The ω-hydroxyceramides of pig epidermis are attached to corneocytes solely through ω-hydroxyl groups

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Abstract The cornified outer cells of mammalian epidermis possess a monolayer of ω-hydroxyceramides that are ester-linked to the exterior of a cross-linked protein envelope. In the present study, conclusive evidence was sought on which of the ceramide hydroxyl groups are involved in the linkage to protein. This was obtained by derivatizing all free hydroxyl groups in isolated solvent-extracted porcine stratum corneum using triisopropylsilyl (TIPS) chloride in pyridine in the presence of silver nitrate. After an 18-h reaction, the tissue was recovered, rinsed, and the derivatized ceramides were then released from protein linkage by hydrolysis with 1M KOH in 95% methanol. This gave a single ceramide product that was shown by nuclear magnetic resonance to contain two triisopropyl groups. Acetylation of the product using acetic anhydride in pyridine resulted in a downfield shift of the NMR signal for the ω-methylene protons, showing that it was the ω-hydroxyl that was free in the initial reaction product, and subsequently was acetylated. These results show that all of the ω-hydroxyceramides of corneocyte lipid envelopes are attached to protein through their ω-hydroxyl groups.— Stewart, M. E., and D. T. Downing. **The ω-hydroxyceramides of pig epidermis are attached to corneocytes solely through ω-hydroxyl groups.** ***J. Lipid Res.*** **2001.** 42: **1105–1110.**

Supplementary key words NMR • protein envelope • epidermal lipids • ω-hydroxyacids

The mammalian stratum corneum consists of layers of flattened corneocytes with multiple lipid lamellae lying between them. Polar solvents remove most of the lipid, but even after exhaustive extraction, the cells remain coated with a bound lipid monolayer, which can be released by mild alkaline hydrolysis (1). The ease of hydrolysis indicates that it is ester bonds that link the lipid with the protein. Analysis has shown that the bound lipid of porcine stratum corneum consists predominantly of ceramides formed from sphingosine in amide linkage with 30- to 34-carbon ω-hydroxyacids (1). This class of molecules has three hydroxyl groups that possibly could participate in an ester link with the corneocyte protein envelope. These are the hydroxyls on the 1 and 3 carbons of the sphingosine moiety and the ω-hydroxyl of the fatty acid moiety.

Shortly after the bound ceramides were discovered, an attempt was made to ascertain which hydroxyl group(s) was involved in the linkage (2). Extracted porcine stratum corneum was treated with acetone in the presence of a trace of acid to form an adduct linking the sphingosine hydroxyl groups. This adduct is stable to alkaline hydrolysis, so it was possible to free the bound lipid and to determine what percentage of the ceramide molecules had reacted with acetone. The result showed that less than 50% of the molecules had formed acetonides, so it was concluded that about half of the bound lipid was bound by a hydroxyl on the 1 or 3 carbon of sphingosine and the remainder by the ω-hydroxyl. However, Nemes et al. (3), using an in vitro system that attached a hydroxyceramide analog to involucrin, found that 90% of the bound ceramide could form an acetonide.

In this article, we revisit the question of how the hydroxyceramide is bound in vivo. In doing this, we looked for a different reagent for derivatizing the free hydroxyl groups. We found that triisopropylsilyl (TIPS) chloride (4) was satisfactory, giving complete reaction and producing derivatives that are stable to mild alkaline hydrolysis. Also, because this reagent reacts with individual hydroxyl groups rather than requiring a 1,2- or 1,3-diol, as in the case of acetone, any free ω-hydroxyl groups would be derivatized. Our results with this reagent indicated that the hydroxyceramides are bound to corneocytes solely by the ω-hydroxyl.

MATERIALS AND METHODS

Porcine stratum corneum

The carcass of a freshly killed pig was carefully shaved, first with animal clippers and then with a rotary shaver. Disks of epidermis were removed by 30-s application of a hot (65°C) aluminum cylinder, followed by scraping with a spatula. The disks of epidermis were then incubated overnight in 0.5% trypsin in water at 20°C to digest the noncornified cells. After a second trypsin digestion, the remaining stratum corneum was rinsed in water and allowed to dry in air. The dry tissue was extracted serially with chloroform–methanol mixtures (2:1, 1:1, and 1:2, by volume) and then twice

Abbreviation: TIPS, triisopropylsilyl.

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more with choroform–methanol (2:1, v/v) and twice with chloroform–methanol (2:1, v/v) containing 1% acetic acid. The exhaustively extracted tissue was then allowed to dry in air.

TIPS derivatization

TIPS chloride converts hydroxyl groups to silyl ethers that are resistant to alkaline hydrolysis (4). In a typical reaction, dry, solvent-extracted stratum corneum (260 mg) was added to a mixture of 4 ml TIPS chloride (Sigma, St Louis, MO) in 20 ml pyridine containing 1 g of silver nitrate. After stirring overnight at 20°C, the tissue was removed, rinsed three times in pyridine, and then allowed to dry in air. The dry, derivatized tissue was then placed in 1M KOH in 95% methanol (50 ml) at 60°C for 1 h. The hydrolysis mixture plus 100 ml of chloroform and 30 ml of water were added to a separatory funnel and shaken. The mixture was acidified with 10 g of citric acid and again shaken. The chloroform layer was withdrawn and the aqueous layer was shaken with fresh chloroform. The combined chloroform extracts were washed with water and then evaporated in a rotary evaporator. The lipid residue was recovered in chloroform.

Lipid purification

The ceramides that were recovered after hydrolysis of the underivatized tissues were isolated by preparative thin layer chromatography on silica gel G plates (Analtech, Newark, DE), using chloroform–methanol–acetic acid (190:9:1, v/v/v) for development. The ceramides that were derivatized with TIPS were isolated by thin-layer chromatography (TLC) using chloroform–ethanol (100:1, v/v) for development. For visualization, the preparative chromatograms were sprayed with 0.01% 8-hydroxy-1,3,6-pyrenetrisulfonic acid trisodium salt in ethanol and dried, then examined under ultraviolet irradiation. The ceramide band in each chromatogram was scraped into a short glass column and the lipid was eluted with chloroform–methanol (2:1, v/v).

Acetylation

To verify the number and location of free hydroxyl groups in the TIPS-derivatized and underivatized hydroxyceramides, the respective hydrolysis products were acetylated using acetic anhydride–pyridine (1:1, v/v) at 20°C for 2 h, and then the reagents were removed with a stream of nitrogen. The acetylation products were isolated by thin layer chromatography using chloroform–ethanol (200:1, v/v) as the developing solvent.

Nuclear magnetic resonance

The ceramides recovered from derivatized and underivatized stratum corneum, and their respective acetylation products, were each dissolved in deuterochloroform or, in the case of the underivatized hydroxyceramides, which are less soluble in chloroform, deuterochloroform–deuteromethanol (2:1, v/v). The solutions were examined by NMR spectroscopy in a Bruker AMX 600 NMR spectrometer.

Thin-layer chromatography

The hydrolysis products of untreated stratum corneum, and of stratum corneum that had been treated with TIPS chloride, were analyzed by analytical thin layer chromatography on silica gel G plates using chloroform–methanol–acetic acid (190:9:1, v/v/v) for development. The chromatograms were visualized by spraying with 50% H_2SO_4 followed by charring on a hotplate at 220°C for 45 min. The standards for the underivatized and TIPS-derivatized hydroxyceramides were obtained using the isolation and identification procedures described above. The acetate groups added for the NMR analysis were removed by alkaline hydrolysis. The ω-hydroxyacid standard was prepared by alkaline hydrolysis of giant-ring lactones that had been isolated from donkey skin surface lipid (5).

RESULTS

TIPS derivatives of porcine corneocyte envelope ceramides

Under the conditions described, the lipids recovered by hydrolysis from porcine stratum corneum that had been treated with TIPS chloride contained only one ceramide product, which was much more mobile on TLC than the corresponding product from underivatized tissue (**Fig. 1**). The recovery of a single product demonstrates that TIPS derivatization of the ceramide free hydroxyl groups went to completion in pig stratum corneum, and that the product survived subsequent alkaline hydrolysis intact. Subsequent vigorous acid hydrolysis of the tissue remaining after the alkaline hydrolysis failed to yield any additional lipid that might have resisted liberation because of steric hindrance by the TIPS groups.

NMR spectra indicated that the derivatized material was the hydroxyceramide in which both of the sphingosine hydroxyls were derivatized. This conclusion was supported

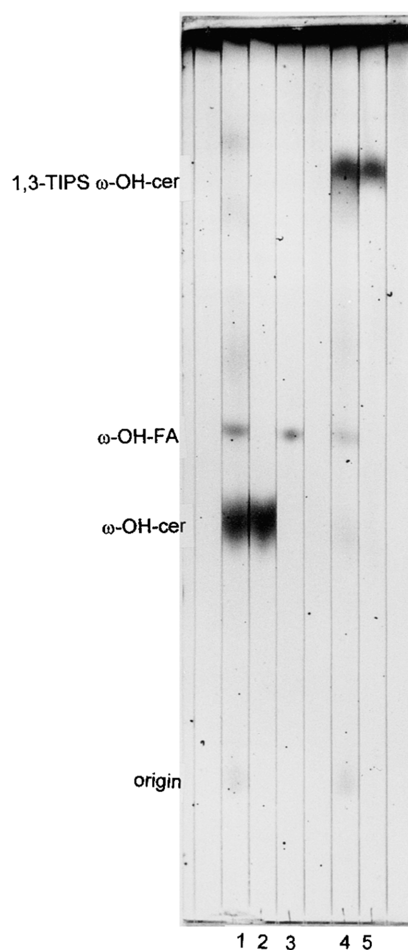

Fig. 1. Thin layer chromatograms of the lipids released from pig stratum corneum, developed with chloroform–methanol–acetic acid (190/9/1, v/v/v) on silica gel G. Lane 1: Bound lipids released from solvent-extracted pig stratum corneum; lane 2: ω-Hydroxyceramide standard; lane 3: ω-Hydroxyacid standard; lane 4: Bound lipids released from pig stratum corneum after TIPS derivatization; lane 5: TIPS derivative of ω-hydroxyceramide after isolation by TLC.

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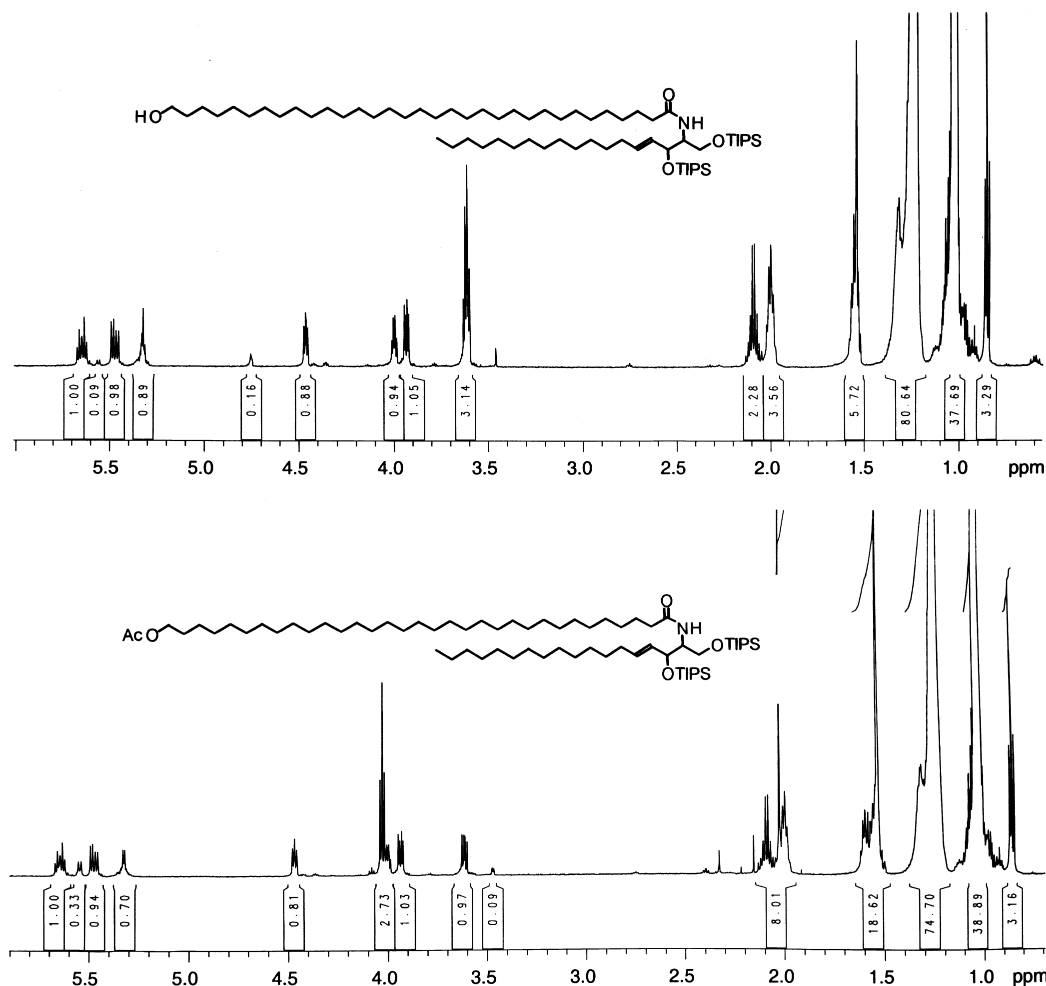

Fig. 2. Top: Proton NMR spectrum of isolated TIPS-derivatized ω-hydroxyceramide released from TIPS-treated pig stratum corneum. Bottom: Spectrum of isolated TIPS-derivatized ω-hydroxyceramide after acetylation.

by a small downfield shift of the protons attached to the respective hydroxyl-bearing carbons at the sphingosine 1 and 3 posititions, as compared to the nonderivatized hydroxyceramide (**Fig. 2** and **Table 1**). In contrast, the methylene protons of the carbon bearing the ω-hydroxyl had the same chemical shift in the derivatized and underivatized hydroxyceramides (Table 1). This result indicates that the ω-hydroxyl had been protected from reaction with TIPS chloride by attachment to the protein of the corneocyte envelope. The status of this ω-hydroxyl group was confirmed by acetylation, which added an NMR signal at 2.02 ppm equivalent to one acetate group (3-proton singlet), and shifted the ω-methylene signal (2-proton triplet) from 3.61 ppm to 4.03 ppm (Fig. 2 and Table 1).

A minor product of hydrolysis (about 10% of the total) occurred in the same chromatographic position before and after derivatization of the tissue with TIPS chloride, with the same TLC mobility as a free ω-hydroxyacid standard. This product is unlikely to have been produced by alkaline hydrolysis of hydroxyceramides, which should be resistant to the mild treatment used. It seems more plausible that the hydroxyacid resulted from the in vivo action of ceramidase on the ω-hydroxyceramides attached to the protein envelope, releasing free sphingosine (6), which would have been removed in the initial lipid extractions.

It was observed that after reaction of the tissue with the TIPS chloride, rinsing of the tissue in pyridine was essential. In experiments where the derivatized tissue was only rinsed in methanol prior to alkaline hydrolysis, a major artifact was produced. The substance ultimately was found by NMR to also bear two TIPS derivatives on the sphingosine hydroxyls, whereas the ω-methylene signal was shifted downfield to the location obtained for the acetylated ω-hydroxyl. However, no unaccounted proton signals

TABLE 1. NMR chemical shifts of derivatives of porcine epidermal ω-hydroxyceramide

Proton Location	1-H_A	1-H_B	2-H	3-H	ω-H	2′-H
ω-Hydroxyceramide	3.60	3.75	3.80	4.08	3.51	2.15
1,3,ω-Triacetate	4.01	4.28	4.43	5.33	4.02	2.00
1,3,ω-TIPS	3.61	3.93	4.00	4.47	3.65	1.99
1,3-TIPS	3.61	3.93	4.00	4.46	3.61	2.02
1,3-TIPS-ω-acetate	3.61	3.95	4.00	4.47	4.03	1.99

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were found in the NMR spectrum, and attempted acetylation left the artifact unchanged, as judged by TLC and NMR. As a result, the structure of the artifact remains unclear, but its production can be completely avoided by removal of the reagents in pyridine after the TIPS derivatization reaction.

DISCUSSION

Structures of the TIPS derivatization products

The structure of the single ceramide product obtained by TIPS derivatization of pig stratum corneum was deduced by comparison of the the proton NMR spectra of the lipid and its acetylation product with the spectra of the corresponding lipids obtained without TIPS derivatization of the tissue. Only the signals from protons on the sphingosine head group were shifted as the result of the derivatization, which added two TIPS groups (as determined by area integration of the NMR signal for the TIPS methyl groups at 1.05 ppm). Subsequent acetylation of the TIPS product shifted only the characteristic triplet signal produced by the ω-methylene protons of the ω-hydroxyacid moiety, providing conclusive evidence that the ω-hydroxyl group was underivatized in the single TIPS product, and must therefore have been protected by its chemical connection to the protein envelope.

Structure of the corneocyte lipid envelope

On the basis of previous results (2), the ω-hydroxyceramides that form the corneocyte lipid envelope were thought to alternate in their orientation, providing for an orderly packing in the resulting monolayer (7, 8). With the present demonstration that only one orientation of the bound hydroxyceramides exists, a new postulate is required to explain the molecular structure of the corneocyte lipid envelope. **Figure 3A** represents a plausible structure for the lipid envelope as it might exist in porcine

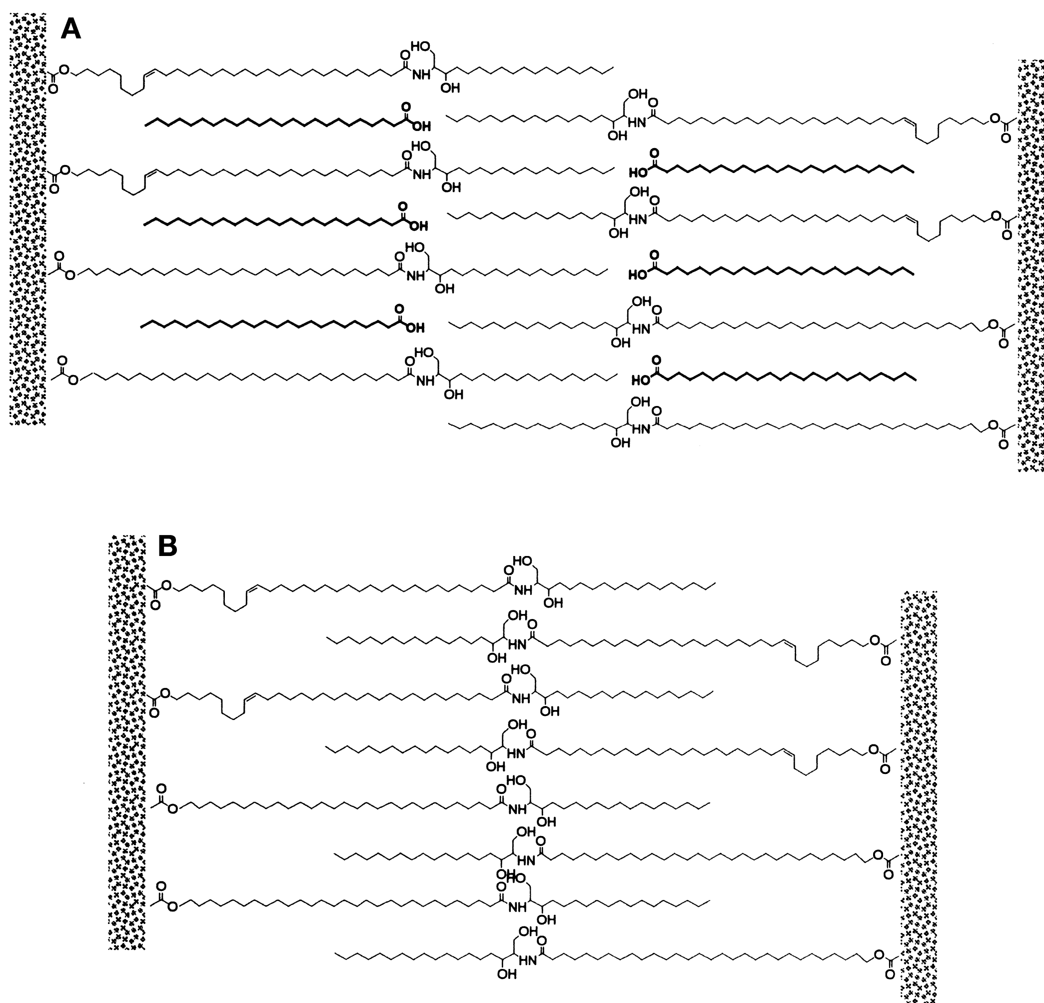

Fig. 3. A: Molecular structure proposed for corneocyte lipid envelopes as they might exist in porcine stratum corneum that has not been extracted with solvents, in regions where intercellular lamellae between the corneocyte envelopes are absent. Unbound fatty acids that may contribute to the structure of the envelopes are shown in bold. B: Structure proposed for the corneocyte lipid envelopes in porcine stratum corneum after extraction of the fatty acids.

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stratum corneum that has not been extracted with solvents, in the regions where the structure is not complicated by the presence of unbound intercellular lipid lamellae (7). As in prior formulations of the lipid envelope (7, 9), sphingosine chains of the ceramides are folded out and interdigitated with those of the corresponding chains of an adjacent corneocyte. This arrangement was proposed previously to explain the apparent lipid monolayer that is seen by electron microscopy (EM) between the lipid envelopes of apposing corneocytes (7, 9), and is retained in the present postulate.

In both the present and previous formulations, orderly packing of the envelope ceramides to form the characteristic *broad-narrow-broad* pattern of lucent bands in the EM (9) requires the involvement of additional unbound lipids. The available unbound lipids have been shown to consist principally of cholesterol (25%), ceramides (45%), and free fatty acids (15%). The free fatty acids are predominantly saturated and are 22 and 24 carbons long (10), which would be suitable for completing the native corneocyte lipid envelopes, as visualized in Fig. 3A.

Removal of the unbound lipids by solvent extraction results in an EM pattern where only two broad lucent bands are seen between apposing corneocytes, representing one lipid envelope from each of the contacting cells (7, 9). Figure 3B shows a possible orientation of the ceramides in the lucent bands between cells in solvent extracted porcine stratum corneum. The sphingosine chains are shown everted and fully interdigitated between adjacent lipid envelopes, in part to explain the tight adherence that resists all attempts to disperse the cells of solvent extracted stratum corneum.

Previous studies showed that the very long chain ω-hydroxyacid moieties of the envelope hydroxyceramides contain saturated (50%) and monounsaturated (45%) components (10). The saturated ω-hydroxyacids are predominantly 30 carbons in length, and the monounsaturates have 32 carbons. The location of the double bonds in the unsaturated acids has not been determined, but it is likely that the long-chain monounsaturates are biosynthesized by chain extension of oleic acid, in which case the unsaturation would be located at the ω9 position. A corresponding location has been shown for the unsaturation in the 32-carbon branched-chain ω-hydroxyacids found as lactones in horse sebum (11). Therefore, the monounsaturated ω-hydroxyacids in mammalian epidermis are assumed to have unsaturation at the ω9 location, as drawn in the representations of the lipid envelope in Fig. 3. The likely biophysical effects of such unsaturation include the fluidization of the distal ends of the hydroxyacid chains, aiding accommodation of the disparate chain lengths in this region. Also, terminal chain fluidization would assist in matching the ω-hydroxyl groups with the carboxyl side chains of the protein to which they become attached.

It should be noted that the bound lipids of human stratum corneum contain two classes of hydroxyceramides. About 60% of the hydroxyceramides contain sphingosine, as in pig, and the other 40% contain 6-hydroxysphingosine, a type of sphingosine that has only been found in humans (12). 6-Hydroxysphingosine is also found in three classes of free ceramides of human skin. The fatty acid moieties of these three classes are: nonhydroxyacids, α-hydroxyacids, or ω-hydroxyacids having a nonhydroxyacid esterified to the ω-hydroxyl group (12, 13).

The nature of the protein substrate bearing the lipid envelopes

The surface of the corneocyte envelope must provide a sufficient surface density of binding sites to accommodate all of the closely packed hydroxyceramide molecules. The density of sites required can be calculated from the cross-sectional area of the lipid chains in the ceramide monolayer. Lipid chains packed in crystalline array are known to have an average cross-sectional area of 19 square Ångstroms (14), and in the proposed formulation, each ceramide effectively provides two such chains, giving it a cross-sectional area of 38 $Å^2$. Therefore, the substrate protein must provide a similar surface density of carboxyl side chains.

In humans, involucrin has been shown to provide binding sites for hydroxyceramide molecules (15). It also has been shown that, in vitro, the epidermal transglutaminase1 enzyme can catalyze the attachment of hydroxyceramides to human involucrin, using glutamine residues as the points of attachment and producing an ester bond (3). Similar information is not available for pigs, in which involucrin has a different amino acid sequence (16, 17). However, pig involucrin, like human involucrin, meets the requirements of a substrate in having a sufficient density of glutamine residues in the repeat region to bind all of the closely packed hydroxyceramide molecules. An alternative possibility is that the hydroxyceramides are bound to glutamate residues, of which pig involucrin also has a sufficient density.

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