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Sphingolipid Activator Proteins Are Required for Epidermal Permeability Barrier Formation*

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The epidermal permeability barrier is maintained by extracellular lipid membranes within the interstices of the stratum corneum. Ceramides, the major components of these multilayered membranes, derive in large part from hydrolysis of glucosylceramides mediated by stratum corneum β-glucocerebrosidase (β-GlcCerase). Prosaposin (pSAP) is a large precursor protein that is proteolytically cleaved to form four distinct sphingolipid activator proteins, which stimulate enzymatic hydrolysis of sphingolipids, including glucosylceramide. Recently, pSAP has been eliminated in a mouse model using targeted deletion and homologous recombination. In addition to the extracutaneous findings noted previously, our present data indicate that pSAP deficiency in the epidermis has significant consequences including: 1) an accumulation of epidermal glucosylceramides together with below normal levels of ceramides; 2) alterations in lipids that are bound by ester linkages to proteins of the cornified cell envelope; 3) a thickened stratum lucidum with evidence of scaling; and 4) a striking abnormality in lamellar membrane maturation within the interstices of the stratum corneum. Together, these results demonstrate that the production of pSAP, and presumably mature sphingolipid activator protein generation, is required for normal epidermal barrier formation and function. Moreover, detection of significant amounts of covalently bound ω-OH-GlcCer in pSAP-deficient epidermis suggests that deglucosylation to ω-OH-Cer is not a requisite step prior to covalent attachment of lipid to cornified envelope proteins.

Sphingolipid activator proteins (SAPs[1] or saposins) are nonenzymatic glycoprotein cofactors required for lysosomal degradation of various glycosphingolipids by exohydrolases (1, 2). Proteolytic cleavage of SAP precursor (pSAP) in acidic compartments releases four homologous polypeptides (*i.e.* SAP-A, SAP-B, SAP-C, and SAP-D), thought to function either by direct activation of their respective enzyme or as biological detergents that lift substrates out of the membrane plane (3–5). One of the activator proteins, SAP-C, has been shown to stimulate lysosomal glucosylceramide (GlcCer) degradation by β-glucocerebrosidase (β-GlcCerase) (6, 7). A deficiency of SAP-C causes a variant form of Gaucher disease (8–10).

In terrestrial mammals, the permeability barrier that restricts excess transepidermal water loss is maintained by unique arrays of lamellar membranes localized in the interstices of the outermost stratum corneum layer of the epidermis. These lamellar arrays derive from the extruded lipid contents of lamellar bodies after fusion of these organelles with the apical plasma membrane of the outermost granular cell. The initially secreted lipids are processed by a set of co-localized lipid hydrolases into a more hydrophobic mixture, enriched in ceramides (Cer). The resultant Cer, comprising a family of compounds with complex chemical structures, together with free fatty acids and cholesterol are the dominant constituents of the stratum corneum (SC) lamellar membranes (11). Two of these species, which possess an ω-hydroxy moiety on the *N*-acyl fatty acid, are covalently bound to structural proteins of the cornified cell envelope forming a hydrophobic surface on terminally differentiated keratinocytes (12, 13).

The postsecretory processing of lamellar body-derived GlcCers to ceramides by β-GlcCerase is essential for maintenance of permeability barrier homeostasis (14–17). Because SAP-C stimulates lysosomal GlcCer degradation *in vivo*, it seems reasonable to predict that expression of pSAP would also be important for epidermal barrier formation. Decreased levels of pSAP have been detected in atopic skin, suggesting a role for SAPs in epidermal function (18). Furthermore, a 20-week pSAP-deficient human fetus, with a phenotype resembling acute neuronopathic (type 2) Gaucher disease, displayed extensive cutaneous lysosomal storage deposits and neutral glycosphingolipid accumulation in dermal fibroblasts (19). A SAP precursor-deficient mouse (pSAP −/−) has recently been created by targeted gene disruption and homologous recombination (20). Because of the deficiency of the complete precursor protein, none of the four SAPs are generated in any cell type. Initial characterization of these mice exhibited two distinct phenotypes, *i.e.* neonatally fatal and later onset. The pathology of the latter is complex, including severe hypomyelination and storage of multiple sphingolipids in the brain (20, 21), as well as an ichthyosiform dermatosis. Thus, in the present work, we focused on the physiologic function of pSAP in the context of cutaneous permeability barrier formation and investigated the effects of pSAP deficiency on epidermal lipid composition and structure.

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[1] The abbreviations used are: SAP, sphingolipid activator protein; pSAP, prosaposin; β-GlcCerase, β-glucocerebrosidase; Cer, ceramide; GlcCer, glucosylceramide; N, non-hydroxy fatty acid; A, α-hydroxy fatty acid; O, ω-hydroxy fatty acid; S, sphingosine; P, phytosphingosine; E, esterified; MALDI, matrix-assisted laser desorption ionization; SC, stratum corneum; SM, sphingomyelin; SMase, sphingomyelinase.

MATERIALS AND METHODS

Animals—Generation of SAP-deficient mice through targeted gene disruption has been described previously (20). Newborn knockout mice (homozygous, $n = 9$; heterozygous, $n = 8$) as well as newborn wild-type littermates ($n = 7$) were studied. Genotyping was performed by polymerase chain reaction. The primers used had the following sequence: forward primer, 5′-GCC CAC AGC GGT GAG TGC-3′, matching on the pSAP genomic DNA on exon 2/intron 2; reverse primer, 5′-CAG-CAA GTT CCC AGC TTC GG-3′, matching on exon 3. With those primers a band of 1300 bp appeared in the pSAP −/− mice, a band of 806 bp appeared in pSAP +/+ mice, and both bands appeared in pSAP +/− mice.

Preparation of Epidermis—Whole skin was removed at autopsy from newborn mice. Epidermis was separated from dermis after floating the skin on Dispase (Boehringer Mannheim, grade II) diluted 1:1 in Hanks' buffer at 4 °C overnight.

Lipid Analysis—Tissue samples were homogenized, lyophilized, and weighed. Epidermal lipids were extracted for 24 h at 37 °C in each of three solvent mixtures (chloroform/methanol/water 1:2:0.5 (v/v/v); chloroform/methanol 1:1 (v/v), and chloroform/methanol 2:1 (v/v). Total lipid extracts were applied to thin-layer Silica Gel 60 plates (Merck Darmstadt, Germany). All plates were washed with chloroform/methanol (9:1, v/v) before sample application. For separation of glucosylceramides, the chromatograms were developed with chloroform/methanol/water (70:30:5, v/v/v). Ceramides were resolved twice using chloroform/methanol/acetic acid (190:9:1, v/v/v) as developing solvent. For quantitative analytical TLC determination, increasing amounts of standard lipids (*N*-stearoyl-sphingosine (kind gift of Beiersdorf AG, Hamburg, Germany), sphingomyelin (Sigma), and glucosylceramide (purified from Gaucher spleen in our laboratory)) were applied. After development, plates were air-dried, sprayed with 8% (w/v) H_3PO_4 containing 10% (w/v) $CuSO_4$, and charred at 180 °C for 10 min, and lipids were quantitated by photodensitometry (Shimadzu, Kyoto, Japan).

Recovery and Analysis of Covalently Bound Lipids—After sequential extraction of epidermal samples as described above, pellets were extracted three times for 5 min at room temperature with methanol followed by two additional extractions for 2 h with 95% methanol at 60 °C. The final fraction was checked for lipid content. Covalently bound lipids were released by incubation with 1 ml of 1 M KOH in 95% methanol for 2 h at 60 °C. Lipids were subsequently recovered by adding 2 ml of water and 2 ml of chloroform. The chloroform layer was removed, and the aqueous phase was extracted once more with 2 ml of chloroform. The organic phases were combined and evaporated under a stream of nitrogen. Lipids were applied to prewashed (chloroform/methanol 9:1, v/v) thin-layer Silica Gel 60 plates (Merck) and separated using 2× chloroform/methanol/acetic acid (190:9:1, v/v/v) as developing solvent. Quantification of lipids was performed as described above. For quantification of fatty acids, increasing amounts of palmitic acid (Fluka, Buchs, Switzerland) were applied to the plates.

MALDI Mass Spectrometry of Lipids—Aliquots of lipid extracts were separated by TLC techniques as described above. Individual sphingolipids were scraped from plates and redissolved in chloroform/methanol 1:1 (v/v). Approximately 50–200 pmol of lipid were mixed with 20 μg of 2,5-dihydroxybenzoic acid (ICN Biochemicals) directly on the target. Mass spectrometric analysis was performed on a TofSpec E (MicroMass, Manchester, UK) mass spectrometer operating at an acceleration voltage of 20 kV with a 337-nm nitrogen laser. External calibration was performed using PEG-1000 (Sigma).

Light and Electron Microscopy—Skin samples were taken at autopsy, either immediately or 24 h after birth, and processed for light and electron microscopy. For light microscopy, samples were fixed by immersion in phosphate-buffered paraformaldehyde (4%), embedded in paraffin, sectioned (5 μm), stained with hematoxylin and eosin, and photographed with a Zeiss Axioplan microscope. For electron microscopy, samples were minced to <0.5 mm, fixed overnight in HEPES buffer (0.16 M, pH 7.3) containing glutaraldehyde/formalin (1.25:0.5%) and 0.1 mM $CaCl_2$, and postfixed in both ruthenium tetroxide, as described previously (22), and 2% aqueous osmium tetroxide, each containing 1.5% potassium ferrocyanide. After fixation, all samples were dehydrated in graded ethanol solutions and embedded in an Epon/epoxy mixture. Ultrathin sections were examined, with or without further contrasting with lead citrate, in an electron microscope (Zeiss 10A, Carl Zeiss, Thornwood, NY) operated at 60 kV.

Statistical Analysis—Statistical evaluation of data was performed using a two-tailed Student's *t* test.

RESULTS

Epidermal Sphingolipids in pSAP Knockout Mice—To address the question of whether the pSAP product SAP-C is involved in the processing of epidermal GlcCer to Cer and therefore important for epidermal barrier formation, we first determined the GlcCer and Cer levels in the epidermis of pSAP knockout mice. After extraction, sphingolipids were separated by TLC and quantitated by photodensitometry. Aliquots of lipid extracts taken in parallel were used to isolate individual sphingolipids by TLC for MALDI mass spectrometric analysis. Although we had proposed likely compositions for sphingolipid structures, we could not distinguish differences in fatty acid and sphingoid chain length distribution from masses of intact molecular ions. In this work, structural data are presented in accordance with the epidermal Cer terminology proposed by Motta *et al.* (23) and modified by Robson *et al.* (24). Briefly, the Cer structures are denoted by the composition of the sphingoid base (either sphingosine (S) or phytosphingosine (P)) and the *N*-acyl fatty acid by the presence of α-hydroxy group (A), ω-hydroxy group (O), or no-hydroxy group (N) and whether the ω position is further acylated (*i.e.* esterified (E)).

Distinct differences in the epidermal lipid composition were evident between pSAP-deficent and wild-type animals. First, the content of each of the three distinct GlcCer fractions that were readily separated by TLC (see mass spectrometric analysis for structural assignments below) was significantly elevated in the epidermis of pSAP −/− *versus* wild-type littermates (Fig. 1, *A* and *C*). In the absence of pSAP, GlcCer(EOS) accumulated 5.2-fold ($p < 10^{-8}$, $n = 9$), whereas the more polar (i.e., lower R_f value) GlcCer A/B and GlcCer(C16-AS) accumulated 2.1-fold ($p < 10^{-4}$) and 2.2-fold ($p < 0.02$), respectively. In heterozygous mice, which demonstrate nearly 50% reduction in the expression of pSAP protein (20), only the GlcCer(EOS) fraction showed significant accumulation (1.4-fold, $p < 0.03$), whereas the other GlcCer fractions remained unchanged. These results suggest not only that the processing of epidermal GlcCers by acidic β-GlcCerase requires the production of pSAP, and presumably mature SAP generation, but that the hydrolysis of the more hydrophobic epidermal GlcCers (*e.g.* GlcCer(EOS)) is more dependent upon the presence of SAP-C.

The storage of GlcCers was accompanied with a decrease in the level of epidermal ceramides. Ceramides were separated into five fractions (Fig. 1*B*) and later identified by MALDI mass spectrometric analysis (see below). With the exception of Cer(C16-AS), all of the other epidermal ceramides were modestly decreased in the SAP −/− epidermis in comparison with wild-type littermates (by approximately 20%) (Fig. 1*D*). In contrast, the least hydrophobic ceramide, Cer(C16-AS), accumulated 1.3-fold over control ($p < 0.05$). Moreover, the Cer levels of heterozygous mouse epidermis were not significantly different from normal littermates (Fig. 1*D*). The diminished levels of epidermal Cer in conjunction with the preferential accumulation of hydrophobic GlcCer (noted above) strongly suggest a role for either pSAP itself or a mature SAP, presumably SAP-C, in the hydrolytic processing of GlcCer-to-Cer in epidermis.

We next identified each of the GlcCer and Cer fractions separated by TLC. Using MALDI mass spectrometric analysis, the most hydrophobic glycolipid, GlcCer(EOS), was found to contain ω-OH fatty acids of various chain lengths (C28–C36) and degrees of saturation with a majority being C32:0 (M + Na, 1224.7 atomic mass units) and C34:1 (M + Na, 1250.6 atomic mass units) (Fig. 2*A*). The ω-OH moiety of this GlcCer fraction is known to be acylated in the epidermis with linoleic acid, as shown here and previously (25). GlcCer A/B (Fig. 2*B*) comprised a complex mixture of GlcCers (*i.e.* GlcCer(NS) GlcCer(NP), and GlcCer(C24,26-AS)). Non-hydroxy fatty acids

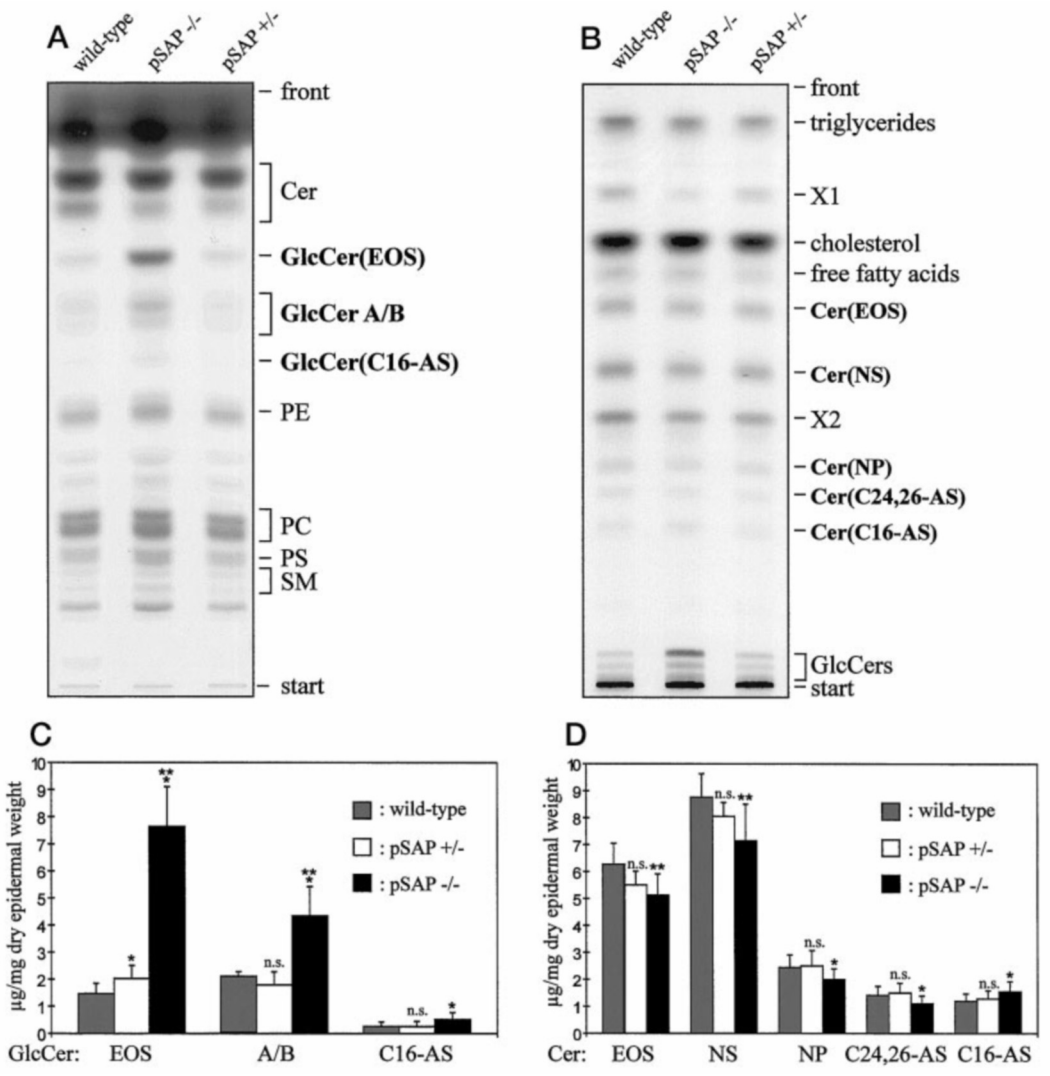

FIG. 1. **Epidermal sphingolipid composition of pSAP knockout mice.** One-dimensional TLC separations of polar epidermal lipids (*panel A; PE*, phosphatidylethalolamin; *PC*, phosphatidylcholine; *PS*, phosphatidylserine, *SM*, sphingomyelin) and epidermal ceramides (*panel B; X1* and *X2*, not identified) are shown. Densitometric quantitations of GlcCer (*panel C*) and Cer (*panel D*) are given. *C* and *D*, data are presented as the mean ± S.E. ($n \geq 7$). Note the accumulation of GlcCers, especially of GlcCer(EOS), in the homozygous knockout mice. GlcCer A/B is a complex mixture of GlcCer(NS), GlcCer(NP), and GlcCer(C24,26-AS). The lipid contents of either heterozygous or homozygous mice differ significantly from wild-type levels at: ***, $p \leq 10^{-5}$; **, $p \leq 0.01$; and *, $p \leq 0.05$. *n.s.*, not significant.

with dominant chain length of C24:0, C25:0, and C26:0 were attached to either sphingosine or phytosphingosine. The less abundant α-hydroxy fatty acids in GlcCer A/B showed a similar pattern with C24:0-OH (M + Na, 850.4 atomic mass units), C25:0-OH (M + Na, 864.4 atomic mass units), and C26:0-OH (M + Na, 878.4 atomic mass units). The presence of significant amounts of C25 and other odd-chain fatty acids in epidermal ceramides has also previously been reported (26, 27). Because of the differences in molecular polarity, it seems likely that the upper part of the double band, GlcCer A/B, represents GlcCers with non-hydroxy fatty acids, whereas the lower part corresponds to the GlcCers containing α-hydroxylated fatty acids. The most hydrophilic glycolipid, GlcCer(C16-AS), was identified as a homogenous GlcCer containing mainly a C16:0-OH fatty acid (M + Na, 738.5 atomic mass units, Fig. 2*C*).

MALDI mass spectrometric analysis of ceramides revealed sphingoid and fatty acid compositions similar to those found in their corresponding glucosylceramide precursors as well. With regard to the ceramide moiety, GlcCer(EOS) appeared to correspond to Cer(EOS) having the same fatty acid pattern, again with a majority being C32:0 (M + Na, 1062.9 atomic mass units) and C34:1 (M + Na, 1088.8 atomic mass units) (Fig. 2*E*). Cer(NS) contained different non-hydroxy fatty acids with chain length between C24 and C26 (Fig. 2*F*). Cer(NP) appeared to be hydroxylated in the sphingoid backbone because it comigrated with synthetic phytoceramide on TLC chromatograms. The molecular masses indeed demonstrated an additional hydroxyl group in Cer(NP) together with various degrees of saturation of long chain fatty acids (Fig. 2*G*). Cer(C24,26-AS) and Cer(C16-AS) both were identified as ceramides containing α-hydroxy fatty acids attached to sphingosine. In Cer(C24,26-AS) C24:0-OH (M + Na, 688.5 atomic mass units), C25:0-OH (M + Na, 702.6 atomic mass units), and C26:0-OH (M + Na, 716.6 atomic mass units) fatty acids were found (Fig. 2*H*). Taken together, Cer(NS), (NP), and (C24,26-AS) comprised the lipid backbones of GlcCer A/B. The most hydrophilic ceramide, Cer(C16-AS), possessed mainly a C16:0-OH fatty acid and therefore corresponds to GlcCer(C16-AS) (Fig. 2*D*).

Processing of sphingomyelin to Cer by acidic sphingomyelinase (SMase) could also contribute to the pool of Cer available for barrier formation within the epidermis. A significant *in vivo* activation of acidic SMase by SAPs could not be concluded from lipid profiles of brain and liver from pSAP knockout mice (20). However, in the epidermis the sphingomyelin level was in-

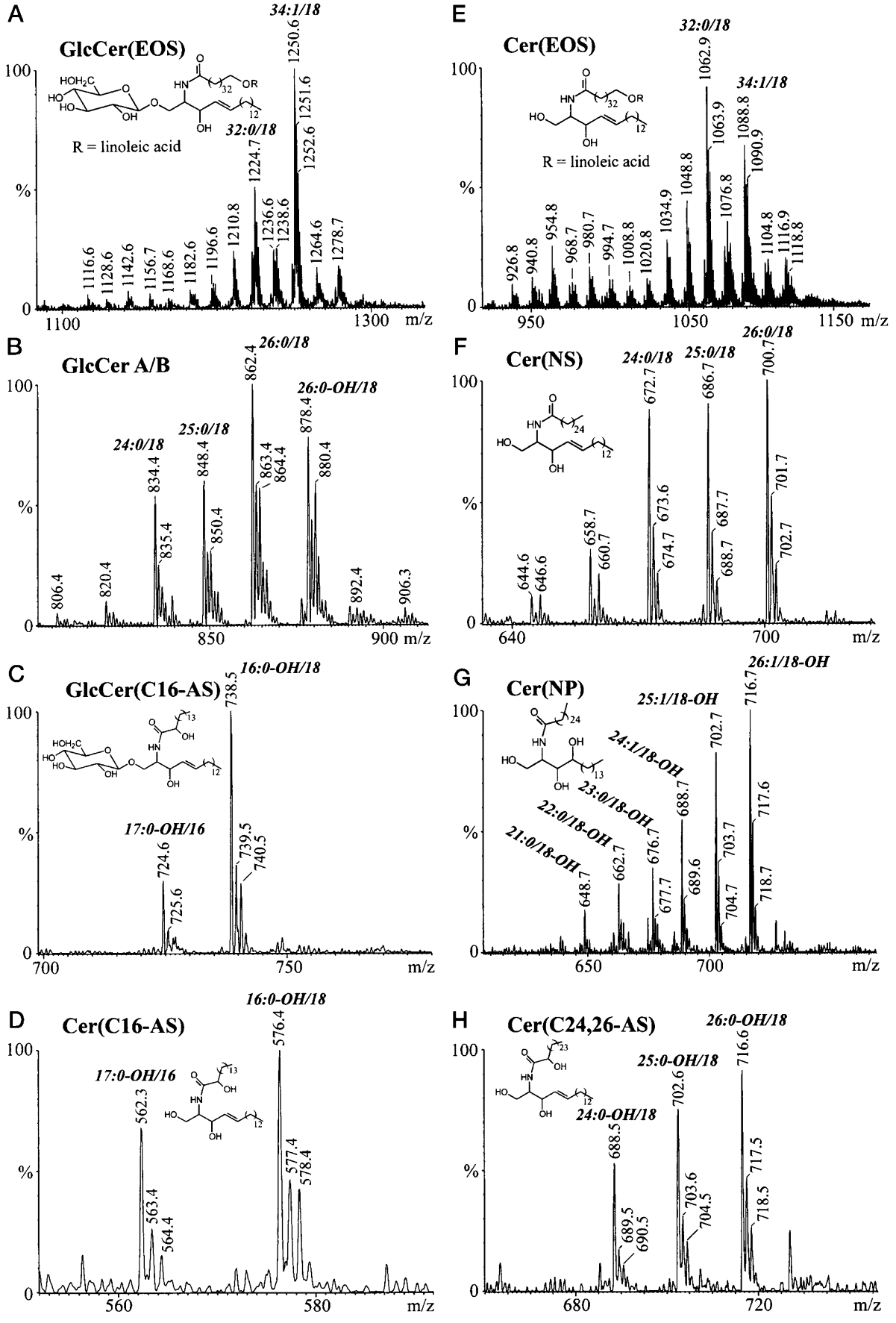

FIG. 2. **MALDI mass spectrometry of epidermal sphingolipids.** Lipids were isolated from either wild-type or pSAP knockout mice by TLC and analyzed in the positive ion mode. All indicated masses represent sodium adducts of intact molecules. The likely compositions of the most prominent peaks are indicated. The fatty acids are indicated first followed by the sphingoid bases. The proposed chemical structures of individual sphingolipids are shown. The lipid backbones of GlcCer A/B are Cer(NS), Cer(NP), and Cer(C24,26-AS).

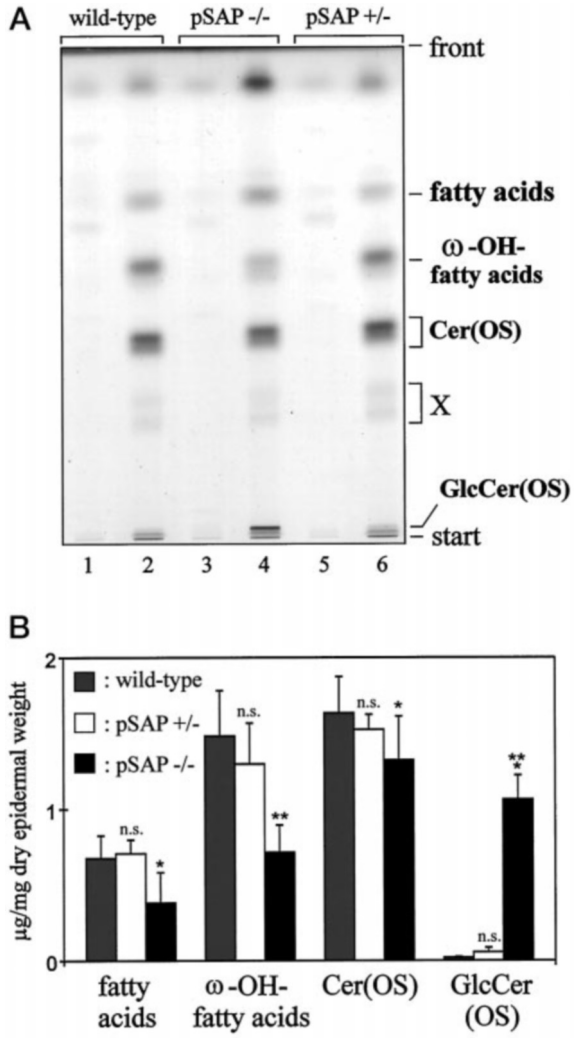

FIG. 3. **Covalently bound lipids in wild-type and pSAP knockout mice.** Ester-linked epidermal lipids were released by alkaline hydrolysis and analyzed by TLC. *A,* representative chromatographical separation of recovered lipids from either wild-type or pSAP knockout mice (*lanes 2*, *4*, and *6*). Note that the samples were devoid of unbound lipids before undergoing alkaline hydrolysis (*lanes 1*, *3*, and *5*). *X*, not identified. *B,* individual lipid levels were quantified by densitometric analysis. Data are presented as the mean ± S.E. ($n \geq 7$). The lipid contents of either heterozygous or homozygous mice differ significantly from wild-type levels at: ***, $p \leq 10^{-5}$; **, $p \leq 0.01$; *, $p \leq 0.05$. *n.s.*, not significant.

creased 1.5-fold over that in wild-type mice ($p < 0.02$, data not shown).

Covalently Bound Lipids in pSAP Knockout Mice—Terminally differentiated keratinocytes (corneocytes) are coated by a monolayer of ω-OH ceramides, ester-linked to proteins of the outer surface of the cornified cell envelope. This lipid-bound envelope is thought to be essential for either corneocyte cohesion and/or organization of extracellular lipid lamellae (28, 29). We characterized these lipids obtained after exhaustive prior extraction of epidermal samples followed by alkaline hydrolysis (12). To ascertain that the recovered lipids were indeed covalently attached, each sample was checked to ensure that there was no residual extractable lipids before undergoing base hydrolysis (Fig. 3*A*, *lanes 1*, *3*, and *5*). A mixture of long chain fatty acids, Cer(OS), and ω-OH fatty acids were released from the wild-type epidermis (Fig. 3*A*, *lane 2*). In addition to these lipids, substantial amounts of GlcCer(OS) were covalently attached to the cornified cell envelope in pSAP −/− mice (Fig. 3*A*, *lane 4*). All three ω-OH species had the same fatty acid chain

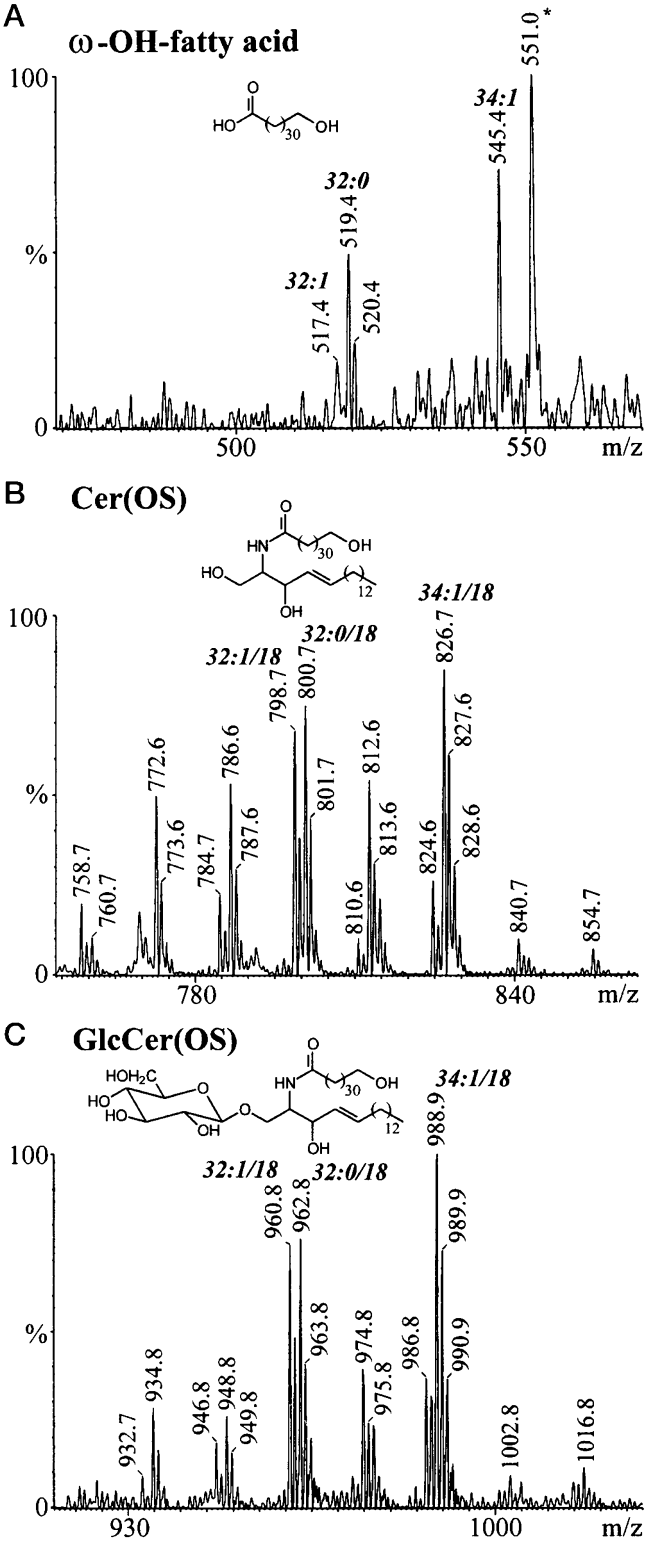

FIG. 4. **Mass spectrometry of ester-linked epidermal lipids.** Bound lipids were released by alkaline hydrolysis and isolated using TLC. Indicated molecular masses represent positively charged sodium adducts of intact molecules. The likely compositions of the most prominent peaks are indicated. The fatty acids are indicated first, followed by the sphingoid bases. The proposed chemical structures of individual lipids are shown. *, unidentified impurity extracted from silica gel.

length distribution with a majority being C32:1, C32:0, and C34:1 as demonstrated by mass spectrometry (Fig. 4). Furthermore, the levels of bound Cer(OS) and ω-OH fatty acids in homozygous knockout mice were significantly decreased to 80%

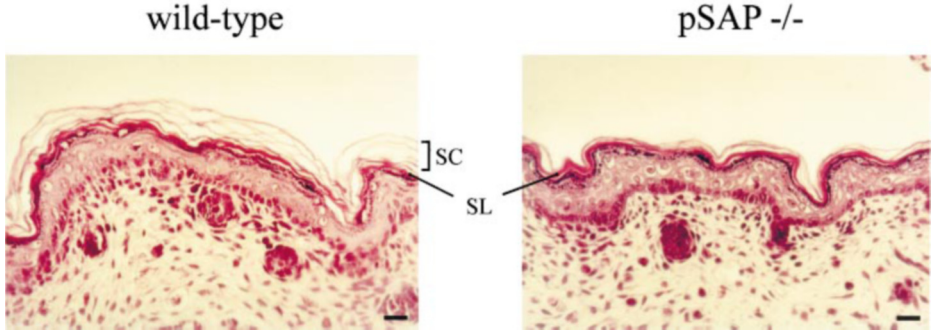

FIG. 5. **Light micrographs of skin sections of pSAP knockout mice in comparison with wild-type mice.** Whole skin was removed from the abdomen of either wild-type or homozygous knockout mice, and paraffin-embedded thin sections were stained using hematoxylin and eosin. *SL*, stratum lucidum. *Bars*, 20 μm.

($p = 0.05$) and 48% ($p = 0.002$) of normal control, respectively. Comparably low levels of covalently bound GlcCer(OS) could also be detected in pSAP +/− as well as in wild-type epidermis. In summary, these data show that in pSAP −/− mice there is a switch from a hydrophobic to a more hydrophilic lipid-bound envelope.

Morphological Alterations in the Epidermis of pSAP Knockout Mice—Newborn pSAP knockout mice displayed an ichthyotic skin phenotype including a red and wrinkled appearance previously observed in a more dramatic fashion in the β-GlcCerase-deficient Gaucher mice (15, 17). These findings are consistent with a defect in the epidermal permeability barrier and suggest underlying defects in the critical membrane domains in the stratum corneum. Therefore, to ascertain the significance of pSAP deficiency in the epidermis, we first analyzed epidermal sections at the light microscopic level (Fig. 5). In the homozygous knockout mice, morphological alterations were limited to the stratum corneum and the stratum lucidum, which represents the interface between the upper stratum granulosum and the lower stratum corneum. The thicker stratum lucidum in the knockout mice in comparison with the wild-type mice could be the result of an accumulation of unprocessed GlcCer. Furthermore, large parts of the stratum corneum were absent in sections of the knockout animals, possibly because of reduced mechanical stability resulting from swelling of intercellular domains.

Alterations in Epidermal Ultrastructure—To further delineate the basis for the cutaneous abnormalities induced by the deficiency of pSAP in the epidermis, micrographs from pSAP knockout, heterozygous, and wild-type littermates were compared (Fig. 6). The pSAP-deficient epidermis (−/−) displayed a striking abnormality in lamellar membrane maturation within the interstices of the lower-to-middle stratum corneum (Fig. 6, *A* and *B*) when compared with that of normal (Fig. 6*C*) littermates. The distinctive membrane unit structures seen in the normal stratum corneum (Fig. 6*C*) were replaced by arrays of foreshortened lamellae in loose, discontinuous aggregates in the pSAP-deficient stratum corneum (Fig. 6, *A* and *B, arrows*), resembling those seen with β-GlcCerase deficiency (14, 15). Although normal quantities of lamellar bodies were present and secreted normally in pSAP-deficient epidermis (Fig. 6*E, arrowheads*), their internal structure appeared to be altered from those in heterozygous or normal littermates (Fig. 6*D* and *inset*); individual organelles often appeared enlarged and engorged with non-lamellar, electron-dense material, which in turn disrupts the internal contents normally seen in wild-type epidermis. Moreover, the secreted lamellar body-derived contents in the intercellular spaces at the stratum corneum-stratum granulosum interface in pSAP −/− animals retained a compact, spherical pattern (Fig. 6*A, asterisks*) rather than progressively unfurling into linear configurations, as occured in the heterozygous and wild-type stratum corneum. These studies indicate that a profound deficiency of pSAP does not disrupt production, trafficking, or secretion of lamellar body contents into the intercellular domains. Instead, pSAP deficiency results in major abnormalities in the following: (*a*) the internal organization of lamellar body contents; (*b*) dispersal after secretion; and (*c*) extracellular processing of extruded lamellar body contents. Heterozygous animals, although expressing approximately 50% of the pSAP protein levels (20), did not display abnormalities in the contents or maturation of the lamellar body, suggesting that sufficient pSAP is expressed to sustain the extracellular hydrolytic activity required to generate mature lamellar membrane organization.

DISCUSSION

In this work, we isolated lipids from murine epidermis and for the first time presented a complete mass spectrometric characterization of both epidermal ceramides and corresponding glucosylceramides. Our data on the ceramides are in good agreement with previous structural characterization of murine Cer(EOS), Cer(NS), and Cer(C16-AS) (26). Because we exclusively obtained intact molecular ions (by MALDI), the fatty acid and sphingosine chain length distributions are not discernable. However, the sphingoid bases from human and pig epidermal ceramides are known to have limited chain length heterogeneities between C16 and C22 (27, 30), consistent with the substrate specificity of epidermal serine palmitoyltransferase (31). We therefore proposed likely compositions of individual sphingolipids. The structural assignment of Cer(NP), referred to here as phytoceramide, is based both on its mass spectrum and its comigration on TLC plates with synthetic phytoceramide. We found similar sphingoid and fatty acid compositions of ceramides and corresponding glucosylceramides. For example, GlcCer(EOS) and Cer(EOS) both exhibited mainly C32:0 and C34:1 ω-OH fatty acids, strongly suggesting a precursor/product relationship between these two related epidermal lipids.

Processing of epidermal GlcCers to ceramides by the action of acidic β-GlcCerase is well known to be essential for cutaneous barrier formation and homoeostasis (14–17). In pSAP −/− mice, an accumulation of all GlcCers was evident, demonstrating that optimal epidermal β-GlcCerase activity *in vivo* requires stimulation by SAPs (*i.e.* SAP-C). Furthermore, the importance of SAP-C mediation is underscored by the observation that degradation of the most hydrophobic GlcCer, GlcCer (EOS), was found to be most impaired in the absence of SAPs. SAP-C is thought to stimulate β-GlcCerase by an allosteric mechanism, resulting in trimeric complex formation between the water-soluble enzyme, the activator protein, and the membrane-associated glycolipid (1, 7). *In vitro* data have demonstrated the ability of SAP-C to form complexes with β-GlcCerase in the presence of phosphatidylserine (32, 33). However, *in vivo* binding of SAP-C to GlcCers may also contribute to the stimulation of GlcCer hydrolysis. Indeed, SAP-C has recently been shown to solubilize membrane-bound GlcCers presum-

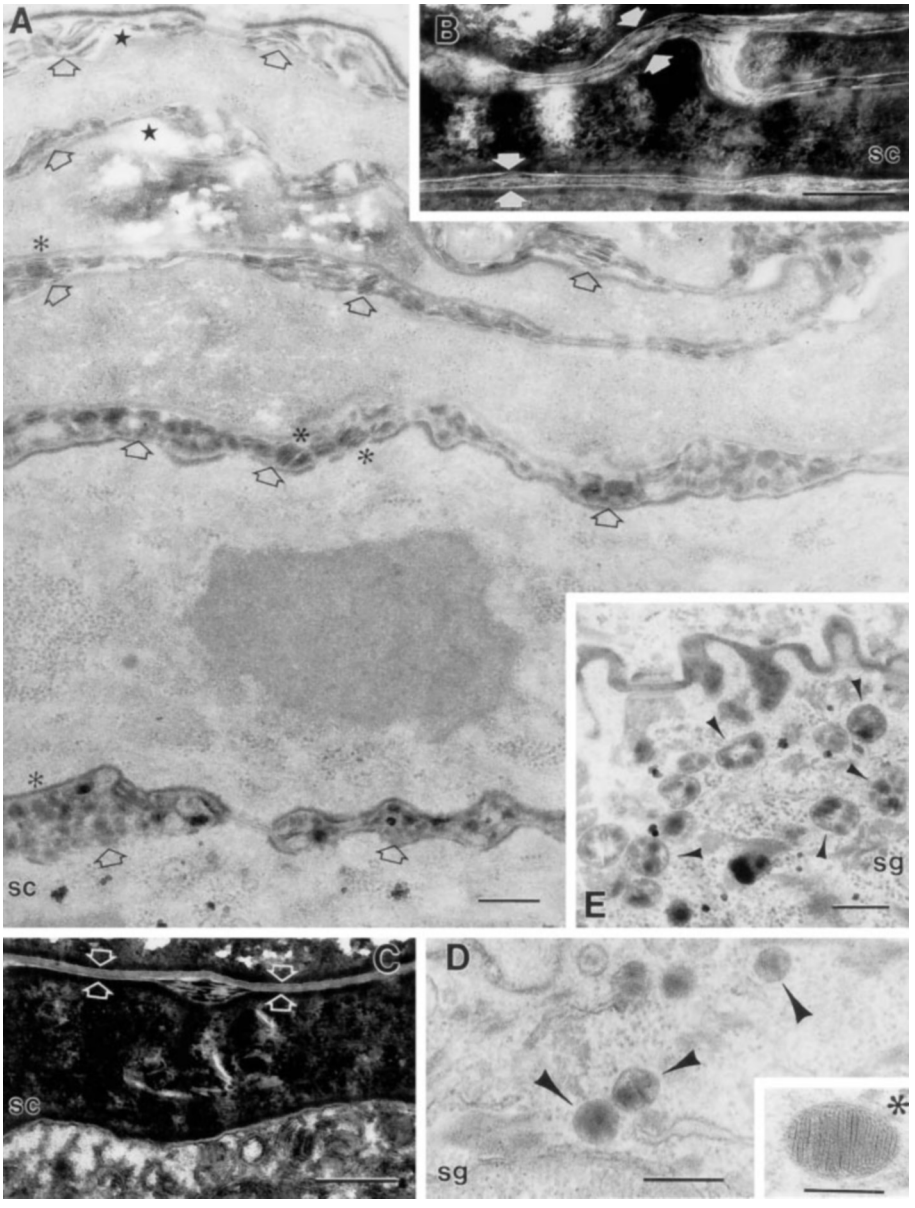

FIG. 6. **Ultrastructure of pSAP knockout epidermis in comparison with wild-type littermates.** In the lower to mid-SC from pSAP-deficient (−/−) mouse epidermis, conventional osmium tetroxide staining reveals extruded lamellar body contents to be highly disorganized (*A, open arrows*), with persistence of osmiophilic material in the interstices out to at least the fourth to fifth SC cell layers (*A, stars*), an area routinely undeposited by osmophilic material in normal wild-type SC tissues (not shown). Moreover, extruded lamellar body contents retain a spherical pattern in the interstices (*A, asterisks*), suggesting that linearization of lamellar body-derived lamellae is delayed or defective. Ruthenium staining (22) further reveals that the pSAP-deficient interstices (*B, between arrows*) lack the regular, compact pattern of lamellar membranes observed in normal, pSAP-replete SC (*C, between open arrows*). Although significant quantities of lamellar bodies are formed in pSAP-deficient epidermis (*E, arrowheads*), the contents have a more condensed appearance when compared with normal wild-type littermates (*D, arrowheads;* and *inset*, *asterisk* shows an enlarged image of a lamellar body from normal epidermis). *Bars* represent 0.25 (*A–E*) and 0.125 μm (*D*, *inset*) with final image magnifications (all are ×103) of: *A*, 46.6; *B*, 68.8; *C*, 62.5; *D*, 64.0; *E*, 44.8; *D, inset*, 120. *sg*, stratum granulosum.

ably facilitating their processing by water-soluble enzyme (7).

Despite the massive accumulation of GlcCers, epidermal Cer levels were only moderately reduced in newborn pSAP −/− mice compared with wild-type littermates. One explanation for the near normal levels of Cer might be increased sphingomyelin (SM)-to-Cer hydrolysis. However, the increased SM content in the pSAP −/− mouse epidermis reported here is also of consequence. In normal epidermis, the levels of both SM and GlcCer are high in nucleated cells but these lipids are nearly absent from the stratum corneum (34). Previous results with β-GlcCerase null allele animals also suggest that the majority of stratum corneum ceramides are derived from the hydrolysis of GlcCer precursors (15). The importance of stratum corneum SMase for barrier homoeostasis has only recently been demonstrated.[2,3] Thus, a portion of the Cer in stratum corneum normally may derive from SM, or this pathway could be up-regulated in the face of a β-GlcCerase blockade. Moreover, although SAP-C has been shown to stimulate acidic SMase activity *in vitro* (35, 36), the role of SAPs in SM hydrolysis *in vivo* has not been adequately addressed. SMase activity is normal in extracts from dermal fibroblasts isolated from a patient with pSAP deficiency (8). However, the increased epidermal SM content in the absence of pSAP expression described here suggests that SAP(s) may have a role in the regulation of epidermal SMase activity. Whether increased epidermal SM contributes to the stratum corneum pathology remains to be determined.

In principle, β-GlcCerase can degrade GlcCer without SAP-C. Furthermore, an excess of β-GlcCerase activity is present in normal epidermis, as Gaucher heterozygous animals with approximately 50% of normal activity do not exhibit SC or epidermal pathology (15). Therefore, it seems likely that during embryonic development substantial amounts of ceramides are provided by slow hydrolysis of GlcCer without any loss by desquamation starting after birth. Finally, ceramides could also derive directly from *de novo* synthesis under pathological conditions or even physiologically. The most polar ceramide, Cer(C16-AS), was found to be elevated in the absence of pSAP, suggesting a compensatory capacity of sphingolipid biosynthetic pathways in keratinocytes.

It is well known that a monolayer of ω-OH-Cer is covalently attached to the surface of corneocytes connecting proteins of the cornified cell envelope to hydrophobic intercellular lipid

[2] E. Proksch and J. M. Jensen, personal communication.
[3] W. M. Holleran and P. M. Elias, unpublished observation.

lamellae (12). Recently, some structural proteins within the epidermis have been shown to be esterified at Glu or Gln residues with Cer(OS) (13). Although little is known about the attachment of lipids to proteins of the epidermal cornified cell envelope, it was assumed that free Cer(EOS) are the direct precursors of bound Cer(OS). We have analyzed covalently bound epidermal lipids in pSAP knockout mice. Most importantly, in pSAP −/− mice a novel lipid was recovered and identified as GlcCer(OS), which had the same fatty acid chain length distribution as found in the bound Cer(OS) and in ω-OH fatty acids as well. Obviously, these lipids are metabolically related to each other. It has been an open question whether Cer(OS) is bound to amino acid side chains either via the ω-hydroxyl group or via C1 of sphingoid bases. Because GlcCer(OS) cannot be bound through C1, where the glucose moiety is located, this finding provides evidence that at least a portion of all three ω-OH species are attached to the cornified cell envelope via the terminal ω-hydroxyl group.

Because we detected small amounts of bound GlcCer(OS) in both pSAP +/− and wild-type mice, this phenomenon appears to be physiologically important. In our model, lamellar body-derived GlcCer(EOS) is, in part, utilized by an enzyme that exchanges esterified linoleic acid against the side chain of a glutamate residue. Resulting bound GlcCer(OS) would subsequently be deglycosylated to bound Cer(OS) by SAP-C-assisted β-GlcCerase and then hydrolyzed to bound ω-OH fatty acid and free sphingosine by SAP-D-assisted ceramidase. This metabolic relationship is supported by identical fatty acid chain length distribution in all three ω-OH species. Stimulation of lipid-bound envelope-associated processing of lipids by hydrolytic enzymes would mechanistically work by an allosteric enzyme activation rather than by solubilization of lipids, which are covalently attached and therefore cannot be lifted by an activator protein. On the other hand, it cannot be excluded that free Cer(EOS) would also be transferred to proteins of the cornified cell envelope by an enzyme, which utilizes either Cer(EOS) or GlcCer(EOS). In pSAP −/− mice, levels of bound ω-OH fatty acid and Cer(OS) were significantly decreased to 48 and 80% of control, respectively. These data support the concept that bound GlcCer(OS) is the likely precursor of bound Cer(OS) and ω-OH fatty acid. Formation of the latter is more severely impaired than formation of Cer(OS) because, in addition to reduced substrate concentration due to SAP-C deficiency, ceramidase is missing its physiological activators, SAP-D (37) and SAP-C.[4] However, it needs to be clarified in future studies whether covalently attached Cer(OS) and GlcCer(OS) are indeed substrates for ceramidase and β-GlcCerase, respectively.

Finally, morphological alterations in the epidermis of pSAP −/− mice were significant and reminiscent of the alterations observed in β-GlcCerase-deficient Gaucher epidermis. Electron microscopy revealed a striking abnormality in SC lamellar membrane organization. Moreover, light micrographs revealed a partial loss of stratum corneum in pSAP −/− mice, which may reflect decreased SC coherence. Thus, the accumulation of the more hydrophilic glucosylated Cer species, rather than Cer, within the extractable SC lipid and/or the lipid-bound envelope is likely responsible for the observed alterations in epidermal structure. However, since pSAP also exists as a surface component of neuronal cells (38), other potential functions for this protein in the epidermis, beyond its precursor role, still need to be explored.

[4] T. Linke and K. Sandhoff, unpublished observations.

REFERENCES

1. Sandhoff, K., Harzer, K., and Fürst, W. (1995) in *The Metabolic and Molecular Basis of Inherited Disease* (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds) McGraw-Hill, New York, pp. 2427–2441
2. Suzuki, K. (1995) *Essays Biochem.* **29,** 25–37
3. Fischer, G., and Jatzkewitz, H. (1978) *Biochim. Biophys. Acta* **528,** 69–76
4. Weiler, S., Kishimoto, Y., O'Brien, J. S., Barranger, J. A., and Tomich, J. M. (1995) *Protein Sci.* **4,** 756–764
5. Vaccaro, A. M., Tatti, M., Ciaffoni, F., Salvioli, R., Barca, A., and Scerch, C. (1997) *J. Biol. Chem.* **272,** 16862–16867
6. Ho, M. W., and O'Brien, J. S. (1971) *Proc. Natl. Acad. Sci. U. S. A.* **68,** 2810–2813
7. Wilkening, G., Linke, T., and Sandhoff, K. (1998) *J. Biol. Chem.* **273,** 30271–30278
8. Harzer, K., Paton, B. C., Poulos, A., Kustermann-Kuhn, B., Roggendorf, W., Grisar, T., and Popp, M. (1989) *Eur. J. Pediatr.* **149,** 31–39
9. Schnabel, D., Schroeder, M., and Sandhoff, K. (1991) *FEBS Lett.* **284,** 57–59
10. Rafi, M. A., de Gala, G., Zhang, X. L., and Wenger, D. A. (1993) *Somatic Cell Mol. Genet.* **19,** 1–7
11. Elias, P. M., and Menon, G. K. (1991) *Adv. Lipid Res.* **33,** 301–313
12. Wertz, P. W., and Downing, D. T. (1987) *Biochim. Biophys. Acta* **917,** 108–111
13. Marekov, L. N., and Steinert, P. M. (1998) *J. Biol. Chem.* **273,** 17763–17770
14. Holleran, W. M., Takagi, Y., Menon, G. K., Legler, G., Feingold, K. R., and Elias, P. M. (1993) *J. Clin. Invest.* **91,** 1656–1664
15. Holleran, W. M., Ginns, E. I., Menon, G. K., Grundmann, J. U., Fartasch, M., McKinney, C. E., Elias, P. M., and Sidransky, E. (1994) *J. Clin. Invest.* **93,** 1756–1764
16. Sidransky, E., Fartasch, M., Lee, R. E., Metlay, L. A., Abella, S., Zimran, A., Gao, W., Elias, P. M., Ginns, E. I., and Holleran, W. M. (1996) *Pediatr. Res.* **39,** 134–141
17. Liu, Y., Suzuki, K., Reed, J. D., Grinberg, A., Westphal, H., Hoffmann, A., Doering, T., Sandhoff, K., and Proia R. L. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 2503–2508
18. Chang-Yi, C., Kusuda, S., Seguchi, T., Takahashi, M., Aisu, K., and Tezuka, T. (1997) *J. Invest. Dermatol.* **109,** 319–323
19. Bradová, V., Smíd, F., Ulrich-Bott, B., Roggendorf, W., Paton, B. C., and Harzer. K. (1993) *Hum. Genet.* **92,** 143–152
20. Fujita, N., Suzuki, K., Vanier, M. T., Popko, B., Maeda N., Klein, A., Henseler, M., Sandhoff, K., Nakayasu, H., and Suzuki, K. (1996) *Hum. Mol. Genet.* **5,** 711–725
21. Oya, Y., Nakayasu, H., Fujita, N., Suzuki, K., and Suzuki, K. (1998) *Acta Neuropathol.* **96,** 29–40
22. Hou, S. Y. E., Stephen, H., Mitra, A. K., White, S. H., Menon, G. K., Ghadially, R., and Elias, P. M. (1991) *J. Invest. Dermatol.* **96,** 215–223
23. Motta, S., Monti, M., Sesana, S.,. Caputo, R, Carelli, S., and Ghidoni, R. (1993) *Biochim. Biophys. Acta* **1182,** 147–151
24. Robson, K. J., Stewart, M. E., Michelsen, S., Lazo, N. D., and Downing, D. T. (1994) *J. Lipid Res.* **35,** 2060–2068
25. Wertz, P. W., and Downing, D. T. (1983) *J. Lipid Res.* **24,** 753–758
26. Madison, K. C., Swartzendruber, D. C., Wertz, P. W., and Downing D. T. (1990) *J. Invest. Dermatol.* **95,** 657–664
27. Wertz, P. W., Miethke, M. C., Long, S. A., Strauss, J. S., and Downing, D. T. (1985) *J. Invest. Dermatol.* **84,** 410–412
28. Downing, D. T. (1992) *J. Lipid Res.* **33,** 301–313
29. Wertz, P. W., Madison, K. C., and Downing, D. T. (1989) *J. Invest. Dermatol.* **92,** 109–111
30. Wertz, P. W., and Downing, D. T. (1983) *J. Lipid Res.* **24,** 759–765
31. Holleran, W. M., Williams, M. L., Gao, W. N., and Elias, P. M. (1990) *J. Lipid Res.* **31,** 1655–1661
32. Berent, S. L., and Radin, N. S. (1981) *Biochim. Biophys. Acta* **664,** 572–582
33. Ho, M. W. (1975) *FEBS Lett.* **53,** 243–247
34. Gray, G. M., and Yardley, H. J. (1975) *J. Lipid Res.* **16,** 441–447
35. Poulos, A., Ranieri, E., Shankaran, P., and Callahan, J. W. (1984) *Biochim. Biophys. Acta* **793,** 141–148
36. Tayama, M., Soeda, S., Kishimoto, Y., Martin, B. M., Callahan, J. W., Hiraiwa, M., and O'Brien, J. S. (1993) *Biochem. J.* **290,** 401–404
37. Klein, A., Henseler M., Klein C., Suzuki K., Harzer K., and Sandhoff K. (1994) *Biochem. Biophys. Res. Commun.* **200,** 1440–1448
38. Fu, Q., Carson, G. S., Hiraiwa, M., Grafe, M., Kishimoto, Y., and O'Brien, J. S. (1994) *J. Mol. Neurosci.* **5,** 59–67