of the protein envelope (Wertz and Downing, 1991). The lipid envelope creates cohesion between the cornified cells and the surrounding intercellular lipids, and may be essential for alignment of these lipids into lamellae (Swartzendruber *et al*., 1989; Wertz *et al*., 1989a).

This review describes our current knowledge and models of the composition and formation of the stratum corneum barrier. We also report current information on

the molecular bases of certain inherited diseases affecting the barrier function.

The cornified cell envelope

For morphologists, the CE is an electron dense band located just beneath the keratinocyte plasma membrane that first appears in the most superficial granular or transitional cells of terminally differentiating stratified squamous epithelia (Brody, 1969; Hashimoto, 1969; Ishida-Yamamoto and Iizuka, 1995) and gradually increases in thickness and density. Mature CEs cover and incorporate desmosomal attachment plaques and utilize proteins of those for CE assembly (Steinert and Marekov, 1995; Robinson *et al.*, 1997; Steinert and Marekov, 1997) forming a morphologically uniform layer about 15 nm thick in the terminally differentiated dead cells (Jarnik *et al.*, 1998).

The biochemist uses the term CE for the most insoluble fraction from stratified squamous epithelia, referring to the mass remaining after exhaustive removal of keratins, lipids and other solubilizable components by detergents, reducing agents (e.g. 2-mercaptoethanol), and concentrated chaotropic agents (urea, guanindine-HCl) (Steinert and Marekov, 1995). The extreme insolubility of CE *in vivo* is a result of macromolecular polymerization of various keratinocyte proteins by both disulfide and isopeptide bond formation. However, the disulfide bonds created by sulfhydryl oxidase (Yamada *et al*., 1987) are lost during CE isolation procedures in order to remove the quantita-tively overwhelming keratin mass (Hohl *et al*., 1991a). Therefore, the *in vitro* analyzed CE means in practice a highly insoluble macromolecular protein polymer held together by isopeptide bonds (Hohl, 1990; Polakowska and Goldsmith, 1991; Reichert *et al*., 1993; Simon, 1994; Eckert *et al*., 1997), and may thus differ somewhat in structure and composition from the *in vivo* structure. Nevertheless isolated CE fragments from many types of epithelia appear as sheets uniformly 15 nm thick (Jarnik *et al*., 1998). The isopeptide bonds are formed by TGases which release ammonia from glutamine residues of substrate proteins to create a thioester acyl-enzyme intermediate, and consecutively transfer the acyl residue to primary amines (Folk and Finlayson, 1977). In biological systems the primary amine is either provided by the ε-amino group of a protein-bound lysine thereby creating an N^{ε}-(γ-glutamyl)lysine isopeptide bond, or by a diamine (most commonly spermidine) thereby forming an N^1,N^8-bis(γ-glutamyl)spermidine bond (Lorand and Conrad., 1984). Although the abundance of spermidine cross-linking is minuscule in CE formation, and has been poorly investigated, this pathway might be more significant in certain pathological conditions such as psoriasis (Martinet *et al*., 1990).

The TGases

Human TGases constitute an evolutionarily related family of Ca^{2+} dependent enzymes. Seven members of the TGase family have been identified in the human genome so far, which are listed in Table 1. Four of these,

Table 1. Human transglutaminases.

Enzyme	Gene locus	Size (kDa)	Expression	Functions	Proteolytic processing?
TGase 1 (keratinocyte TGase)	14q11.2	92	epidermis	CE formation	Yes
TGase 2 (tissue TGase)	20q11.2	77	ubiquitous	Apoptosis?	No
TGase 3 (epidermal TGase)	20q11.2	77	epidermis	CE formation	Yes
TGase 4 (prostate TGase)	3p21-22	75	prostate	clotting of the seminal plasma	Yes
Factor XIII	6p24-25	80	platelets, histiocytes, megakaryocyts	blood clotting	Yes
Band 4.2	15q15	72	erythroblasts erythrocytes	structural protein	Inactive
TGase X	?	81?	keratinocytes?	?	?

TGases 1, 2, 3 and × are commonly expressed in epithelia such as the epidermis (Kim *et al.*, 1991; Aeschlimann *et al.*, 1998), although to date only TGases 1 and 3 have proven importance in CE assembly (Candi *et al.*, 1995; Tarcsa *et al.*, 1997; Tarcsa *et al.*, 1998 Candi *et al.*, 1999). It has also been proposed that the cross-linking by these enzymes coordinates mechanically the association between the CE and the underlying intracellular keratin intermediate filaments (Candi *et al.*, 1998a), and perhaps also in the bundling of keratin filaments (Clement *et al.*, 1998).

The *TGM1* gene encoding the TGase 1 enzyme is located on chromosome 14q11.2 (Yaminishi *et al.*, 1990; Kim *et al.*, 1992). The synthesis of TGase 1 is regulated in cultured keratinocytes by various stimuli, including phorbol esters, retinoids and corticosteroids (Floyd and Jetten, 1989; Liew and Yamanishi, 1992; Yamada *et al.*, 1994) and by intercellular Ca^{2+} concentrations, presumably by AP-1 mediated gene regulatory signals (Dlugosz and Yuspa, 1994; Mariniello *et al.*, 1995). In epithelia such as the epidermis, TGase 1 expression is induced shortly after commitment to terminal differentiation (Michel *et al.*, 1992), although a minor degree of TGase 1 expression is detectable in undifferentiated basal keratinocytes (Schroeder *et al.*, 1992; Kim *et al.*, 1995a). The bulk of the TGase 1 enzyme is bound to the plasma membranes by its constitutively *N*- and *S*-fatty acylated 10 kDa amino terminal part (Rice *et al.*, 1990; Phillips *et al.*, 1993; Steinert *et al.*, 1996a). During terminal differentiation, some of the full-length TGase 1 enzyme undergoes proteolytic cleavage into fragments of apparent electrophoretic mobility of 10/67/33 kDa which are held together by secondary forces and thus remain membrane-bound (Steinert *et al.*, 1996b). This 10/67/33 kDa complex shows a 200-fold higher specific activity in standard *in vitro* TGase assays (Kim *et al.*, 1994; Kim *et al.*, 1995b). Also, some of the 67 kDa fragment harboring the catalytic activity may cycle off the membranes and thus might contribute to cross-linking at sites remote from the plasma membrane. However, the mechanisms by which the TGase 1 enzyme is proteolytically activated remain unexplored.

The *TGM2* and *TGM3* genes encoding the TGase 2 and 3 enzymes are located close to each other on chromosome 20q11.2 (Wang *et al.*, 1994). Expression of TGase 3 in cultured keratinocytes is triggered by elevated extracellular Ca++ presumably through adjacent cooper-ating Ets and Sp1 transcription factors (Lee *et al.*, 1996). TGase 3 is translated as a soluble inactive proenzyme of 77 kDa, and is subsequently activated by proteolysis at a flexible loop sequence into an amino-terminal 50 kDa domain harboring the active site and the carboxy-terminal 27 kDa portion (Negi *et al.*, 1985; Kim *et al.*, 1990; Kim *et al.*, 1993). No diseases have yet been linked to muta-tions in the *TGM3* gene.

Structural protein components of CEs

Table 2 lists proven CE protein constituents.

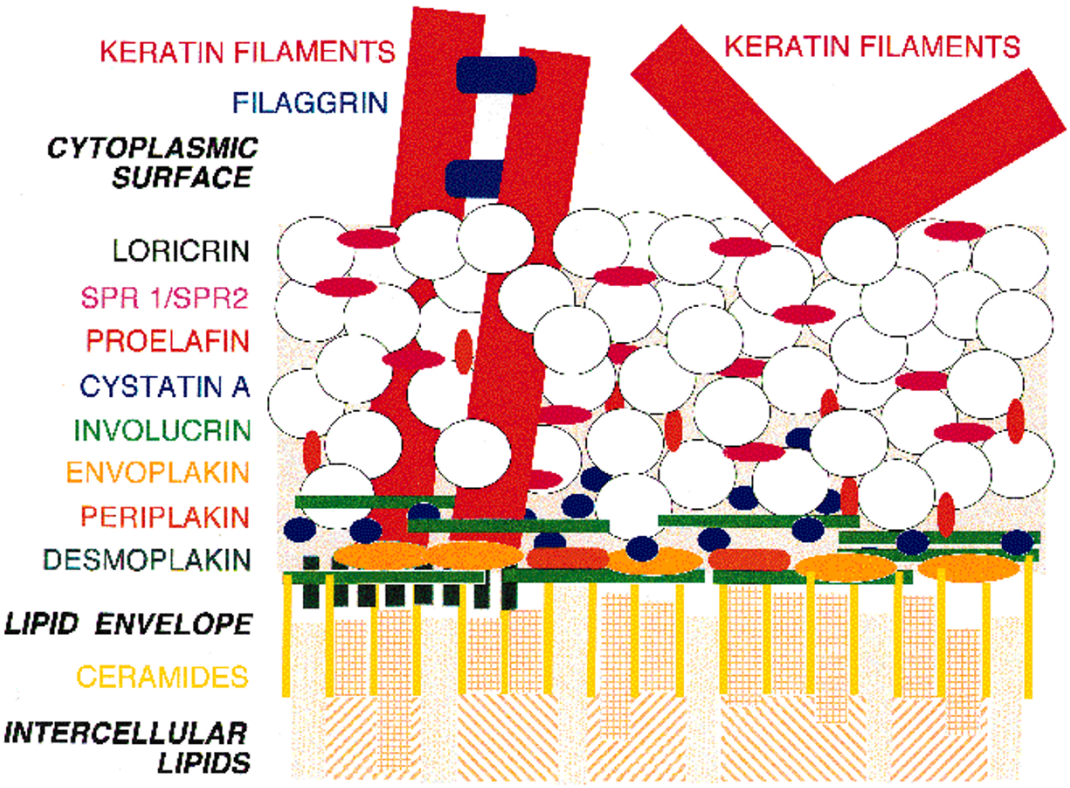
Involucrin is ubiquitously expressed in stratified squa-mous epithelia, thus suggesting it is commonly involved in CE formation. The involucrin gene is located in a cluster with the genes for numerous other CE proteins in the so-called epidermal differentiation complex (EDC) region on chromosome 1q21 (Volz *et al.*, 1993; Mischke *et al.*, 1996). The involucrin gene has a single exon encoding the entire protein (Eckert and Green, 1986) and shows astonishing polymorphism in humans (Simon *et al.*, 1991; Djian *et al.*, 1995). Mammalian involucrins evolved from a common ancestor gene by tandem duplications of a 45-60 base pair sequence in prosimians

Table 2. CE precursor proteins

Name	Gene locus	Size (kDa)	Relative abundance in human foreskin CE	Cross-linking sites identified *in vivo*?
Involucrin	1q21 (EDC)	65	2-5%	Yes
Loricrin	1q21 (EDC)	26	80%	Yes
SPRs	1q21 (EDC)	6-26	3-5%	Yes
Cystatin A	3cen-q21	12	2-5%	Yes
Proelafin	20q12-q13	10	<1%	Yes
(Pro)filaggrin	1q21 (EDC)	>400	<1%	Yes
Type II keratins	12q13	56-60	<1%	Yes
Desmoplakin	6p21-ter	330/250	<1%	Yes
Envoplakin	17q25	210	<1%	Yes
Periplakin	16p13.3	195	<1%	Yes
S100 proteins	1q21 (EDC)	12	<1%	No
Annexin I	9q12-q21.2	36	<1%	No

and which changed to a 30 base pair sequence in higher monkeys (Tseng and Green, 1988; Green and Djian, 1992). For example, pig involucrin has 13 repeats of 16 amino acids, of which seven are glutamine and three are glutamic acid residues. Human involucrin has 37 repeats of 10 residues of which three are glutamines and two are glutamates. Thus the whole human involucrin protein contains ~25% glutamine and ~20% glutamate residues (Eckert and Green, 1986). Presumably, the expansion of these repeat sequences was driven by the evolutionary benefit of increasing the number of potential TGase substrate glutamine residues for cross-linking, and perhaps of lengthening the molecule. This repeat region is flanked on both the amino and carboxy termini by domains which have been remarkably conserved throughout mammalian evolution and indeed show significant sequence homology to similar regions on other EDC-encoded CE structural proteins such as loricrin and the small proline rich proteins (Gibbs *et al*., 1993). Involucrin is a rod-like, elongated protein of ~45 nm long and 1.5 nm in diameter. Thus involucrin is ideally suited for cross-bridging widely separated CE components (Yaffe *et al*., 1992), although only a small fraction of its 150 glutamines appear to be utilized *in vivo* (Steinert and Marekov, 1997). The repeat region is mostly α-helical in humans but other secondary structures have also been proposed for the prosimian repeats (Downing, 1992). Expression of involucrin appears at the onset of terminal differentiation in epithelia (Eckert *et al*., 1993). In cultured keratinocytes *in vitro*, involucrin expression is induced

Figure 1. Schematic model of the structure of the CE and the lipid barrier. Modified from ref. 27. An initial scaffold 2-3 nm thick is built at the cell periphery by deposition of involucrin (long green rods) onto periplakin, envoplakin and perhaps desmosomal components such as desmoplakin. In the CE of the epidermis as shown, the subsequent reinforcement proteins are composed mostly of loricrin and interconnecting SPRs. In the CEs of the forestomach, trichohyalin is also used (Steinert *et al*., 1998b). In the CEs of other internal stratified squamous epithelia, the reinforcement proteins are mostly SPRs. In the hair fiber cuticle, other so far uncharacterized cysteine-rich proteins are used (Zahn *et al*., 1997). The KIF cytoskeleton is thought to be directly crosslinked to the cytoplasmic surface of the CE in order to mechanically integrate cornified cell structure (Candi *et al*., 1998a). In all cases examined so far, the protein envelope of the CE is uniformly 10 nm thick (Jarnik *et al*., 1998). In the epidermal CE, a lipid envelope of about 5 nm thick is also present, which is formed from a monomolecular layer of unusually large ceramides (vertical yellow rods). These may serve to interdigitate with other intercellular corneocyte lipids to complete the skin barrier.



by calcitriol (Su *et al*., 1994), corticosteroids (Cline and Rice, 1983), phorbol esters (Takahashi and Iizuka, 1993), and Ca^{2+} (Younus and Gilchrest, 1992), by regulatory elements located within about the first 2.5 kb upstream of the cap site (Welter *et al*., 1995; Lopez-Bayghen *et al*., 1996; Welter *et al*., 1996; Crish *et al*., 1998). CEs from 'dry' epithelia such as human foreskin epidermis contain about 5% involucrin (Steinert and Marekov, 1997). However, it is much more abundant in the CEs recovered from cultured keratinocytes (Rice *et al*., 1979; Yaffe *et al*., 1993; Steven and Steinert, 1994; Robinson *et al*., 1996) or internal 'wet' epithelia [P.M.S., unpublished].

Loricrin accounts for >70% of the protein mass of epidermal CEs, but rather less (30-50%) in the CEs of certain internal epithelia such as the esophagus, palate, buccal mucosa (Hohl *et al*., 1991a; Yoneda and Steinert, 1993), and is not expressed in many other internal epithelia. Expression of loricrin is induced by phorbol esters, cell confluence, and Ca^{2+} (Hohl *et al*., 1991b; Dlugosz and Yuspa, 1993), presumably through signals acting through an AP1 site (DiSepio *et al*., 1995; Rossi *et al*., 1998), and occurs very late during the terminal differentiation program of these tissues. Loricrin is an insoluble protein under physiological conditions, apparently due to its high content of glycine, serine and cysteine: indeed it has the highest glycine content of any protein known in biology (Mehrel *et al*, 1990; Hohl *et al*., 1991a; Yoneda *et al*., 1992). Owing to its high rate of expression and low solubility, loricrin forms spherical inclusions, called L-bodies in newborn mouse skin, human foreskin and acrosyringium, but it is diffusely distributed in the cytoplasm of adult epithelia (Steven *et al*., 1990; Ishida-Yamamoto *et al*., 1993; Ishida-Yamamoto *et al*., 1996). Loricrin contains three glycine rich domains which are thought to form uniquely flexible glycine loops (Steinert *et al*., 1991), interspersed by glutamine-rich motifs and flanked by lysine- and glutamine-rich amino and carboxy terminal domains (Mehrel *et al*., 1990; Hohl *et al*., 1991a). *In vitro* cross-linking experiments using recombinant human loricrin have demonstrated that the TGase 1 and 3 enzymes utilize different glutamine and lysine residues, implying that both enzymes have distinctly complementary and essential functions in the utilization of loricrin for CE assembly *in vivo* (Candi *et al*., 1995).

Small proline rich proteins (SPRs, cornifins, pancornulins) are a family of 11-14 closely related proteins. Three classes of SPRs have been identified: SPR1 (two members), SPR2 (8-11 members) and SPR3 (1 member) (Kartasova and van de Putte, 1988; Kartasova *et al*., 1988a; Marvin *et al*., 1992; An *et al*., 1993; Gibbs *et al*., 1993; Greco *et al*., 1995; Hohl *et al*., 1995; Austin *et al*., 1996; Kartasova *et al*., 1996; Steinert *et al*., 1998a; Song *et al*., 1999). The various members of the SPR classes display wide variations of expression in different epithelia. For example, SPR1a (cornifin α) and certain SPR2 proteins are expressed in dry epithelia such as the epidermis; distinctly different members of the SPR2 class are expressed in internal epithelia; and SPR3 is abundantly expressed in mucosal epithelia exposed to mechanical stress, such as the esophagus and rodent forestomach, while it is absent in the epidermis (Fujimoto *et al*., 1993; Hohl *et al*., 1995; Steinert *et al*., 1998b; Song *et al*., 1999). However, most members are induced in response to UV damage and phorbol esters (Kartasova and van de Putte, 1988; Kartasova *et al*., 1988b; Gibbs *et al*., 1990) or malignancy (Yaar *et al*., 1995). SPR1 and 2 transcription is induced by Ca^{2+} through a complex array of interacting AP1, Sp1, ets and other transcription factors (Fischer *et al*., 1996; Sak *et al*., 1998). All SPRs are built from a variable number of eight (in SPR1 and SPR3) or nine (in SPR2) amino acid residue proline-rich repeats. The number of repeats ranges from three in human SPR2 to 23 in human SPR3, so that the mass of SPRs varies between 6 kDa to 25 kDa (Fujimoto *et al*., 1993; Gibbs *et al*., 1993; Austin *et al*., 1996; Kartasova *et al*., 1996; Steinert *et al*., 1998b; Song *et al*., 1999). The repeats are flanked by short glutamine-, lysine- and proline-rich amino and carboxy terminal domains showing distant homology to the head and tail regions in involucrin and loricrin (Gibbs *et al*., 1993). Recombinant human SPR2 (Candi *et al*., 1999) and SPR1 (Tarcsa *et al*., 1998) proteins have been studied *in vitro*. Circular dichroism measurements indicate a random coil secondary structure for the termini, but a limited protein turn conformation for the repeat motifs. Both are powerful TGase substrates, and many adjacent glutamine and lysine residues become cross-linked *in vitro* and *in vivo* (Steinert *et al*., 1998a). Interestingly different residues on the amino termini are used by the two enzymes, indicating that both enzymes are also required for the appropriate assimilation of SPRs into the CE *in vivo*. In addition, double cross-linking experiments have shown that the TGase 3 enzyme first cross-links the SPRs into short oligomers, which are later affixed to the CE by the TGase 1 enzyme (Candi *et al*., 1999). We have noted a correlation between the amount of SPRs present in CEs and the presumed requirements of epithelia for resistance to physical trauma. For example, trunk epidermis contains only traces of SPRs; foreskin epidermis contains about 5% SPRs; human palm/sole or rodent footpad and lip epidermis contain 10-15% SPRs; rodent esophagus and forestomach epithelia contain >20% SPRs; and human buccal and gingiva epithelia contain near 50% SPRs. We have proposed the SPRs serve as cross-bridging proteins and in this way directly modulate the biomechanical properties of the CE and the entire epithelium in which they are expressed (Steinert *et al*., 1998a; Steinert *et al*., 1998b).

Cystatin A (keratolinin) expression is inducible in cultured keratinocytes by Ca^{2+}, phorbol esters and forskolin (Takahashi *et al*., 1997). In epidermis it is

expressed in the spinous layer (Jarvinen *et al*., 1987). Cystatin A is a substrate for epidermal TGases and has been identified as a minor cross-linked component of CEs (Zettergren *et al*., 1984; Takahashi *et al*., 1992; Steinert and Marekov, 1997). The protein is a known cysteine protease inhibitor (Jarvinen *et al*., 1987; Takahashi *et al*., 1994; Shibuya *et al*., 1995) and it has been suggested that this feature might be relevant for the bacteriostatic properties of the skin (Takahashi *et al*., 1994).

Elafin (elastase specific inhibitor, SKALP; skin derived anti-leukopeptidase) is a minor component of CEs from normal adult epidermis, is transiently expressed in fetal and neonatal epidermis, but is highly expressed in wounded or psoriatic skin and in cultured keratinocytes (Molhuizen *et al*., 1993; Schalkwijk *et al*., 1993; Alkemade *et al*., 1994; Nonomura *et al*., 1994; Pfundt *et al*., 1996). The protein is translated as preproelafin, which is curtailed to proelafin after the removal of the 25 amino acid signal peptide. Proelafin is composed of an amino terminal 34 amino acid proline-rich sequence ("cementoin") and the 57 amino acid long elafin (Wiedow *et al*., 1990; Schalkwijk *et al*., 1991; Saheki *et al*., 1992; Sallenave *et al*., 1993; Wiedow *et al*., 1993). Though elafin has potent elastase and proteinase-3 inhibitory properties (Wiedow *et al*., 1990; Wiedow *et al*., 1993), the biological relevance of this is unknown in keratinocytes. The fate of proelafin after synthesis is somewhat obscure, as the protein is stored in secretory granules and is later extruded from the cells (Pfundt *et al*., 1996). However, TGases utilize glutamine residues in the cementoin sequence as sub-strates (Molhuizen *et al*., 1993), and proelafin has been identified as a cross-linked component of epidermal CEs (Steinert and Marekov, 1995; Steinert and Marekov, 1997).

Profilaggrin is a major differentiation product of ortho-keratinizing epithelia such as the epidermis (Gan *et al*., 1990), and its gene is encoded in the EDC (McKinley-Grant *et al*., 1989). Profilaggrin is a polyprotein consisting of numerous filaggrin units flanked by distinctive amino and carboxy terminal domains (Presland *et al*., 1992; Markova *et al*., 1993). Interestingly, the amino terminus of profilaggrin contains two functional calcium binding EF hand motifs (Presland *et al*., 1992; Markova *et al*., 1993). Filaggrin is released by proteolysis from profilaggrin (Presland *et al*., 1997), and functions to bind keratin intermediate filaments into tight arrays typically seen in corneocytes (Dale *et al*., 1978; Mack *et al*., 1993). Some filaggrin becomes cross-linked to CE proteins (Richards *et al*., 1988; Steinert and Marekov, 1995; Simon *et al*., 1996), presumably together with and at the same time as the keratins. It has also been proposed that the amino terminal parts of profilaggrin are also incorporated into CEs after proteolytic cleavage from the filaggrin units (Presland *et al*., 1997).

Keratin intermediate filaments (KIF) are of course the major protein of the corneocyte, and some keratin proteins become cross-linked to the peripheral CE during terminal differentiation (Steinert and Marekov, 1995; Steinert and Marekov, 1997; Candi *et al*., 1998a). KIF and their roles in health and disease have been reviewed in detail elsewhere (Parry and Steinert, 1995; Steinert, 1996). In living nucleated epithelial cells the KIF cytoskeleton is attached to the cell periphery at desmosomal junctions, which provides mechanical stability throughout the epithelium (Holbrook and Wolff, 1993; Garrod *et al*., 1996). Although the exact details are unknown, the connection of the KIF to desmosomes occurs through several desmosomal proteins, including desmoplakin and envoplakin (Garrod *et al*., 1996; Green and Jones, 1996; Fuchs *et al*., 1997). In the course of terminal differentiation and CE formation, the structural integrity of desmosomes is destroyed at the same time as many normal house keeping cellular constituents are absorbed. During this process, KIF become cross-linked to the CE primarily through a single lysine residue located in the amino-terminal head domain of the type II keratins 1, 2e, 5, 6, typically expressed in stratified squamous epithelia (Candi *et al*., 1998a). In this way, the KIF cytoskeleton becomes integrated mechanically with the CE to form a stable insoluble structure for the corneocyte.

Desmoplakin is a major intracellular desmosomal plaque protein. Two isoforms, DPI and DPII, result from alternative splicing. In stratified squamous epithelia both isoforms are expressed (Virata *et al*., 1992). A large body of evidence indicates that the KIF meet at the site of the desmosome where they may interact directly or indirectly through various other intermediary proteins, with the terminal domains of desmoplakin (Green *et al*., 1990; Virata *et al*., 1992; Kouklis *et al*., 1994; Garrod *et al*., 1996; Green and Jones, 1996; Fuchs *et al*., 1997). There is direct evidence from protein sequencing that desmo-plakin becomes crosslinked to a variety of other CE proteins, although interestingly, the crosslink connection with the type II keratins seems to be indirectly through other proteins (Steinert and Marekov, 1995; Steinert and Marekov, 1997; Candi *et al*., 1998a). Several other desmosomal proteins including desmoglein 3, desmo-collins 3A/3B, plakoglobin, and plakophilin were found among proteolyzed CE fragments (Robinson *et al*., 1997). However, the sites of isopeptide bond formation were not identified in them, and the conclusion that they are in fact integral CE components awaits verification. Two other proteins, envoplakin and periplakin, which are structural homologues of desmoplakin, are located at or between the desmosome junctions of stratified squamous epithelia (Ruhrberg *et al*., 1996; Ruhrberg *et al*., 1997). These become cross-linked components of mature CEs, and indeed, envoplakin seems to mediate linkages between desmoplakin and KIF (Steinert and Marekov, 1997; Candi *et al*., 1998a).

The S100 proteins are akin to the amino-terminal domain of profilaggrin in that they contain two calcium binding EF hand motifs (Engelkamp *et al*., 1993; Volz *et al*., 1993; Moog-Lutz *et al*., 1995; Mischke *et al*., 1996). Many of their genes are located in the EDC region of chromosome 1q21. Among these, S100A10 (calpactin, light chain) and S100A11 (S100C, calgizzardin) are expressed and incorporated into CE in cultured keratinocytes (Robinson *et al*., 1997). On binding calcium, these proteins expose hydrophobic domains on their surface and bind to several proteins, including annexin I (Seemann *et al*., 1996).

Annexin I (lipocortin 1) is a member of the multigene family of annexins expressed in all eukaryotic kingdoms except fungi (Morgan and Fernandez, 1997). Annexins are structurally defined by an ancient conserved domain of four homologous repeats responsible for ion channel activity and calcium dependent binding to anionic phospholipids, the cytoskeleton and extracellular matrix proteins (Liemann and Lewit-Bentley, 1995). Annexin I is abundantly expressed in most tissues, although its precise role remains to be elucidated. Annexin I was found to be a component of CEs from cultured keratinocytes (Moore and Sartorelli, 1992; Robinson *et al*., 1997), a finding not yet corroborated by *in vivo* data. As annexins I and II were shown to associate with S100 proteins on calcium binding (Mailliard *et al*., 1996; Seemann *et al*., 1996), this mechanism may play a role in docking certain early CE protein components to the plasma membrane (Robinson and Eckert, 1998).

The order of CE assembly

As CEs consist of many different proteins, the relative ratios of which vary widely between different epithelia, it would be parsimonious to assume that these proteins are cross-linked to one another in a random fashion, when keratinocyte Ca^{2+} concentrations reach sufficiently high levels to activate TGases (Michel *et al*., 1987; Reichert *et al*., 1993). This random copolymerization, or so-called 'dustbin' hypothesis, does not explain the structure of CE, and moreover, seems inconsistent with the known orderly expression of the various protein components. Several studies indicate that the CE is formed by sequential deposition of consecutively-expressed proteins, apparently starting with the fixation of involucrin on the intracellular surface of the plasma membrane (Eckert *et al*., 1993; Steinert, 1995; Steinert and Marekov, 1997). Initially the CE appears as a thickened electron dense band between the desmosomes, which later overlayers the desmosomal attachment plaques, which presumably are not only masked but also degraded during the terminal differentiation process (Green and Jones, 1996). Expres-sion studies have shown that involucrin deposition at the cell periphery precedes that of most other CE proteins (Rice *et al*., 1979; Watt and Green, 1981; Simon and Green, 1984; Crish *et al*., 1993; Murthy *et al*., 1993; Yaffe *et al*., 1993; de Viragh *et al*., 1994; Jarnik *et al*., 1998; Song *et al*., 1999). Further, immunogold labeling and ultrastructural data have suggested that a monomolecular layer of involucrin is first deposited as a scaffold (Steinert and Marekov, 1997; Jarnik *et al*., 1998). In addition, sequential digestion and protein sequencing of foreskin CEs has revealed that involucrin is cross-linked to other peripheral CE proteins including desmoplakin, envoplakin, and perhaps periplakin. Finally, we have shown that involucrin is covalently bound to ω-hydroxyceramides from the exterior surface of the CE, indicating that involucrin must have been deposited in the intimate vicinity of the cell membrane at an early time (Marekov and Steinert, 1998). A transporting system has been proposed for positioning involucrin to the cell membrane (Robinson and Eckert, 1998). This model hypothesizes cross-linking of CE building blocks first to S100 proteins, which then dock to annexins to attach to the inner memb-rane surface in a calcium and phospholipid dependent manner. An alternative model has been proposed. We (Nemes *et al*., 1999) have shown that involucrin can bind to the plasma membrane in a calcium and phosphatidyl-serine dependent manner and serve as substrate for membrane-bound TGase 1. Thus involucrin and TGase 1 might form the initial scaffold of the CE without the need for any other transporter or organizer proteins.

Current models suggest that a monomolecular layer of involucrin is then used as a scaffold for the subsequent attachment of other 'reinforcement' proteins (Hohl, 1990; Reichert *et al*., 1993; Eckert *et al*., 1997; Steinert and Marekov, 1997; Jarnik *et al*., 1998). In the case of the epidermis, these reinforcement proteins include loricrin and SPRs, which together comprise about 85% of the total mass of the CE (Steinert and Marekov, 1997; Steinert *et al*., 1998b). The forestomach also includes significant amount of trichohyalin (Steinert *et al*., 1998b). The CEs from other internal epithelia which do not express loricrin have much higher contents of SPRs instead [P.M.S., un-published]. The CEs of the hair cuticle use as yet unknown cysteine-rich proteins (Zahn *et al*., 1997). These differ-ences presumably reflect the different barrier function requirements of different epithelia (Steinert *et al*., 1998b). In addition, there is considerable functional redundancy in CE proteins and their subsequent cross-linking. For example, the complete lack of loricrin in the knock out mouse model resulted in a surprisingly mild phenotype: newborns had an abnormal epidermis with diminished barrier function, but this improved by five days after birth. This improvement was concurrent with increased expression of SPRs (de Viragh *et al*., 1997).

The mortar

Terminal differentiation of keratinocytes is accompanied by vigorous lipid metabolism and synthesis of keratinization-specific lipids in the granular layer (Swartzendruber *et al.*, 1989; Wertz *et al.*, 1989a; Schurer *et al.*, 1991; Wertz and Downing, 1991; Downing *et al.*, 1993; Elias, 1996). Newly synthesized lipids are accumulated and temporarily stored in small cytoplasmic inclusions known as lamellar bodies, in which the lipids are arranged as multilayered stacks (Landmann, 1980). These layers are held together by extremely long ω-acylceramides spanning over several lipid layers (Abraham *et al*., 1988). The lamellar body lipids consist predominantly of acylated/glucosylated/hydroxylated ceramides, cholesterol and its acyl and sulfate esters, and free fatty acids (Schurer *et al.*, 1991; Downing *et al.*, 1993; Elias, 1996). In the upper granular layer the lamellar bodies are extruded into the intercellular space, forming broad multilamellar lipid sheets (Landmann, 1986). Ultrastructural examination of lamellar bodies as well as intercorneocyte lipids display a parallel pattern of electron dense and lucent bands (Madison *et al*., 1988; Swartzendruber *et al.*, 1995). The dense bands correspond to the polar head groups of the lipid layers, while lucent bands are occupied by the apolar hydrocarbon chains (Swartzendruber *et al.*, 1989). On extrusion, the bounding membrane of the lamellar bodies is fused with the plasma membrane of the keratinocytes (Ricardo Martinez and Peters, 1971). This process apparently coincides with the initiation of CE assembly inside the cells. One minor but important component of the extruded lipids are the ω-hydroxyceramides, which become covalently attached to the outer surface of the protein envelope of the CE forming an ~5 nm thick lipid envelope coat surrounding each corneocyte (Swartzendruber *et al.*, 1987; Wertz and Downing, 1987a; Wertz *et al.*, 1989b). The ceramides are attached by way of ester bonds to glutamic acid and glutamine residues of several CE proteins, including involucrin, envoplakin and periplakin (Wertz *et al.*, 1989b; Marekov and Steinert, 1998). The protein-bound ω-hydroxyceramides are built from sphingosine coupled to highly saturated, uniquely long (C_{30-36}) chain fatty acids having a chain terminal ("ω") hydroxyl group (Wertz and Downing, 1991). This functional group is presumably involved in the ester bond formation, although incomplete conversion of protein-bound ceramides to their acetonides by acidic acetone indicated that sphingosine hydroxyls may also be used (Wertz and Downing, 1987a).

Isolation and sequencing of ceramide-peptide ester adducts from proteolyzed foreskin epidermal CEs located possible lipid attachment sites in involucrin and desmosomal proteins. These included both glutamate and glutamine residues (Marekov and Steinert, 1998). Thermodynamic considerations necessitate high-energy intermediate formation to drive formation of ester bonds in biology. Ester formation on glutamate residues presumably involves transferases using nucleotide triphosphate as the energy source, and indeed many transferases are present in lamellar body exudate (Downing *et al*., 1993; Elias, 1996). However, glutamines are an intrinsically activated derivative of glutamic acid and the release of ammonia from its carboxamido groups provides sufficient entropy increase to drive ester formation. TGases (precisely: glutamine-amine aminotransferases) are known to utilize the lysis of glutamine carboxamido moieties to drive thermodynamically difficult reactions, including activation of alcohol moieties to form esters (Gross and Folk, 1974; Folk and Finlayson, 1977; Lorand and Conrad., 1984). Thus we propose that TGases may also participate in lipid envelope formation by covalent attachment by esterification of ω-hydroxyceramides to glutamines of the protein envelope.

The long chain ceramides comprising the lipid envelope attached covalently to the surface of the CE function in large part by interdigitation with the intercorneocyte lipids in a Velcro-like fashion. As part of its obviously important water barrier function, this attachment presumably permits 'fixation' of the cornified cells after disappearance of desmosomal linkages, and may have a role in inhibiting the clumping, vacuolization or other derangement of the lipid lamellae (Wertz *et al*., 1989a; Wertz, 1997), especially in hair cuticle cells (Zahn *et al*., 1997).

Defects of the skin barrier

Broken bricks

More than 10 different diseases involving the genes encoding KIF (which comprise the bulk of epithelial cells) are now known and have been described in detail elsewhere (Parry and Steinert, 1995; Steinert, 1996). Included in these is a novel mutation involving the loss by mutation of a single lysine residue in the head domain of the keratin 1 gene, resulting in non-epidermolytic palmar-plantar keratoderma (Unna-Thost disease). This disease is characterized by pathological thickening of the stratum corneum of the palms and soles (tylosis) (Kimonis *et al*., 1994). The lysine residue has been shown to be essential for the crosslinking of KIF to CE structural pro-teins, and its loss appears to interfere with the orderly structure of the corneocyte (Candi *et al*., 1998a).

In addition, a few genetic diseases caused by defects in the genes encoding either CE structural proteins or TGase 1 are now known. Defective forms of loricrin dis-rupt the terminal differentiation program of keratinocytes and cause skin diseases. Frameshift mutations, resulting in loss of key glutamine and lysine

residues for cross-linking, and expression of an aberrantly highly positively charged protein which accumulates in the nucleus instead, cause the autosomal dominant diseases Vohwinkel's keratoderma (keratoderma hereditaria mutilans (Vohwinkel, 1929; Gibbs and Frank, 1966; Korge *et al*., 1997; Lam *et al*., 1997) or progressive symmetric erythrokeratoderma (Ishida-Yamamoto *et al*., 1997). In these diseases the CE is thinner than normal and contains less loricrin (Ishida-Yamamoto *et al*., 1997; Korge *et al*., 1997; Lam *et al*., 1997). Patients with Vohwinkel's keratoderma have diffuse palmoplantar hyperkeratosis with small "honeycomb" depressions and progressively develop constricting bands on their fingers. Irregularly shaped keratoses on the backs of feet and hands, elbows and knees and variable deafness are also part of the syndrome. Progressive symmetric erythrokeratoderma is characterized by wide-spread erythematous keratotic plaques (Ishida-Yamamoto *et al*., 1998).

Genetic defects of the *TGM1* gene encoding TGase 1 cause the devastating life threatening disease lamellar ichthyosis, which manifests as large brown plate-like scaling throughout the body, accompanied by ectropion and/or eclabium, scarring alopecia and diminished skin barrier function (Huber *et al*., 1995a; Russell *et al*., 1995). Several mutations have been identified which cause non-sense, frameshift or splice site changes affecting either the active site of the enzyme (Parmentier *et al*., 1995; Huber *et al*., 1997; Petit *et al*., 1997; Candi *et al*., 1998b) or its posttranslational proteolytic processing (Candi *et al*., 1998b). In all of these cases, the amount of TGase 1 activity is greatly diminished or lost (Hohl *et al*., 1998; Raghunath *et al*., 1998). Several of these phenotypic changes are also apparent in the mouse *TGM1* gene knock-out model (Matsuki *et al*., 1998). Thus the TGase 2, 3 and X enzymes also co-expressed in the epidermis are unable to replace the missing TGase 1 activity. Interestingly, however, other internal epithelia which also express TGase 1 and other TGases are not affected in lamellar ichthyosis. Thus it is possible that debilitating epidermal involvement may be due to the inability of TGase 1 to attach ceramide lipids to the CE. In addition, there are a variety of other autosomal recessive ichthyoses that are unlinked to the *TGM1* locus, indicating that defects in other genes cause a phenotype similar to lamellar ichthyosis (Huber *et al*., 1995b; Bale *et al*., 1996; Hennies *et al*., 1998). One possibility is that these genes encode proteins involved in the posttranslational proteolytic activation of the TGase 1 enzyme (Candi *et al*., 1998b). Finally, to date, no disease has been linked to any of the other TGase genes expressed in epithelia.

Weak mortar

Production of an effective lipid barrier in the skin (and other epithelia) involves an extraordinarily complex set of machinery and a very large (indeed, unknown) number of genes. Generally, deficiencies in barrier function result in an ichthyosiform disease. Even minor depletion of the lipid barrier causes dry skin, a common manifestation of which is the scaling caused by exaggerated application of hygienic detergents. Essential fatty acid deficiency causes excess scaling in rats (Wertz *et al.*, 1987b) and also in humans on long-term intravenous alimentation lacking linolate (Friedman, 1986). The systemic application of HMG-CoA reductase inhibitors impedes epidermal cholesterol synthesis and might cause ichthyosiform symptoms (Williams, 1992). Excessive depletion of the cornified layer triggers hyperproliferation often leading to abnormal scaling (Fartasch, 1997).

Many subtypes of ichthyoses have been distinguished on the basis of ultrastructural (Anton-Lamprecht, 1994) or other criteria based on abnormal intercellular deposition of apolar lipids, cholesterol, polar lipids, etc (Williams and Elias, 1987; Traupe, 1989). The exact genetic defect in the vast majority of these classified diseases is not yet known, although some have been identified. As discussed above, lamellar ichthyosis is caused by mutations in the *TGM1* gene encoding the TGase 1 enzyme; the disease may result from the inability to both crosslink structural proteins and attach ceramides. X-linked ichthyosis is due to cholesterol sulfate accumulation owing to a deficiency of the arylsulfatase C/cholesterol sulfatase gene (Shapiro *et al*., 1978; Kubilus *et al*., 1979; Baden *et al*., 1980). How abnormally high levels of cholesterol sulfate cause barrier dysfunction has not yet been clearly elucidated (Zettersten *et al*., 1998), although the *TGM1* gene may be involved (Kawabe *et al*., 1998). Some ichthyoses are the direct result of genetic defects of lipid metabolism, as exemplified by Refsum's disease (phytanic acid accumul-ation owing to phytanoyl-CoA hydroxylase deficiency) (Steinberg *et al*., 1978; Jansen *et al*., 1997), and Sjogren-Larsson's syndrome (pathological lipid metabolism owing to fatty aldehyde dehydrogenase deficiency (De Laurenzi *et al*., 1996; De Laurenzi *et al*., 1997). Similarly, maple syrup urine disease in cattle is caused by an inherited deficiency in the enzyme branched chain alpha-ketoacid dehydrogenase, which leads to accumulated branched chain amino acids (valine and isoleucine). A hair fiber barrier defect is also evident because of loss of a key lipid, 18-methyleicosanoic acid, which is a downstream metabolite of the enzyme (Zhang *et al*., 1990).

Conclusion

In recent years substantial progress has been made to identify the protein and lipid components involved in skin barrier function. Nevertheless, several major problems still await resolution in order to provide a complete understanding of the biochemical mechanisms of barrier

formation as well as the temporal and geometric interactions of the individual components. Thus, much additional basic research is essential to understand the bases of genetic diseases barrier function before rational therapy procedures can be developed.

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