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Biochimica et Biophysica Acta 1768 (2007) 521–529
Biochimica et Biophysica Acta BBA
www.elsevier.com/locate/bbamem

New arrangement of proteins and lipids in the stratum corneum cornified envelope

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Received 26 July 2006; received in revised form 15 November 2006; accepted 16 November 2006
Available online 23 December 2006

Abstract

A new arrangement of proteins and lipids of stratum corneum (SC) cornified envelope (CE) is proposed. The chemical analysis of CE revealed the presence of free fatty acids (FFA), ceramides (Cer), and important percentages of glutamic acid/glutamine (Glx) and serine (Ser) residues. The molecular structure of these components suggests the existence of covalent links not only between Cer and Glx but also between FFA and Ser. The protein distribution of extracellular surface of CE, i.e., the proteins that could be involved in the bonds with lipids, was studied using post- and pre-embedding immunolabeling electron microscopy. Some loricrin (protein rich in Ser) was detected in the outermost part of the CE protein layer. The external arrangement of some domains of this protein may give rise to form linkages with FFA, yielding further insight into the CE arrangement in which Cer–Glx bonds and FFA–Ser bonds would be involved. Although the importance of fatty acids in the cohesion and barrier function of SC has been widely demonstrated, their role could be associated not only to the presence of these lipids in the intercellular lamellae but also in the CE, in the same way that Cer.

Keywords: Immunolabeling-transmission electron microscopy; TLC-FID iatroscan; Ceramides; Loricrin

1. Introduction

The barrier function of the skin is provided by the stratum corneum (SC), the uppermost layer of the skin [1]. SC composition, structure and function have been theme of a number of works [1–8]. This tissue consists of 10–15 layers of annucleated, keratin-rich corneocytes embedded in a lipid matrix organized in lamellae. The major components of the lipid matrix are ceramides, cholesterol and free fatty acids and this unusual composition is responsible for the skin barrier function and its regulation [5]. The corneocytes are surrounded by a proteinaceous structure called the cornified envelope (CE). This structure consists of a layer of highly crosslinked insoluble proteins covalently bound to a layer of lipids. These covalent bonds have been described as ester linkages in which the carboxyl group of the repetitively arrayed glutamic acid (Glu) residues of involucrin (protein rich in Glu) is bound to the hydroxyl group of the ceramides (Cer) [6–8]. This description is widely accepted by the scientific community. However, it does not consider the presence of free fatty acids (FFA) detected in the CE by some authors [9,10]. This fact would involve an additional link of these lipids with the amino acids of the CE proteins, and hence, a specific distribution of the proteins sited on the extracellular surface of the CE. Several publications have focussed on the protein composition and structure of the CE [11–15]. Protein precursors of the CE have been shown to include involucrin [16] and loricrin [17,18]. Involucrin is a rod-shaped molecule containing 20% Glu and 25% glutamine (Gln) residues, making it an ideal substrate for attachment of the Cer sheath through ester bonds [16]. However, amino acid analysis followed by mathematic modelling have indicated that

Abbreviations: CE, cornified envelope; Cer, ceramide; Chol, cholesterol; Cys, cisteine; FFA, free fatty acid; Gln, glutamine; Glu, glutamic acid; Gly, glycine; PBS, phosphate buffered solution; SC, stratum corneum; Ser, serine; TEM, transmission electron microscopy; TG1, transglutaminase 1; TLC-FID, thin layer chromatography coupled to a flame ionization detector

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doi:10.1016/j.bbamem.2006.11.023

involucrin is a minor constituent, whereas loricrin amounts to between 65% and 70% (w/w) in human and from 80% to 85% in mouse CE [17]. In this sense, Jarnik [13] proposed a molecular model of the mature structure of the CE where involucrin lies in the plane of the CE and the loricrin molecules engage in side-by-side interactions underlying involucrin.

In earlier works we studied the structure and composition of the SC, as well as the effect of different agents, such as organic solvents and surfactants, on this structure [19–23]. The exhaustive extraction and analysis of the SC compounds allowed us to clarify some aspects related to the arrangement of the lipids and proteins in the CE, and to the involvement of the CE components in the SC functions. Thus, this work proposes the analysis of the CE components and the study of the protein distribution of the extracellular surface of the CE. Our results report a new arrangement of CE in which not only Cer–Glu bonds but also FFA–Ser bonds are involved. The relevance of Cer in the organization of SC have been deeply studied [24,25] and the importance of the fatty acids in the cohesion and barrier function of the SC has been widely demonstrated [26], their key role could be associated with the presence of these lipids not only in the intercellular lamellae but also in the CE in the same way as the Cers.

2. Materials and methods

Trypsin and phosphate buffered solution (PBS) were purchased from Sigma Co. The chemicals for the sample fixation: glutaraldehyde, paraformaldehyde and cacodylate buffer were supplied by EMS, and the glycerol by Merck. The primary antibodies, anti-loricrin and anti-involucrin were purchased by Covance, USA, and by Neomarkers, USA, respectively. The secondary gold-conjugated antibodies were supplied by British Biocell, UK, and by Aurion, The Netherlands.

2.1. Stratum corneum and cornified envelope isolation

Sections of human fresh skin obtained from cosmetic surgery in the Department of Dermatology, University Hospital "Principes de España", Barcelona, Spain, were placed in water at 65° for 4–5 min, and the epidermis was scraped off in sheets. These sheets were placed in 100 ml of 0.5% trypsin in PBS (pH 7.4, 4 °C, overnight). The SC pieces were then collected, rinsed with distilled water, and suspended in a large volume of water. The pieces were transferred to a round flask with fresh trypsin/PBS solution, rotated at 100 rpm (25 °C, 2 h) and washed with distilled water [27,28]. To isolate the CE of the SC pieces, an incubation of the SC with 8 mM *N*,*N*-dimethyldodecylamine oxide (Merk) and 2 mM sodium dodecylsulfate (Merk) in PBS buffer (45 °C, 4 h) was performed. After centrifugation and redispersion, the supernatants were drawn off and the pellet was incubated with fresh surfactant solution (45 °C, 24 h) resonicated and centrifuged again. Finally, the pellet was washed by resuspension in PBS and in distilled water. The pellet contained corneocytes and envelopes. These two components were separated by resuspension in a solution of cesium chloride of density 1.280. Only the denser component, the corneocytes, sedimented on centrifugation and the lighter component, the CE, could be drawn off in the supernatant. This process was repeated twice more until the supernatants were then diluted with water and recentrifuged to recover the light component containing the CE [29].

2.2. Extraction and analysis of lipids

SC samples were successively extracted for 120 and 60 min with mixtures of chloroform/methanol (2:1, 1:1 and 1:2, v/v) and finally extracted with methanol overnight to extract the lipids organized by polar forces. The different extracts were then combined, concentrated to dryness, weighed and redissolved in chloroform/methanol (2:1) prior to analysis [28]. After extractions, the material remaining (delipidized SC) was treated at 60 °C for 1 h with 1.0 M NaOH in methanol/water 9:1 to break the covalent bonds (through ester linkages) between the lipids and the amino acids building the CE proteins. To remove these lipids, the resulting suspensions were acidified to pH 4.0 with 2.0 M HCl and after filtration through a coarse sintered glass, they were twice extracted with chloroform/methanol. The extracts were then concentrated to dryness and redissolved in a small volume of chloroform/methanol (2:1) to eliminate the interference caused by the presence of amino acids in the lipid analysis [9]. CE samples were submitted to the same hydrolysis and extraction procedure.

The lipids of the different extracts (native SC, delipidized SC and CE) were analyzed by thin layer chromatography coupled to a flame ionization detector (TLC-FID) using the IATROSCAN MK-5; Iatron Laboratory, Inc., Tokyo, Japan. Samples were spotted on silica-gel chromarods and developed with a nonpolar mobile phase: hexane-diethyl ether-formic acid (53/17/0.3) 10 cm. After a partial scan of 85% to detect and eliminate the nonpolar lipids, a second development was performed using a polar mobile phase: chloroform–hexane–methanol–acetone (55/5/3/7) 10 cm [30]. The same procedure was applied to different standard solutions to obtain calibration curves for the quantification of each compound.

2.3. Amino acid analysis

In order to analyze the amino acid content of the SC and CE samples, sheets of both components were hydrolyzed with 6 N HCl for 24 h at 110 °C. Determination of the amino acid composition of these hydrolyzed samples was carried out by HPLC using an Automated Amino Acid Analyzer (Biotronik LC 5001, Hucoa Erlös, Frankfurt, Germany).

2.4. Immunoelectron microscopy experiments

2.4.1. Post-embedding immunogold labeling

Fresh samples of SC were fixed by high pressure freezing using a Leica EM Pact at 2100 bar and −196 °C. The subsequent freeze substitution (at −90 °C for 3 days) was performed in a Leica AFS using methanol containing 0.5% uranyl acetate as substitution medium. Infiltration was carried out in Lowicryl HM20. Immunogold staining was performed on ultrathin sections. For double labeling experiments the primary antibodies were applied simultaneously. The sections were incubated with polyclonal anti-loricrin and monoclonal anti-involucrin antibodies for 1 h. As secondary antibodies, 15 nm gold-conjugated goat-anti-rabbit IgG and 10 nm gold-conjugated goat-anti-mouse IgG were used to label loricrin and involucrin, respectively. In order to improve the spatial resolution of the method, additional post-embedding experiments were carried out by using antibody fragments F(ab′)2 instead the whole antibody molecule (IgG). It is known that the F(ab′)2 fragment is one third the size of a full IgG molecule [31,32]. Finally these sections were post-stained with uranyl acetate and studied with a Jeol 1010 TEM.

2.4.2. Pre-embedding immunogold labelling

Small pieces of SC were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in PBS pH 7.4. After this chemical fixation the material was three times rinsed in 50 mM glycine /PBS for 10 min at 25 °C and gentle shaking. Blockage was carried out by incubation in 5% BSA/PBS 1 h at 25 °C. Samples were quickly rinsed in PBS and then incubated overnight at 4 °C in agitation with the primary antibodies: anti-involucrin and anti-loricrin. After that, the samples were rinsed in PBS at 4 °C. The samples were then incubated with secondary gold-conjugated antibodies overnight at 4 °C and gentle shacking. In the same way of the post-embedding experiments the secondary 15 nm gold-conjugated goat-anti-rabbit IgG and 10 nm gold-conjugated goat-anti-mouse IgG were used to label loricrin and involucrin, respectively. The tissue was extensively rinsed in PBS and postfixed in 2.5% glutaraldehyde in PBS 15 min and rinsed again in PBS. The material was then osmicated in 2% OsO_4/PBS during 1 h at 25 °C, dehydrated in graded ethanol (70, 90 and 100% during 20 min two times each) and infiltrated in Ethanol/Epon resin (3:1 o/n; 1:1, 2 h; 1:3, 3 h, RT and Epon twice during 2 h at RT. The specimen blocks were polymerized at 60 °C during 48 h. The ultrathin sections were post-stained with uranyl acetate and studied with a Jeol 1010 TEM.

3. Results

The lipid and amino acid analysis of the SC showed 82.3% (w/w) of protein material and 17.7% (w/w) of lipids. The CE proved to be 5% in weight with respect to the whole SC, and was made of 90% (w/w) of proteins and 10% (w/w) of lipids, in agreement with published data [33]. The TLC-FID analysis showed that lipids from the intercellular spaces of SC consisted mainly of Cer, Chol, FFA, small amounts of cholesteryl sulfate, cholesteryl esters, triglycerides and an absence of phospholipids. Fig. 1 shows the chromatograms of the lipids developed with the nonpolar mobile phase (A, C and E) and with the polar mobile phase (B, D and F) corresponding to extractions from native SC (A and B), to extractions after mild alkaline hydrolysis of delipidized SC (C and D) and also to isolated and hydrolyzed CE (E and F). Chromatograms A and B show the intercellular lipids extracted by organic solvents. This result agrees with earlier works [27,28]. The chromatograms for lipids from delipidized SC and CE samples (C–F spectra) reveal the presence of additional lipids. One of them with mobility of FFA (chromatograms C and E), and two more peaks with mobility similar to Cer 2 and Cer 6 (chromatograms D and F). The two latter peaks were associated with the omega hydroxyl Cers covalently bound to the CE, i.e. Cer A and Cer B described by Ponec [10].

To evaluate if FFA detected after mild alkaline hydrolysis (chromatograms C and E) could originate from the hydrolysis of bound Cers, samples of synthetic Cer 2 and Cer 3 were submitted to hydrolysis at mild alkaline and strong acid conditions. In the first case, the chromatograms for the samples of Cer 2 and Cer 3 showed only one peak that corresponded to the mobility of each Cer. The chromatograms in the second case

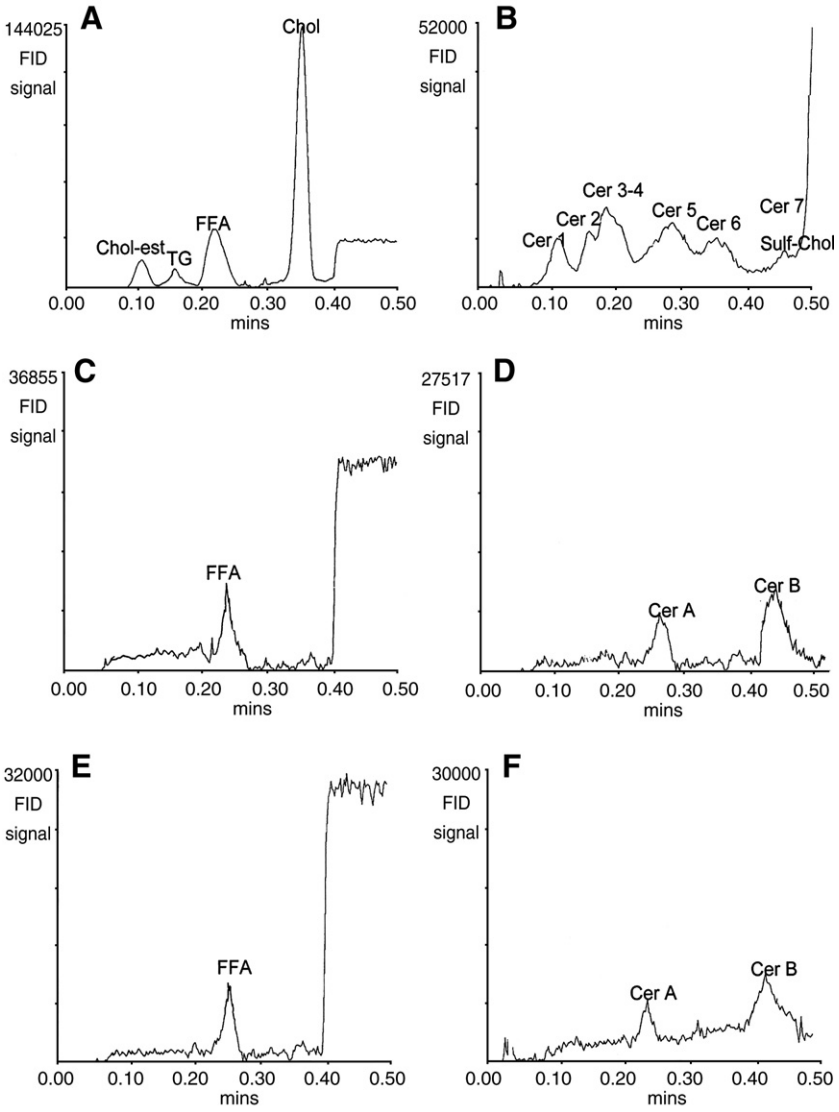

Fig. 1. TLC-FID chromatograms of the different lipids. The chromarods were developed with the nonpolar mobile phase (A, C, and E) and with the polar mobile phase (B, D, and F) corresponding to extractions from native SC (A and B), to extractions after mild alkaline hydrolysis of delipidized SC (C and D) and also to isolated and hydrolyzed CE (E and F).

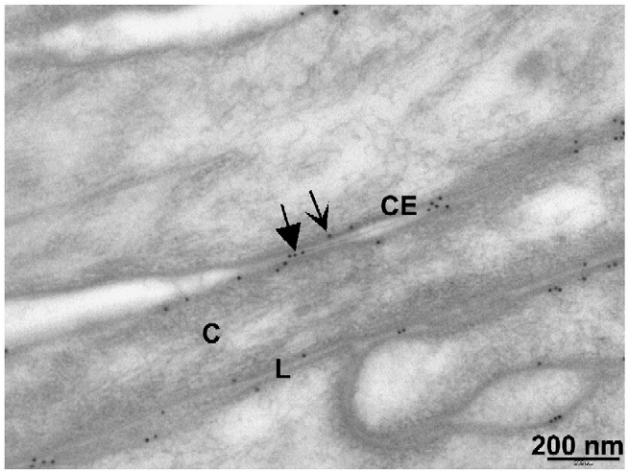

Fig. 2. TEM micrographs of post-embedding immunogold labeled SC using loricrin antibody. Areas for corneocytes (C), lipids (L) and cornified envelope (CE) are shown. Gold particles are observed in both intracellular (open arrow) and extracellular (black arrow) sides of the CE. This indicates that the loricrin could be sited with these two orientations.

showed two peaks with mobilities of each Cer and FFA. Thus, strong acid conditions are needed to break the amide linkage present in the Cers.

This finding supports that the fatty acids found after mild alkaline hydrolysis of the CE do not come from the Cers.

The amino acid analyses of the SC and the CE proteins showed that glutamic acid-and-glutamine (Glu/Gln), Ser and glycine (Gly) residues are the predominant species in both structures. The weight percentages of these amino acids (respect to the total amount of amino acids in the CE) are 14.7% Glu/Gln, 11.1% Ser and 10.1% Gly. The amino acid sequencing detected in the CE justifies the presence of a number of structural proteins, which have been described mainly as involucrin, loricrin, envoplakin, periplakin [7,17,34], and more recently as periphilin [35].

The protein distribution of the extracellular surface of the CE was studied by immunolabeling-TEM using involucrin and loricrin antibodies. TEM micrographs of post-embedding immunogold stained SC are shown in Figs. 2 and 3. These figures show the samples incubated using the whole antibody molecule (IgG). The antibodies showed an appropriated labeling of both proteins (involucrin and loricrin) which are located in the CE [34]. In Fig. 2, a micrograph of SC labeled only with loricrin antibody is shown. This micrograph shows clearly the areas corresponding to the corneocytes (C), and to the lipids (L). The micrograph of Fig. 2 shows gold particles in both intracellular (open arrow) and extracellular (black arrow) sides of the CE, which is located at the edges of corneocytes (noted as CE). This indicates that the loricrin could be sited with these two orientations. Fig. 3 shows some pictures for the double labeling of loricrin and involucrin. At low magnifications the shape of the corneocytes can be seen following the gold particles (Fig. 3A). Higher magnifications (Fig. 3B, C) show the disposition of both proteins. Involucrin labeling (small gold granules) was observed at intra and extracellular sides. The extracellular domains have been described as those linked to the Cers of the CE (arrowhead). Loricrin arranges just below involucrin at the intra cellular side (large gold granules, open arrow). However, some loricrin was also detected on the outermost part of the CE (large gold granules, arrow), indicating the external disposition of some loricrin domains that may be

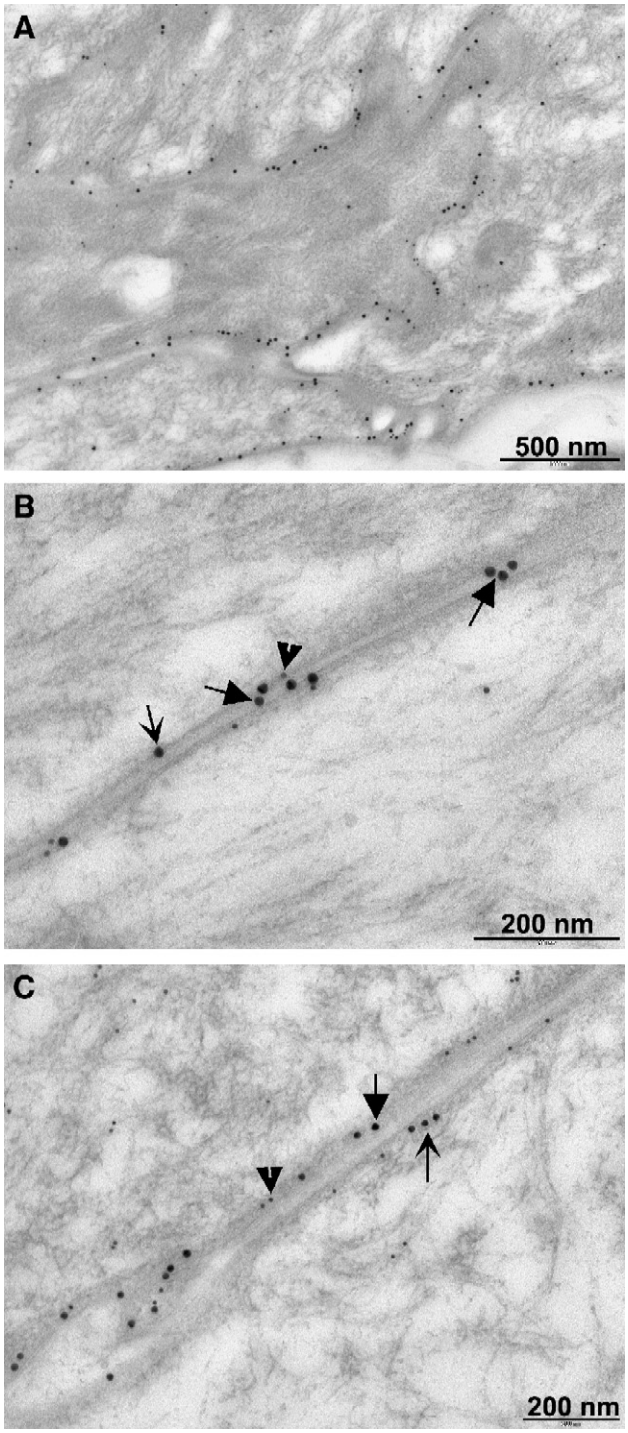

Fig. 3. TEM micrographs of double labeling post-embedding immunogold stained SC using loricrin (15-nm gold) and involucrin (10-nm gold). At low magnifications the shape of the corneocytes can be seen following the gold particles (A). Higher magnifications (B, C) show the disposition of the involucrin (arrowhead). The loricrin is arranged just below the involucrin (open arrow) and also in the outermost part of the protein layer of the CE (black arrow).

accessible to form linkages with CE lipids. To reduce the distance antigen-gold particle and to improve the spatial resolution F(ab′)2 fragments were also used as secondary antibodies. It is known that the antibody molecule IgG is 15 nm in diameter and the protein–colloidal gold complex is about 10–15 nm, so that the conjugated label has a diameter of 25 to 50 nm. The use of the F(ab′)2 fragments, with about 4.5 nm in size, reduces the total label diameter to 14–20 nm [31,32]. Fig. 4 shows samples labeled with these shorter antibodies. Loricrin labeling (large gold particles) at intra (open arrow) and extracellular (black arrow) sites of the CE was again observed, confirming the loricrin localization visualized in Fig. 3. The intra and extracellular disposition of the involucrin is indicated by the small gold granules. Samples corresponding to the pre-embedding experiments are depicted in Fig. 5. In this pre-embedding procedure the sample was not permeabilized and/or sectioned previously to the antibodies incubation to label only the proteins exposed to the extracellular site of the CE. Both loricrin (large gold granules) and involucrin (small gold granules) label are observed in the surface and in the outside rim of the SC samples, indicating again the extracellular disposition for both proteins.

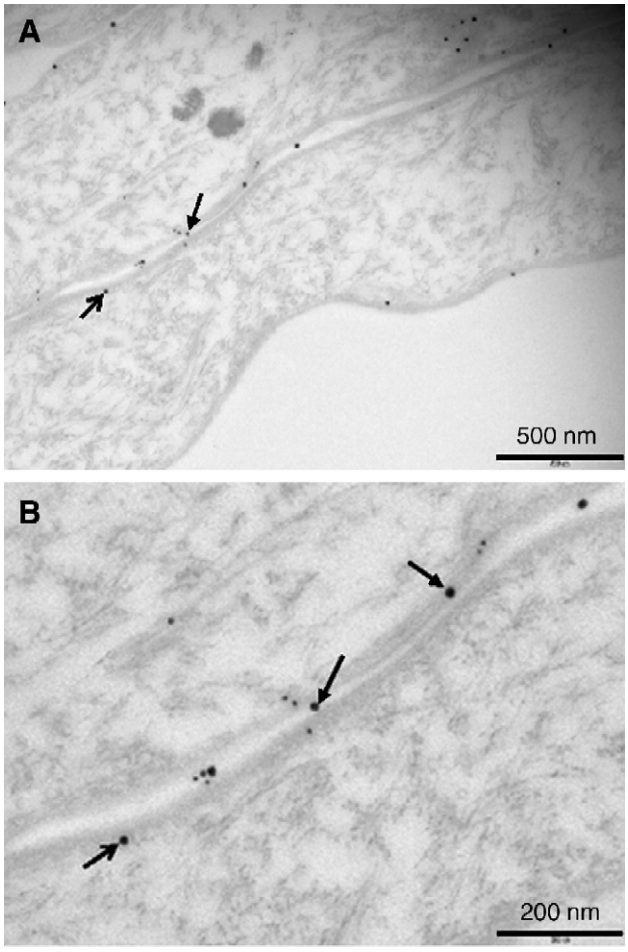

Fig. 4. TEM micrographs of double labeling post-embedding immunogold stained SC using F(ab′)2 fragment as secondary antibodies. Loricrin 15-nm gold and involucrin 10-nm gold. Open and black arrows indicate intra an extracellular location of loricrin, respectively.

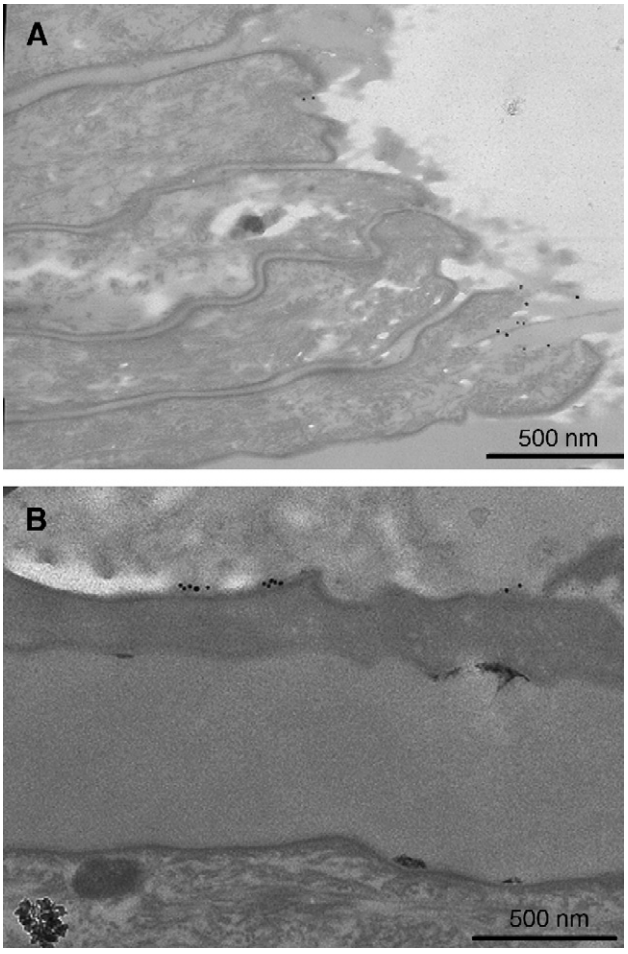

Fig. 5. TEM micrographs of double labeling pre-embedding immunogold stained samples using loricrin (large gold granules) and involucrin (small gold particles) antibodies.

Considering the lack of cryoprotectants and chemical fixation compounds in the preparation of the samples fixed by high pressure freezing (Figs. 2, 3 and 4), it is interesting to note the good preservation of the structure in comparison with samples fixed using chemical fixation (Fig. 5). Clearly, and in agreement with other authors, the use of high pressure freezing combined with freeze substitution is fundamental in the sample ultrastructural preservation [36].

4. Discussion

The similarity between the chromatograms for delipidized SC (chromatograms C and D, Fig. 1) and for the CE (chromatograms E and F, Fig. 1) was expected given that the only lipids present in the delipidized SC are precisely the CE lipids. These lipids were covalently bound to the amino acid residues with the result that a mild alkaline hydrolysis followed by further chloroform/methanol extractions was needed to detect them. As reported by Wertz [9], the hydrolysis broke the covalent bonds between the lipids and the amino acid residues building the CE.

Although omega-hydroxyl Cers have been described as the main bound lipids of the CE [37] our experiments reveal the

additional presence of fatty acids in the CE. At this point a question may be raised: Could the FFA found after alkaline hydrolysis come from the breakage of some part of the hydroxyl Cers instead of from the CE? The study of the stability under hydrolysis conditions of the linkages fatty acid-sphingosine present in the synthetic Cers 2 and 3 rejects that fatty acids found after mild alkaline hydrolysis of CE originate from the Cers. This fact, the resistance to extraction by organic solvents and the rapid liberation by alkali of the fatty acids supports that they are covalently bound to some amino acid residue in the CE. The description of covalent links between proteins and FFA in structures with a function similar to that of the CE is not new. Similar events take place in the hair and wool cuticles. The cell membrane complex located between the cuticular cells contains fatty acids covalently linked to cysteine (Cys) residues from the protein framework of epicuticule [38,39]. Other authors have reported that the fatty acids are linked through a thioester bond to Cys residues, such as Cys-bound palmitate on the insulin receptor [40] or, through an oxygen ester bond to Ser or threonine residues such as the Ser-bound palmitate in the gp37 protein of Rous sarcoma virus [41]. These ester bonds are similar in nature to that described in the CE for the Cer–Glu, in which the hydroxyl group of the Cer is bound by ester linkages to the carboxyl group of the Glu of involucrin (protein rich in Glu) [8,42].

Concerning the amino acid analysis, the amount of Glu/Gln detected in our experiments agrees with the results reported by other authors [18]. These Glu/Gln residues would provide carboxyl sites for the Cer attachment. It is interesting to note the large amount of Ser residue in the CE (11.1% in weight), which is one of the main amino acids of the loricrin. The structure of the Ser, which contains a free hydroxyl-group, would be appropriate to form esters bonds with the carboxyl group of the FFA detected in the hydrolyzed fraction. As in the case of Cer–Glu links, an alkaline hydrolysis would be necessary to break these linkages. Thus, our results indicate the existence in the CE of specific covalent bonds between FFA and Ser residues, in addition to that previously described between Cer and Glu residues. Fig. 6 shows two representative schemes of these ester bonds in the CE. The accepted mechanism is shown in Fig. 6A, where the omega-hydroxyl group of the Cer is bound to the hydroxyl from the carboxyl group of the Glu. The present work proposes a new complementary mechanism (Fig. 6B) in which the situation is the opposite. The FFA contributes with the hydroxyl of carboxyl group to link with the Ser, which involves the free hydroxyl group. The large percentage of the protein loricrin, which is rich in Ser residue [43], in the CE supports our hypothesis. Thus, we propose a new model for the arrangement of the lipid molecules in the CE, where not only Cer but also FFA could be involved. Fig. 7 displays the

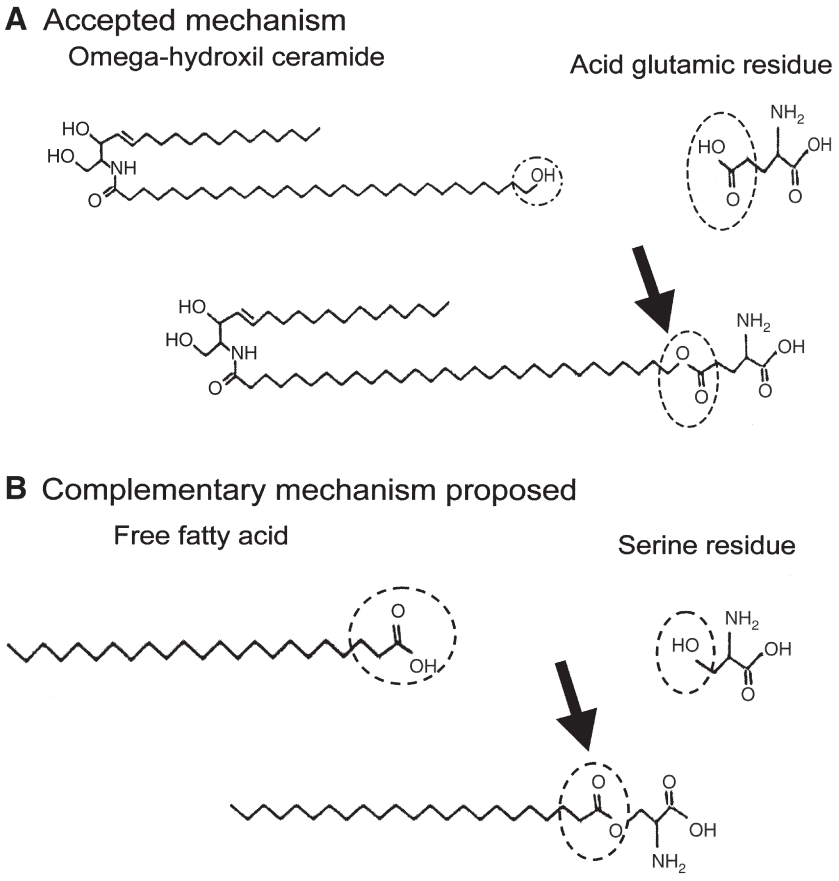

Fig. 6. Representative schemes of the two possible ester bonds in the CE. (A) Accepted mechanism in which the omega-hydroxyl group of the Cer is bound to the hydroxyl from the carboxyl group of the Glu. (B) New mechanism in which the FFA contributes with the hydroxyl of carboxyl group to link with the Ser that involves the free hydroxyl group.

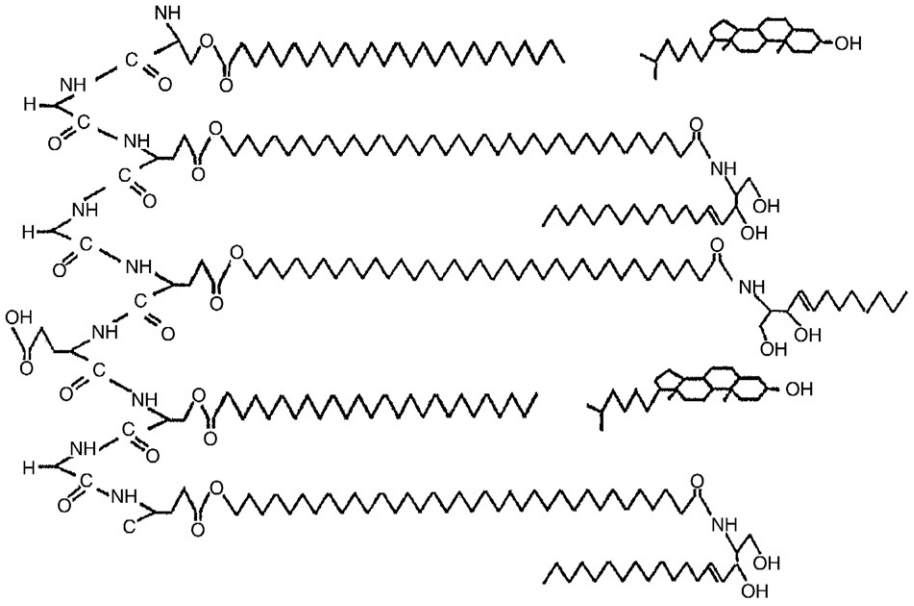

Fig. 7. Representative scheme of the assembly model for the CE. In this scheme the different options of ester links, Cer–Glu and FFA–Ser are shown.

assembly model for the CE showing the different options of ester links.

The data presented in this work point to an involvement of the Ser from loricrin protein in the covalent bond with lipids in the CE. This arrangement would imply the presence of loricrin at the extracellular surface of the CE, fact that our immunolabeling-TEM results confirmed. The use of Fab fragments increased the spatial resolution and the pre-embedding method complemented the information reported by the post-embedding experiments. Thus, pre-embedding procedure avoided the penetration of the antibodies allowing only the immunolabel of the proteins located at the extracellular site of the CE. The presence of label for loricrin protein indicates their possible external disposition.

This external disposition of the loricrin represents a new insight in the arrangement of the CE. Jardik [13] proposed that the involucrin lies mainly in the extracellular plane of the CE and that the loricrin molecules engage in side-by-side interactions just below the involucrin. Our results indicate an additional involvement of the loricrin in the CE based on the link of this protein with the CE lipids, specifically FFA. It is known that the loricrin is synthesized at a very late stage of epidermal differentiation [43]. Other envelope components may be expressed at earlier stages of differentiation and assembled into a scaffold upon which loricrin is cross-linked as a final step in envelope formation. In this regard, loricrin may be cross-linked to other components such as involucrin or even lipids from the CE, as our results indicate and other authors suggest [43].

Recently, in vitro studies using synthetic lipid vesicles have suggested that membrane-bound transglutaminase 1 (TG1) enzyme could engage the terminal omega-hydroxyl group of Cer to form an ester linkage in the CE [44]. However, CE with abundant, covalently bound omega-hydroxyl Cers are present in patients with an absence of TG1 activity [45]. This suggests that TG1 is not the essential, or even the normal enzymatic mechanism responsible for links lipid–protein of the CE [15,44]. The outside part of corneocytes was previously described as a Glu surface in which this amino acid was exposed to provide a carboxyl surface for the Cer attachment. However, the amount of Glu in the CE was not sufficient to support this Glu-surface hypothesis [42]. The deamination of Gln to Glu because of the TG1 seems to produce a surface on which all of the exposed residues are carboxyl groups fulfilling the requirements of the hypothesis [8]. In the absence of TG1 activity the situation would be then unfavourable to the formation of a complete CE. In the light of our results, an increased synthesis of FFA would be expected to form covalent bonds with the Ser from loricrin as a compensatory mechanism. This mechanism probably occurs in varying degrees also in healthy individuals. In addition, the existence of loricrin–FFA links may involve other enzymes that not only facilitate the Ser–FFA assembly but could also compensate the lack of TG1 in the formation of involucrin-Cer links. Thus, FFA in the CE due to the formation of a Glu–Ser-surface should be regarded as a new approach to the structure of this SC compartment supporting and complementing that reported by Downing [42].

This new arrangement of the CE in which not only Cer–Glu bonds but also FFA–Ser bonds are involved suggests a new role of the FFA in the CE functions. Thus, despite the important part played by the fatty acids in the cohesion and barrier function of the SC, the key role of these lipids should be attributed to the presence of FFA not only in the intercellular lamellae but also in the CE in the same way as Cers.

Acknowledgement

The authors would like to thank Pedro González from Transtechnics S.L. for providing technical and financial support for this project. We are also grateful to Dr. Jaime Notario for

providing human skin samples from the Universitary Hospital "Principes de España", Barcelona, Spain. We also thank Sonia Ruíz and Marta Taulés for helping in TEM work and Mr. G. von Knorring for expert technical assistance.

References

[1] R. Mendelsohn, C.R. Flach, D.J. Moore, Determination of molecular conformation and permeation in skin via IR spectroscopy, microscopy, and imaging, Biochim. Biophys. Acta 1758 (7) (2006) 923–933.
[2] D.J. Moore, R.G. Snyder, M.E. Rerek, R. Mendelsohn, Kinetics of membrane raft formation: fatty acid domains in stratum corneum lipid models, J. Phys. Chem., B 110 (5) (2006) 2378–2386.
[3] J.R. Hill, P.W. Wertz, Molecular models of the intercellular lipid lamellae from epidermal stratum corneum, Biochim. Biophys. Acta 1616 (2) (2003) 121–126.
[4] M. de Jager, W. Groenink, J. van der Spek, C. Janmaat, G. Gooris, M. Ponec, J. Bouwstra, Preparation and characterization of a stratum corneum substitute for in vitro percutaneous penetration studies, Biochim. Biophys. Acta 1758 (5) (2006) 636–644.
[5] J.A. Bouwstra, F.E. Dubbelaar, G.S. Gooris, A.M. Weerheim, M. Ponec, The role of ceramide composition in the lipid organisation of the skin barrier, Biochim. Biophys. Acta 1419 (2) (1999) 127–136.
[6] P.W. Wertz, K.C. Madison, D.T. Downing, Covalently bound lipids of human stratum corneum, J. Invest. Dermatol. 92 (1989) 109–111.
[7] L.N. Marekov, P.M. Steinert, Ceramides are bound to structural proteins of the human foreskin epidermal cornified cell envelope, J. Biol. Chem. 273 (1998) 17763–17770.
[8] M.E. Stewart, D.T. Downing, The omega-hydroxyceramides of pig epidermis are attached to corneocytes solely through omega-hydroxyl groups, J. Lipid Res. 42 (2001) 1105–1110.
[9] P.W. Wertz, D.T. Downing, Covalent attachment of omega-hydroxyacid derivatives to epidermal macromolecules: a preliminary characterization, Biochem. Biophys. Res. Commun. 137 (1986) 992–997.
[10] M. Ponec, .E. Boelsma, A. Weerheim, Covalently bound lipids in reconstructed human epithelia, Acta Derm.-Venereol. 80 (2000) 89–93.
[11] R.D. Leapman, M. Jarnik, A.C. Steven, Spatial distributions of sulfur-rich proteins in cornifying epithelia, J. Struct. Biol. 120 (2) (1997) 168–179.
[12] H.P. Baden, J. Kubilus, S.B. Philips, J.C. Kuedar, S.R. Tahon, A new class of soluble basic protein precursors of the cornified envelope of mammalian epidermis, Biochim. Biophys. Acta 925 (1) (1987) 63–73.
[13] M. Jarnik, M.N. Simon, A. Steven, Cornified envelope assembly: a model based on electron microscopic determinations of thickness and projected density, J. Cell Sci. 111 (1998) 1051–1060.
[14] P.J. Koch, P.A. de Viragh, E. Scharer, D. Bundman, M.A. Longley, J. Bickenbach, Y. Kawachi, Z. Suga, Z. Zhou, M. Huber, D. Hohl, T. Kartasova, M. Jarnik, A.C. Steven, D.R. Roop, Lessons from loricrin-deficient mice: compensatory mechanisms maintaining skin barrier function in the absence of a major cornified envelope protein, J. Cell Biol. 151 (2000) 389–400.
[15] P.M. Elias, M. Schmuth, Y. Uchida, R.H. Rice, M. Behne, D. Crumrine, K. R. Feingold, W.M. Holleran, D. Pharm, Basis for the permeability barrier abnormality in lamellar ichthyosis, Exp. Dermatol. 11 (2002) 248–256.
[16] N.D. Lazo, D.T. Downing, A mixture of alpha-helical and, 3_{10}-helical conformations for involucrin in the human epidermal corneocyte envelope provides a scaffold for the attachment of both lipids and proteins, J. Biol. Chem. 274 (1999) 37340–37344.
[17] D. Hohl, T. Mehrel, U. Lichti, M.L. Turner, D.R. Roop, P.M. Steinert, Characterization of human loricrin, J. Biol. Chem. 266 (1991) 6626–6636.
[18] A.C. Steven, P.M. Steinert, Protein composition of cornified cell envelopes of epidermal keratinocytes, J. Cell Sci. 107 (1994) 693–700.
[19] O. López, M. Cócera, A. de la Maza, L. Coderch, J.L. Parra, Different stratum corneum lipid liposomes as models to evaluate the effect of the sodium dodecyl sulfate, Biochim. Biophys. Acta 11508 (1–2) (2000) 196–209.
[20] O. López, M. Cócera, P. Walther, E. Wehrli, J.L. Parra, A. de la Maza, Liposomes as protective agents of stratum corneum against octyl glucoside: a study based on high-resolution, low-temperature scanning electron microscopy, Micron 132 (2001) 201–205.
[21] C. López-Iglesias, O. López, M. Cócera, D. Bellido, P. Walther, F. Gaill, A. de la Maza, Fixation for freeze fracture of stratum corneum: chemical fixation and propane-jet or high pressure methodologies, Biol. Cell 93 (2001) 325–329.
[22] O. López, M. Cócera, C. López-Iglesias, P. Walther, L. Coderch, J.L. Parra, A. de la Maza, Reconstitution of liposomes inside the intercellular lipid domain of the stratum corneum, Langmuir 18 (2000) 7002–7008.
[23] O. López, C. López-Iglesias, M. Cócera, P. Walther, J.L. Parra, A. de la Maza, Influence of chemical and freezing fixation methods in the freeze-fracture of stratum corneum, J. Struct. Biol. 146 (2004) 302–309.
[24] J.A. Bouwstra, G.S. Gooris, F.E.R. Dubbebar, A.M. Weerheim, A.P. Ijzerman, M. Ponec, Role of ceramide 1 in the molecular organization of the stratum corneum lipids (1998).
[25] M.W. Jager, G.S. Gooris, M. Ponec, J.A. Bouwstra, Lipid mixtures prepared with well-defined synthetic ceramides closely mimic the unique stratum corneum lipid phase behaviour, J. Lipid Res. 46 (2005) 2649–2656.
[26] T. Herrmann, F. Van der Hoeven, H.J. Gröne, A.F. Stewart, L. Langbein, I. Kaiser, G. Liebisch, I. Gosch, F. Buchkremer, W. Drobnick, G. Schmitz, W. Stremmel, Mice with targeted disruption of the fatty acid transport protein 4(Fato4, Slc27a4) gene show features of lethal restrictive dermopathy, J. Cell Biol. 161 (2003) 1105–1115.
[27] P.W. Wertz, D.C. Swartzendruber, D.J. Kitko, K.C. Madison, D.T. Downing, The role of the corneocytes lipid envelopes in cohesion of the stratum corneum, J. Invest. Dermatol. 93 (1989) 169–172.
[28] O. López, A. de la Maza, L. Coderch, J.L. Parra, Formation and characterization of liposomes from lipid/protein material extracted from pig stratum corneum, J. Am. Oil Chem. Soc. 73 (1996) 443–448.
[29] D.C. Swartzendruber, D.J. Kitko, P.W. Wertz, K.C. Madison, D.T. Downing, Isolation of corneocyte envelopes from porcine epidermis, Arch. Dermatol. Res. 280 (1988) 424–429.
[30] J. Fonollosa, M. Martí, A. de la Maza, J.L. Parra, L. Coderch, TLC-FID analysis of the ceramide content of internal wool lipids, J. Planar Chromatogr. 13 (2000) 119–122.
[31] J.F. Hainfeld, A small gold-conjugated antibody label: improved resolution for electron microscopy, Science 236 (4800) (1987) 450–453.
[32] J.F. Hainfeld, R.D. Powell, New frontiers in gold labeling, J. Histochem. Cytochem. 48 (4) (2000) 471–480.
[33] H. Schaefer, T.E. Redelmeier, Skin barrier, Principles of Percutaneous Absorption, Karger, Basel, 1996, p. 55.
[34] P.M. Steinert, L.N. Marekov, Direct evidence that involucrin is a major early isopeptide cross-linked component of the keratinocyte cornified cell envelope, J. Biol. Chem. 272 (1997) 2021–2030.
[35] S. Kazerounian, S. Aho, Characterization of periphilin, a widespread, highly insoluble nuclear protein and potential constituent of the keratinocyte cornified envelope, J. Biol. Chem. 278 (2003) 36707–36717.
[36] H.H. Epperlein, H. Schwarz, T. Piendl, J. Lofberg, D. Studer, H. Spring, M. Muller, Improved preservation of the subepidermal extracellular matrix in axolotl embryos using electron microscopical techniques based on cryoimmobilization, J. Struct. Biol. 118 (1997) 43–61.
[37] M. Behne, Y. Uchida, T. Seki, P. Ortiz de Montellano, P.M. Elias, W.M. Holleran, Omega-hydroxyceramides are required for corneocyte lipid envelope (CLE) formation and normal epidermal permeability barrier function, J. Invest. Dermatol. 114 (2000) 185–192.
[38] L.N. Jones, D.E. Rivett, The role of 18-methyleicosanoic acid in the structure and formation of mammalian hair fibres, Micron 28 (1997) 469–485.
[39] J.R. Smith, J.A. Swift, Lamellar subcomponents of the cuticular cell membrane complex of mammalian keratin fibres show friction and hardness contrast by AFM, J. Microsc. 206 (2002) 182–193.
[40] M.B. Omary, I.S. Trowbridge, Covalent binding of fatty acid to the transferring receptor in cultured human cells, J. Biol. Chem. 256 (1981) 4715–4718.
[41] A. Gebhardt, J.V. Bosch, A. Ziemiecki, R.R. Friis, Rous sarcoma virus p19 and gp35 can be chemically crosslinked to high molecular weight complexes. An insight into virus assembly, J. Mol. Biol. 174 (1984) 297–317.

[42] D.T. Downing, Lipid and protein structures in the permeability barrier of mammalian epidermis, J. Lipid Res. 33 (1992) 301–313.
[43] T. Mehrel, D. Hohl, J.A. Rothnagel, M.A. Longley, D. Bundman, C. Cheng, U. Lichti, M.E. Bisher, A.C. Steven, P.M. Steinert, S.H. Yuspa, D.R. Roop, Identification of major keratinocyte cell envelope protein, loricrin, Cell 61 (1990) 1103–1112.
[44] Z. Nemes, L.N. Marekov, L. Fésus, P.M. Steinert, A novel function for transglutaminase 1: Attachment of long-chain omega-hydroxyceramides to involucrin by ester bond formation, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 8402–8407.
[45] P. Djian, K. Easley, H. Green, Targeted ablation of the murine involucrin gene, J. Cell Biol. 151 (2000) 381–387.