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Acylceramide Head Group Architecture Affects Lipid Organization in Synthetic Ceramide Mixtures

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The lipid organization in the upper layer of the skin, the stratum corneum (SC), is important for the skin barrier function. This lipid organization, including the characteristic 13 nm lamellar phase, can be reproduced *in vitro* with mixtures based on cholesterol, free fatty acids and natural as well as synthetic ceramides (CER). In human SC, nine CER classes have been identified (CER1–CER9). Detailed studies on the effect of molecular structure of individual ceramides on the SC lipid organization are only possible with synthetic lipid mixtures, as their composition can be accurately chosen and systematically modified. In the present study, small-angle X-ray diffraction was used to examine the organization in synthetic lipid mixtures of which the synthetic ceramide fraction was prepared with sphingosine-based CER1 or phytosphingosine-based CER9. The latter acylceramide contains an additional hydroxyl group at the sphingoid backbone. The results show that a gradual increase in CER1 level consistently promotes the formation of the 13 nm lamellar phase and that partial replacement of CER1 by CER9 does not affect the phase behavior. Interestingly, complete substitution of CER1 with CER9 reduces the formation of the long periodicity phase and results in phase separation of CER9.

Key words: barrier/ceramides/sphingoid base/stratum corneum/X-ray diffraction
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Ceramides (CER), cholesterol (CHOL), and long-chain free fatty acids (FFA) are the most abundant lipid species present in the stratum corneum (SC) and are responsible for proper function of the skin barrier. Human SC contains at least nine extractable CER types (Wertz *et al*, 1985; Robson *et al*, 1994; Stewart and Downing, 1999; Ponec *et al*, 2003), classified as CER1 to CER9. The molecular structures of the CER are illustrated in Fig S1. The CER can be subdivided into three main groups, based on the nature of their backbone (sphingosine (S), phytosphingosine (P) and 6-hydroxysphingosine (H)). Through an amide bonding, long-chain non-hydroxy (N) or α-hydroxy (A) fatty acids with varying acyl chain lengths are chemically linked to the sphingosine bases. The acylceramides CER1 (EOS), CER4 (EOH), and the recently identified CER9 (EOP) are unique in structure as they contain linoleic acid linked to an ω-hydroxy fatty acid (EO) with a chain length of 30–32 carbon atoms.

X-ray diffraction studies demonstrated the presence of two co-existing lamellar phases in human SC: the short periodicity phase (SPP) with a repeat distance of approximately 6 nm and the long periodicity phase (LPP) with a repeat distance of approximately 13 nm (Bouwstra *et al*, 1991a, b, 1995). The molecular organization of the LPP together with its predominantly orthorhombic lipid packing in the presence of substantial amounts of CHOL are exceptional and are therefore considered to play an important role in the skin barrier function (Bouwstra *et al*, 2002).

X-ray diffraction studies on lipid mixtures prepared with CHOL, FFA, and CER isolated from human or pig SC (natCER (natural ceramides)) revealed that the lipid organization in these mixtures closely mimics that observed in native SC. These studies have markedly contributed to our present knowledge on the SC lipid organization and the role the main lipid classes play in the SC lipid organization (Bouwstra *et al*, 1996, 2001; McIntosh *et al*, 1996). The role of the head group architecture or acyl chain length on the lipid phase behavior, however, cannot be studied using natCER mixtures, as it is impossible to isolate the individual CER subclasses in large quantities. For this reason, we decided to investigate the lipid organization in mixtures prepared with synthetic ceramides (synthCER), which offers the possibility to substitute one single ceramide subclass for another one. A first prerequisite, however, for the possibility to use synthCER is that their phase behavior reflects that observed in SC. In previous studies, we have demonstrated that the SC lipid organization can be reproduced to a high extent with equimolar mixtures of CHOL, FFA, and a limited number of synthCER, namely CER1, CER3, and bovine brain CER type IV (referred to as ΣCERIV) mixed in a 1:7:2 molar ratio, provided that an appropriate equilibrium temperature is chosen during sample preparation (de Jager *et al*, 2004a,b). In synthetic CER1 and CER3, non-hydroxy fatty acids of a uniform acyl chain length are linked to the (phyto)sphingoid backbone, whereas the sphingosine-based ΣCERIV contains α-hydoxy fatty acids

Abbreviations: CER, ceramides; CHOL, cholesterol; FFA, free fatty acids; LPP, long periodicity phase; natCER, natural ceramides; SC, stratum corneum; SPP, short periodicity phase; synthCER, synthetic ceramides

with varying acyl chain lengths, with C18 (22%) and C24 (42%) as the most abundantly present (de Jager *et al*, 2004b). For achievement of a similar lipid organization as observed in the SC, the presence of FFA and an optimal ratio of CER3 and ΣCERIV are crucial (de Jager *et al*, 2004b).

The present study focuses on the role the head group architecture of the acylceramides plays on the phase behavior of synthetic lipid mixtures. In human SC, approximately 8.3% of the total CER fraction comprises CER1 (sphingosine base), whereas CER9 (phytosphingosine base) accounts for 6.4% (Ponec *et al*, 2003). For this reason, we studied synthCER mixtures containing only one acylceramide (CER1 or CER9) as well as mixtures in which both acylceramides are present, by partially substituting CER1 with CER9. The use of CER9 with an ω-hydroxy fatty acid chain length of either 30 or 27 carbon atoms also allowed us to study the role of the acyl chain length on the lipid organization. The major finding of the present study is that partial replacement of CER1 by CER9 does not affect the lipid organization, whereas complete substitution of CER1 with CER9 inhibits the formation of the LPP and results in phase separation of CER9.

Results

LPP is formed in equimolar CHOL:synthCER:FFA mixtures prepared with CER1 or CER9 The diffraction pattern of a CHOL:[CER1:CER3:ΣCERIV]:FFA mixture prepared at a molar ratio of 1:[0.1:0.7:0.2] is shown in Fig 1*A*. The LPP with a repeat distance of 12.2 nm is indicated by the presence of six reflections ($q = 0.52$, 1.03, 1.54, 2.06, 3.08, and 3.59 nm^{-1}). The reflections at $q = 1.16$ and 2.33 nm^{-1} correspond to the first and second order diffraction peaks of a lamellar phase with a periodicity of 5.4 nm (SPP). The two strong reflections at 1.71 and 3.43 nm^{-1} reveal the presence of a phase with a periodicity of 3.7 nm, ascribed to crystalline CER3 in a V-shaped formation (Dahlen and Pascher, 1979; de Jager *et al*, 2003; Raudenkolb *et al*, 2003). The presence of crystalline CHOL in separate domains can be deduced from the peaks at 1.87 and 3.74 nm^{-1}.

Complete substitution of CER1 with CER9(C30) results in the diffraction pattern depicted in Fig 1*A*. Obviously, the LPP with a repeat distance of 12.1 nm is also formed in the presence of CER9(C30), although the intensities of its reflections in comparison with the SPP reflections are weaker than observed in the CER1-containing mixture. A weak reflection at 0.70 nm^{-1} suggests that a small fraction of CER9(C30) phase separates (see the next section for a more detailed description of this phase). In addition, crystalline CHOL and CER3 in a V-shaped formation are present in the lipid mixture.

Replacement of CER9(C30) with CER9(C27) in the mixture results in a marked reduction of LPP formation (data not shown). In addition, the repeat distance of this phase shifts to 11.7 nm. The formation of the other above-mentioned phases is not affected by the reduced ω-hydroxy fatty acid chain length of CER9(C27).

LPP is not formed in mixtures prepared with CER9 in the absence of FFA Figure 1*B* shows the diffraction pattern of the equimolar CHOL:synthCER mixture, prepared at a 1:7:2 ratio of CER1, CER3, and ΣCERIV. Five equidistant peaks suggest the presence of the LPP (12.2 nm), whereas the SPP (5.3 nm) is indicated by two reflections. In addition, phase-separated CER3 and CHOL can be detected. Compared with the diffraction curve of the equimolar CHOL:synthCER:FFA mixture prepared with CER1, the intensities of the reflections attributed to the LPP and SPP decreased as compared with those of crystalline CER3 and CHOL.

Complete substitution of CER1 with CER9(C30) results in the diffraction pattern depicted in Fig 1*B*. Compared with the mixture containing CER1, three major changes are observed in the presence of CER9(C30): (i) No reflections can be detected that might be ascribed to the LPP, indicating that no LPP is formed. (ii) Four reflections at 0.70, 1.41, 2.10, and 2.79 nm^{-1} reveal the presence of a phase with a repeat distance of 9.0 nm. This corresponds to the length of two opposing CER9(C30) molecules in a hairpin formation and is similar to the repeat distance found for hydrated pig CER1 (McIntosh *et al*, 1996). Taking into account the diffraction patterns depicted in Fig 2*B* (see below), it is

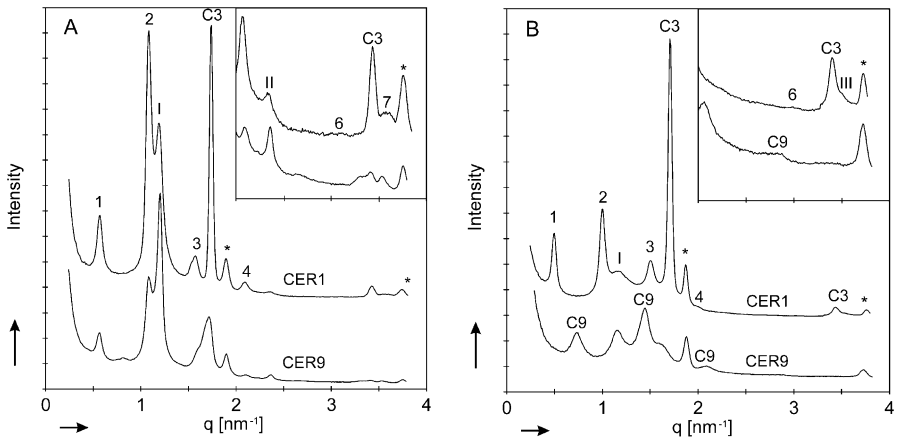

Figure 1
Free fatty acids (FFA) promote long periodicity phase (LPP) formation in mixtures containing 10% ceramide (CER)1 or 10% CER9. The inset is a magnification of the reflections in the q-range between 2 and 4 nm^{-1}. The Arabic and Roman numbers indicate the diffraction orders of the LPP and short periodicity phase (SPP), respectively. C3 refers to the reflections of crystalline CER3 located at 1.71 and 3.42 nm^{-1}, whereas C9 refers to the reflections of crystalline CER9 located at 0.70, 1.41, 2.10, and 2.79 nm^{-1}. The asterisk (*) indicates the reflections of crystalline cholesterol (CHOL) located at 1.87 and 3.74 nm^{-1}. (*A*) Diffraction patterns of CHOL:synthCER:FFA mixtures prepared with CER1 or CER9. The various orders of the LPP are located at $q = 0.52$ nm^{-1} (1), 1.03 nm^{-1} (2), 1.56 nm^{-1} (3), 2.08 nm^{-1} (4), 3.09 nm^{-1} (6), and 3.56 nm^{-1} (7). The various orders of the SPP are located at $q = 1.17$ nm^{-1} (I) and 2.34 nm^{-1} (II). (*B*) Diffraction patterns of CHOL:synthCER mixtures prepared with CER1 or CER9. The various orders of the LPP are located at $q = 0.52$ nm^{-1} (first), 1.03 nm^{-1} (second), 1.56 nm^{-1} (3), 2.08 nm^{-1} (4), and 3.09 nm^{-1} (6). The various orders of the SPP are located at $q = 1.17$ nm^{-1} (1) and 3.51 nm^{-1} (III). (*B*)

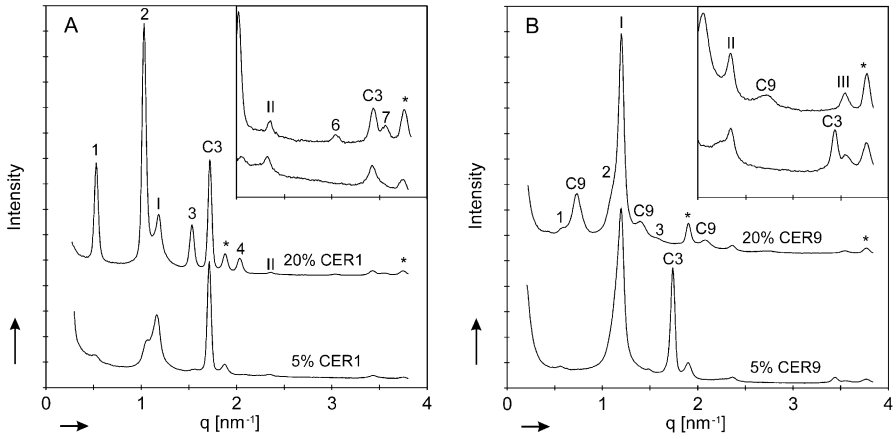

Figure 2
Long periodicity phase (LPP) formation in equimolar cholesterol (CHOL): synthetic ceramides (synthCER): free fatty acids (FFA) mixtures depends on acylceramide content. The inset is a magnification of the reflections in the q-range between 2 and 4 nm^{-1}. The Arabic and Roman numbers indicate the diffraction orders of the LPP and short periodicity phase (SPP), respectively. C3 refers to the reflections of crystalline CER3 located at 1.71 and 3.42 nm^{-1}, whereas C9 refers to the reflections of crystalline ceramide (CER)9 located at 0.70, 1.40, 2.10, and 2.80 nm^{-1}. The asterisk (*) indicates the reflections of crystalline CHOL located at 1.87 and 3.74 nm^{-1}. (*A*) Diffraction patterns of CHOL:synthCER:FFA mixtures prepared with 5 and 20% CER1. The various orders of the LPP are located at $q = 0.52$ nm^{-1} (1), 1.03 nm^{-1} (2), 1.56 nm^{-1} (3), 2.08 nm^{-1} (4), 3.09 nm^{-1} (6), and 3.56 nm^{-1} (4). The various orders of the SPP are located at $q = 1.17$ nm^{-1} (I) and 234 nm^{-1} (II). (*B*) Diffraction patterns of CHOL:synthCER:FFA mixtures prepared with 5 and 20% CER9. The various orders of the LPP are located at $q = 0.52$ nm^{-1} (1), 1.03 nm^{-1} (2), and 1.56 nm^{-1} (3). The various orders of the SPP are located at $q = 1.17$ nm^{-1} (I), 2.34 nm^{-1} (II), and 3.51 nm^{-1} (III).

conceivable to assign this phase to crystalline CER9(C30) in separate domains. (iii) The 3.7 nm phase, attributed to crystalline CER3, is not present. Instead, a shoulder at 1.57 nm^{-1} might designate the presence of a new phase with a periodicity of 4.0 nm. No higher-order reflections, however, can be detected. The exact composition and nature of this phase is not known.

The diffraction pattern of the mixture containing CER9(C27) is almost identical to the one obtained with CER9(C30) (data not shown). No LPP is present in the mixture and several reflections indicate the presence of a phase with a repeat distance of 8.7 nm, ascribed to phase-separated CER9. The slightly shorter periodicity of this phase can be explained by the shorter ω-hydroxy fatty acid chain length of CER9(C27).

LPP formation in equimolar CHOL:synthCER:FFA mixtures depends on acylceramide content To assess the effect of the relative acylceramide content on the lipid organization in equimolar mixtures of CHOL, synthCER, and FFA, the amount of CER1 or CER9 in the synthCER fraction was gradually increased from 0% to 20% mol per mol, thereby maintaining the ratio between CER3 and ΣCERIV constant. As CER9(C27) is less effective in promoting the formation of the LPP, these experiments were only performed with CER1 and CER9(C30). Below, the changes in lipid organization as a function of acylceramide type and relative amount are presented.

In the absence of CER1 and CER9, the characteristic LPP is not present (data not shown). Instead, three strong reflections indicate the presence of a 5.4 nm lamellar phase (SPP). In addition, crystalline CER3 and CHOL are present in the mixture, both indicated by two reflections.

In the equimolar CHOL:synthCER:FFA mixture in which the synthCER fraction contains 5% CER1, only a small fraction of lipids forms the LPP, as deduced from the presence of four weak reflections in the diffraction pattern (see Fig 2*A*). The diffraction curve of the mixture prepared with 10% CER1 is illustrated in Fig 1*A* (see the previous section for a more detailed description of the phases present). Evidently, the fraction of lipids that forms the LPP increased. A raise in CER1 content to 15% or 20% results in diffraction patterns in which the intensities of the LPP reflections further increase as compared with the peak intensities of the SPP, CER3 and CHOL. Even in the presence of 20% CER1 (see Fig 2*A*), no peaks can be detected that might be ascribed to crystalline CER1 in separate domains.

The diffraction pattern of the equimolar CHOL:synthCER:FFA mixture of which the synthCER fraction contains 5% CER9 (see Fig 2*B*) is similar to the one obtained with 5% CER1. The majority of lipids forms the SPP, although weak reflections indicate that a minute fraction of lipids forms a phase with a periodicity of 12.0 nm. In addition, crystalline CER3 and CHOL are present in the mixture. The diffraction curve of the mixture prepared with 10% CER9 (see Fig 1*A*) has already been explained in the previous section. At this concentration, the LPP is clearly present and a small fraction of CER9 phase separates. When the amount of CER9 in the synthCER fraction is further raised to 15% or 20% (see Fig 2*B*), some striking changes in the lipid organization are observed: (i) The reflections attributed to the LPP markedly reduce in intensity as compared with the peak intensities of the other phases. Moreover, only the first three order diffraction peaks of the LPP can be detected. This indicates that at increased CER9 content, the formation of the LPP is inhibited. (ii) An increased amount of CER9 phase separates into separate domains, indicated by strong reflections at $q = 0.70$, 1.40, 2.10, and 2.80 nm^{-1}, which reveal the presence of a 9.0 nm phase. (iii) The reflections ascribed to phase-separated CER3, which is present at a CER9 level of 5% and 10%, completely disappear when the amount of CER9 in the mixture is increased.

No change in LPP formation upon partial substitution of CER1 with CER9 in equimolar CHOL:synthCER:FFA mixtures As native SC contains both CER1 (8.3%) and CER9 (6.4%), we also prepared lipid mixtures in which both acylceramides were present in ratios similar to that found in human SC. The mixtures were prepared with a synthCER fraction that contained 5% CER1 and 10% CER9 and *vice versa*, thereby maintaining the ratio between CER3 and ΣCERIV constant. In both mixtures, six diffraction peaks

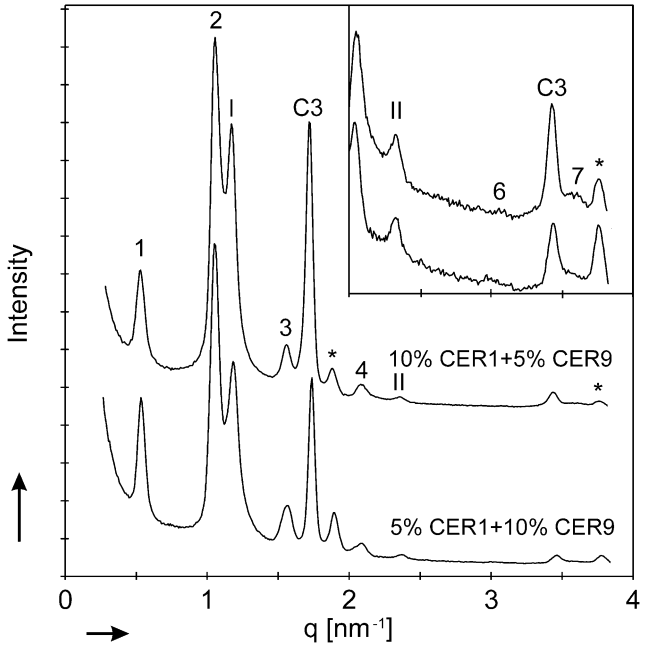

Figure 3
No substantial change in lipid organization in cholesterol (CHOL): synthetic ceramides (synthCER):free fatty acids (FFA) mixtures in which CER1 is partially substituted with CER9. The inset is a magnification of the reflections in the q-range between 2 and 4 nm^{-1}. The Arabic and Roman numbers indicate the diffraction orders of the long periodicity phase (LPP) and short periodicity phase (SPP), respectively. C3 refers to the reflections of crystalline CER3 located at 1.71 and 3.42 nm^{-1}. The asterisk (*) indicates the reflections of crystalline CHOL located at 1.87 and 3.74 nm^{-1}. The various orders of the LPP are located at $q = 0.52\ nm^{-1}$ (1), $1.03\ nm^{-1}$ (2), $1.56\ nm^{-1}$ (3), $2.08\ nm^{-1}$ (4), $3.09\ nm^{-1}$ (6), and $3.56\ nm^{-1}$ (7). The various orders of the SPP are located at $q = 1.17\ nm^{-1}$ (I) and $2.34\ nm^{-1}$ (II).

clearly indicate the presence of the LPP, whereas the SPP is identified by two diffraction peaks (see Fig 3). In addition, crystalline CER3 and CHOL are present. The relative intensities of the reflections attributed to the various phases in both diffraction patterns are similar. Moreover, the diffraction patterns resemble closely the one obtained with the mixture prepared with 15% CER1.

Discussion

In human SC, approximately 8.3% of the total CER fraction comprises CER1, whereas CER4 and CER9 account for 5.0% and 6.4%, respectively (Ponec *et al*, 2003). From the studies performed with mixtures based on natCER, we know that CER1 plays a prominent role in the formation of the LPP, characteristic for the SC lipid phase behavior. After the exclusion of CER1 from mixtures prepared with CER isolated from human SC, still a small fraction of lipids forms the LPP (Bouwstra *et al*, 2001). This indicates that CER4 or CER9 or both contribute to a certain extent to the formation of the LPP. Until now, however, the role CER4 and CER9 play in the lipid organization could not be studied, as it is impossible to isolate these acylceramides in sufficient quantities from human SC. As we now have available synthetic CER9, this is the first study in which we could systematically study the role of CER9, and CER9 in combination with CER1 on the lipid organization. Several important features were noticed: (i) CER1 promotes the formation of the LPP to a higher extent than CER9. (ii) The maximum solubility of CER9 in the lipid lamellar phases is lower than that of CER1. (iii) CER1 promotes incorporation of CER9 into the lamellar phases.

What can we learn from these observations? The presence of an additional hydroxyl group at the sphingoid base of acylceramides impedes the formation of the LPP. This is specially observed in equimolar CHOL:synthCER mixtures in which CER9 is not able to induce the formation of the LPP. Instead, strong reflections in the diffraction pattern reveal the presence of crystalline CER9 in separate domains. A possible explanation for this finding is that the larger and more hydrophilic head group present in CER9 inhibits the formation of the LPP. The situation changes when FFA were added to the CHOL:synthCER mixtures: Phase separation of CER9 is reduced and the LPP is formed. Unlike the synthCER fraction, the FFA fraction shows a high variation in acyl chain lengths. This may suggest that incorporation of CER9 into the lamellar phases in synthetic lipid mixtures requires the presence of acyl chains with varying lengths. Additional studies with mixtures prepared with FFA of a uniform chain length, however, should be performed to elucidate this. Furthermore, it needs to be emphasized, that the simplified synthetic lipid mixtures used might be more sensitive to phase separation than natCER present in native SC, due to limited variability in head group architecture and acyl chain length in the synthCER fraction.

Interestingly, the chain length of the ω-hydroxy acid in CER9 seems to have a major influence on the lamellar ordering of the lipids, as CER9(C27) is less effective in promoting the formation of the LPP than CER9(C30). Possibly, the presence of a shorter ω-hydroxy acid in CER9(C27) is less efficient in spanning the two broad lipid layers within the suggested tri-layer unit of the LPP (Bouwstra *et al*, 1998). This might result in a reduced stability of the LPP and a shift of the repeat distance to lower values (11.7 nm).

Another difference observed between CER1 and CER9 is the dependence of the formation of the LPP on the acylceramide level. Although a raise in CER1 content from 0% to 20% leads to a consistent enhancement of the LPP formation, a more complex phase behavior is observed in mixtures prepared with CER9. There, a raise in CER9 content up to 10% promotes the formation of the LPP. A further raise in the CER9 content reduces the formation of the LPP and results in the formation of crystalline CER9 in separate domains. Phase separation of CER9 is already observed in mixtures in which the synthCER fraction is prepared with 10% CER9. This means that even in the presence of FFA, the amount of CER9 that can be incorporated into the LPP is limited.

The findings that only a minute fraction of lipids organizes into the LPP when the synthCER fraction contains 5% CER9 and that no LPP is present in the absence of CER1 and CER9 confirm and extend the previous results obtained with mixtures prepared with natCER (Bouwstra *et al*, 2001). Based on the results of the present study, we can speculate that CER9 is indeed able to promote the formation the LPP

in natCER mixtures, which are excluded from CER1. From our results, however, we cannot deduce whether the relative amount of CER9 present in the SC is insufficient to effectively promote the formation of the LPP or that its larger and more hydrophilic head group hampers the formation of the LPP. Although the differences in phase behavior between CER1 and CER9 in synthCER mixtures are much more pronounced at increased acylceramide levels, CER1, always, more effectively promotes the formation of the LPP than CER9, even at a relative amount of 5%. The question whether CER4, which contains a 6-hydroxysphingosine head group that is even larger than the phytosphingosine head group of CER9, is also able to promote the formation of the LPP remains unanswered.

In spite of the finding that CER9 is less efficient in enhancing the formation of the LPP than CER1, partial substitution of CER1 with CER9 does not significantly affect the lipid organization. When extrapolating these findings to the *in vivo* situation, one can expect that small variations in the relative content of individual acylceramides will not substantially change the SC lipid organization. The situation, however, is usually more complex *in vivo*. In lamellar ichthyosis and atopic dermatitis, a marked reduction in CER1 in the lesion parts of the SC is observed (Yamamoto *et al*, 1991; Lavrijsen *et al*, 1995; Di Nardo *et al*, 1998; Bleck *et al*, 1999). In addition, in various skin disorders simultaneous changes in composition and content of various lipid classes are observed. All these changes may explain the reduced LPP formation in these skin disorders.

In conclusion, lipid mixtures prepared with synthCER offer an attractive tool to unravel the importance of molecular structure of individual CER on the SC lipid organization. The primary finding of the present study is that the head group architecture of acylceramides considerably affects the lipid organization. Compared with CER1, containing a sphingosine base, CER9 with its phytosphingosine base is less efficient in promoting the formation of the characteristic LPP and the level CER9 in the LPP is limited.

Materials and Methods

Materials Palmitic acid, stearic acid, arachidic acid, behenic acid, tricosanoic acid, lignoceric acid, cerotic acid, CHOL, and bovine brain ceramide type IV were purchased from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany). *N*-(30-linoleoyloxy-triacontanoyl)-sphingosine (synthetic ceramide 1(C30)-linoleate), *N*-(30-linoleoyloxy-triacontanoyl)-phytosphingosine (synthetic ceramide 9(C30)-linoleate), *N*-(27-linoleyl-heptacosanoyl)-phytosphingosine (synthetic ceramide 9(C27)-linoleate), and *N*-Tetracosanoyl-phytosphingosine (synthetic ceramide 3(C24)) were generously provided by Cosmoferm B.V. (Delft, The Netherlands). All organic solvents used were of analytical grade and manufactured by Labscan (Dublin, Ireland).

Preparation of the lipid mixtures For the preparation of the CHOL:synthCER:FFA mixtures, the following fatty acid mixture was used: C16:0, C18:0, C20:0, C22:0, C23:0, C24:0, and C26:0 at molar ratios of 1.3, 3.3, 6.7, 41.7, 5.4, 36.8, and 4.7, respectively. This is similar to the FFA ratio found in SC (Wertz and Downing, 1991). Appropriate amounts of individual lipids dissolved in chloroform:methanol (2:1) were combined to yield mixtures of approximately 1.5 mg dry weight at the desired composition with a total lipid concentration of 7 mg per mL. A Camag Linomat IV (CAMAG, Muttenz, Switzerland) was used to spray the lipid mixtures onto mica. This was done at a rate of 4.3 μL per min under a continuous nitrogen stream. The samples were equilibrated for 10 min at 80°C (CHOL:synthCER:FFA) or 95°C (CHOL:synthCER) and subsequently hydrated with an acetate buffer of pH 5.0. Finally, the samples were homogenized by 10 successive freeze–thawing cycles between -20°C and room temperature, during which the samples were stored under gaseous argon.

Small-angle X-ray diffraction (SAXD) All measurements were performed at the European Synchrotron Radiation Facility (ESRF, Grenoble) using station BM26B (Bras, 1998). The X-ray wavelength and the sample-to-detector distance were 1.24 Å and 1.7 m, respectively. Diffraction data were collected on a two-dimensional multiwire gas-filled area detector. The spatial calibration of this detector was performed using silver behenate. The samples were mounted in a sample holder with mica windows. Diffraction patterns of the lipid mixtures were obtained at room temperature for a period of 5 min. All measurements were performed at least in duplicate during two different sessions.

Small angle X-ray diffraction provides information about the larger structural units in the sample, namely the repeat distance of a lamellar phase. The scattering intensity I (in arbitrary units) was measured as a function of the scattering vector q (in reciprocal nm). The latter is defined as $q = (4\pi\sin\theta)/\lambda$, in which θ is the scattering angle and λ is the wavelength. From the positions of a series of equidistant peaks (q_n), the periodicity, or d-spacing, of a lamellar phase was calculated using the equation $q_n = 2n\pi/d$, n being the order number of the diffraction peak. The Leiden/Amsterdam Center for Drug Research of the University of Leiden approved all described studies.

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Supplementary Material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/JID/JID23477/JID23477sm.htm

Figure S1

The molecular structures of the ceramide (CER) present in human stratum corneum (SC). Two nomenclatures systems are given. The numbering system is based on chromatographic migration, whereas the other system (introduced by Motta *et al*, 1993) is based on the molecular structure.

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