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The expression and regulation of enzymes mediating the biosynthesis of triglycerides and phospholipids in keratinocytes/epidermis

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Key words: glycerol-3-phosphate acyltransferase, 1-acylglycerol-3-phosphate acyltransferase, lipin, diacylglycerol acyltransferase, human keratinocytes, epidermis

Abbreviations: AGPAT, 1-acylglycerol-3-phosphate acyltransferase; CL, cardiolipin; DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase; G-3-P, glycerol-3-phosphate; GPAT, glycerol-3-phosphate acyltransferase; LPA, lysophosphatidic acid; NEM, N-ethylmaleimide; PA, phosphatidic acid; PAP, phosphatidate phosphatase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphoinositol; PS, phosphatidylserine; TAG, triacylglycerol

Triglycerides and phospholipids play an important role in epidermal permeability barrier formation and function. They are synthesized de novo in the epidermis via the glycerol-3-phosphate pathway, catalyzed sequentially by a group of enzymes that have multiple isoforms including glycerol-3-phosphate acyltransferase (GPAT), 1-acylglycerol-3-phosphate acyltransferase (AGPAT), Lipin and diacylglycerol acyltransferase (DGAT). Here we review the current knowledge of GPAT, AGPAT, Lipin and DGAT enzymes in keratinocytes/epidermis focusing on the expression levels of the various isoforms and their localization in mouse epidermis. Additionally, the factors regulating their gene expression, including calcium induced differentiation, PPAR and LXR activators, and the effect of acute permeability barrier disruption will be discussed.

Introduction

The principal function of the skin is to provide a protective barrier against the transtcutaneous loss of water and electrolytes, which is essential for the survival of a mammal in a terrestrial environment. This epidermal permeability barrier is localized to the stratum corneum, the outermost layer of the skin.1 Stratum corneum consists of corneocytes, terminally differentiated keratinocytes which provide the mechanical strength of the skin, and a hydrophobic, lipid-enriched extracellular matrix, which provides the barrier to the movement of water and electrolytes. These extracellular lipids are primarily composed of cholesterol, ceramides and free fatty acids. On a total lipid mass basis, human stratum corneum contains 50% ceramides, 25% cholesterol and 15% free fatty acids.1 These lipids are delivered to the extracellular spaces of the stratum corneum by the secretion of lamellar body contents by differentiated keratinocytes.2 Lamellar bodies contain cholesterol, glucosylceramides and phospholipids, and following lamellar body secretion the glucosylceramides are metabolized to ceramides, a reaction catalyzed by β-glucocerebrosidase, and the phospholipids are metabolized to free fatty acids, a reaction catalyzed by secretory phospholipases.1-4 Inhibition of cholesterol, fatty acid, ceramide or glucosylceramide synthesis in the epidermis results in abnormal lamellar body formation indicating that the synthesis of sufficient quantities of these lipids (ceramide, glucosylceramide, cholesterol, fatty acids) is required for normal permeability barrier homeostasis.1

The free fatty acids produced in the epidermis serve as precursors for both phospholipids and ceramides, which are essential components for lamellar body formation. In human stratum corneum, fatty acids are predominantly straight chained, with C22 and C24 chain lengths being the most abundant.3 These fatty acids include the essential fatty acid linoleate, which is present in acylceramides, and the absence of linoleate (in essential fatty acid deficiency animals) leads to abnormal structure and function of the epidermal permeability barrier,5-9 indicating that the essential fatty acids are required for maintaining permeability barrier homeostasis. Recently, mice deficient in comparative gene identification-58 (CGI-58), a lipid droplet associated protein that facilitates triglyceride hydrolysis, have been shown to develop a severe permeability barrier defect.10 This along with studies in DGAT-2 deficient mice (see below) indicates that the synthesis and breakdown of triglycerides is also required for permeability barrier homeostasis.

Whereas much is known about the enzymes of sphingolipid and cholesterol synthesis in the epidermis/keratinocytes, little is known about the enzymes required for triglyceride and phospholipid synthesis. In this review, the focus will be on the expression levels and factors that regulate the key enzymes responsible for triglyceride and phospholipid biosynthesis in keratinocytes/epidermis.
The initial committed step in triglyceride/phospholipid synthesis via the glycerol phosphate pathway is the acylation of glycerol-3-phosphate by glycerol-3-phosphate acyltransferase (GPAT, EC 2.3.1.15).\(^{13-15}\) The resulting product lysophosphatic acid is further acylated by 1-acylglycerol-3-phosphate acyltransferase (AGPAT, EC 2.3.1.51) to produce phosphatidate.\(^{13,16}\) Phosphatidate is a branch point in lipid synthesis: it can serve as a precursor for either acidic phospholipids (PI, PG, CL) or diacylglycerol biosynthesis. The conversion of phosphatidate to diacylglycerol is catalyzed by lipins, a group of hydrolyases which have phosphatidate phosphatase (PAP1) enzyme activity. Finally, diacylglycerol is converted to triacylglycerol through the action of diacylglycerol acyltransferase (DGAT), or serves as precursor for phospholipid (PC, PS, PE) synthesis.\(^{17,18}\)

**The Expression and Regulation of Key Enzymes in the Biosynthetic Pathway of Triglyceride/Phospholipid in Keratinocytes/Epidermis**

GPATs. In eukaryotic cells, several GPAT isoforms exist that differ in subcellular localization, sensitivity to N-ethylmaleimide (NEM) inactivation and substrate preferences.\(^{12-14}\) So far, four GPAT genes (GPAT-1, -2, -3 and -4) have been cloned and their products demonstrated to have GPAT activities.\(^{13,14}\) GPAT-1 encodes a NEM-resistant enzyme, which is located to the outer membrane of mitochondria with a substrate preference for saturated palmitoyl-CoA. GPAT-2 encodes a NEM-sensitive mitochondrial isoform, but it is expressed in limited tissues and to date only has been found in testis and liver. Both GPAT-3 and GPAT-4 are ER-associated, NEM-sensitive enzymes, which utilize a broad range of long-chain fatty acyl-CoAs, including both saturated and unsaturated species, as substrates.\(^{12-14}\)

In both cultured human keratinocytes and mouse epidermis, GPAT-2 mRNA is not detected in fetal rat epidermis.\(^{19}\) The mRNA levels of GPAT-1 and GPAT-3 are found to increase on day 19, reaching a peak on day 20, and declining thereafter. GPAT-4 mRNA levels also change from days 17–22, but to lesser extent.\(^{19}\) These results indicate that the expression of GPAT-1 and GPAT-3 significantly increases during the late stage of fetal epidermal development, concurrent with the formation of lamellar bodies and SC extracellular lipid membranes.\(^{21}\)

**Table 1.** C\(_s\) values of GPAT, Lipin and DGAT isoforms in human keratinocytes and mouse epidermis.

| GPAT Isoforms | CHKs | Mouse Epidermis |
|--------------|------|-----------------|
| GPAT1        | 24–25 | 24–25          |
| GPAT2        | nd*  | nd              |
| GPAT3        | 30–32 | 23–24          |
| GPAT4        | 27–28 | 27–28          |
| Lipin-1      | 23–24 | 28–30          |
| Lipin-2      | 27–28 | 25–28          |
| Lipin-3      | 25–27 | 23–27          |
| DGAT1        | 29–30 | 30–33          |
| DGAT2        | 34–35 | 23–25          |

nd*, not detected. C\(_s\) values are inversely proportional to the amount of target mRNA in the sample (i.e., the lower the C\(_s\) level the greater the amount of mRNA in the sample). C\(_s\) ≤ 29 indicate abundant target mRNA in the sample. CHKs, cultured human keratinocytes.

**Biosynthetic Pathway of Triglycerides and Phospholipids**

In mammals, triglycerides are synthesized through two major pathways, the glycerolphosphate (Kennedy) pathway and the monoacylglycerol pathway while phospholipids are synthesized via the glycerolphosphate (Kennedy) pathway.\(^{11,12}\) The glycerol phosphate pathway is responsible for the majority of de novo biosynthesis of triglycerides in most cell types, while the monoacylglycerol pathway is very important in triglyceride synthesis in the small intestine.\(^{11,12}\) Here we focus on the glycerol phosphate pathway, in which the acylation of glycerol 3-phosphate occurs through a stepwise addition of activated fatty acyl groups, each of which is catalyzed by distinct enzymes (Fig. 1).
It is well recognized that high calcium induces keratinocyte differentiation. In response to high calcium, GPAT-3 mRNA levels markedly increase whereas both GPAT-1 and GPAT-4 mRNA levels decrease by ~50% at most time points in primarily cultured human keratinocytes. In parallel, total GPAT activity increases (two-fold) in keratinocytes cultured in high calcium medium versus that in low calcium control. Further analysis reveals that NEM-sensitive GPAT activity, which is encoded by GPAT-3, increases about three-fold, with no alteration in NEM-resistant activity, which is encoded by GPAT-1. Together, these studies demonstrate that GPAT isoforms are differentially regulated during keratinocyte differentiation, with GPAT-3 expression increasing, leading to an increase in total GPAT activity, attributable to the GPAT-3-encoded NEM-sensitive GPAT activity.

PPAR-α, -δ, -γ and LXR-α, -β, are expressed in keratinocytes/epidermis and activation of these nuclear hormone receptors has numerous effects on keratinocyte function. When keratinocytes are treated with PPAR and LXR activators, GPAT-3 gene expression increases significantly, with PPAR-γ > PPAR-δ > LXR > PPAR-α. Activators of RXR also slightly increase GPAT-3 mRNA levels. In contrast, GPAT-1 and GPAT-4 mRNA levels are not altered by PPAR or LXR activators. The increase in GPAT-3 gene expression by PPAR-γ or PPAR-δ activators occurs at the level of transcription since treatment of keratinocytes with either PPAR-γ or PPAR-δ activators does not alter GPAT-3 mRNA stability. However, PPAR activators increase the expression of GPAT-3 promoter constructs. Finally, the increase in GPAT-3 gene expression by PPAR activators leads to a significant increase in microsomal GPAT activity (which is consistent with an increase in GPAT-3), resulting in an increase in glycerolipid synthesis. Thus, PPAR or LXR activators specifically upregulate GPAT-3 expression but not other GPAT isoforms in cultured human keratinocytes.

Acute permeability barrier disruption by either tape-stripping or acetone treatment did not alter GPAT in mouse epidermis.

AGPATs. The end product of GPAT action is lysophosphatidic acid, which is further acylated to phosphatidic acid by adding an acyl group to the sn-2 position of the glycerol backbone, catalyzed by AGPAT enzymes (Fig. 1). AGPAT is also known as lysophosphatidic acid acyltransferase. Like GPAT, multiple AGPAT isoforms have been identified in mammals, each encoded by a distinct gene. These AGPAT isoforms display tissue-specific variation in expression and activity as well as different substrate preferences, suggesting tissue-specific functions. New AGPAT isoforms have been identified recently and the current understanding of the biological role of AGPATs is far from complete. Originally, five isoforms were found in mice and six in human, with mouse AGPAT-1-5 having validated enzyme activity. Additionally, mouse AGPAT-2 has two-fold higher enzyme activity than AGPAT-3, -4 and -5 in an in vitro system by direct comparison. Recently, AGPAT-10 has been reported. Since AGPAT-6 has microsomal GPAT activity and is localized to the ER, it is now renamed as GPAT-4. AGPAT-8, which is highly related to AGPAT-5, has been shown to have acyl-CoA: lysocardiolipin acyltransferase activity and AGPAT activity. AGPAT-7 and AGPAT-9 are reported as members of the lysophosphatidylcholine acyltransferase family, with only weak AGPAT activity. Finally, AGPAT-10, which is identical in sequence to GPAT-3 and localized to the ER, has been reported to have both AGPAT and GPAT activity.

In mouse epidermis, AGPAT-3, -4 and -5 have relatively high constitutive expression levels, while AGPAT-1 and -2 have low constitutive expression levels. Localization studies demonstrated that all five isoforms of AGPAT are expressed in all nucleated layers of mouse epidermis. Of note is in other tissues, including brain, heart, lung, liver, kidney and spleen, both AGPAT-1 and -2 are expressed at relatively high levels. Similarly, all five AGPATs are expressed in cultured human keratinocytes. AGPAT-3, -4, and -5 are expressed at high constitutive levels while AGPAT-1 and -2 expression levels are relatively low. Like GPATs, the physiological role of these different isoforms of AGPAT in keratinocytes/epidermis remains unclear.

The expression pattern of AGPATs during fetal rat skin barrier development has been determined. The mRNA levels for both AGPAT-2 and -5 increase progressively in the epidermis at days 17–22, peaking on day 19, while mRNA levels for AGPAT-1, -3 and -4 remain unchanged. There is a parallel increase in the total AGPAT activity on day 19, which may be due to the increased expression of AGPAT-2 and -5.

Unlike GPAT3, which is induced during keratinocytes differentiation, AGPAT expression is not altered by exposure to high calcium treatment for 6–10 days. Additionally, treatment with PPAR or LXR activators also had a minimal effect on AGPAT expression.

Acute disruption of the permeability barrier by tape-stripping results in a rapid increase in the mRNA levels of AGPAT-1, -2 and -3. AGPAT-5 mRNA also increases after tape-stripping but to a lesser extent. In contrast, the mRNA levels of AGPAT-4 remain unchanged. These observations were verified by using another method (acetone) to disrupt the barrier, and similarly, the mRNA levels of AGPAT-1, -2, -3 and -5 increase by 1 hour after acetone treatment with no change in the mRNA levels of AGPAT-4. In parallel with the increase in mRNA levels, there is a corresponding increase in total AGPAT enzyme activity in mouse epidermis following tape-stripping. Thus, acute disruption of the permeability barrier stimulates an increase in both mRNA levels and enzyme activity of AGPATs in mouse epidermis. Since upregulation of AGPAT mRNA after tape-stripping can be partially reversed by artificial restoration of the permeability barrier by occlusion with an impermeable membrane, it can be postulated that the increase in the expression of AGPAT is linked to permeability barrier requirements.

Lipins. At the third step of triglyceride/phospholipid biosynthesis, phosphatidic acid, produced by the action of AGPAT enzymes, is diphosphorylated to form diacylglycerol for the synthesis of triglycerides and phospholipids (PC, PE and PS). This diphosphorylation process is catalyzed by phosphatidate phosphatase (PAP1), which is localized in the cytosol and transiently translocates to the ER membrane to encounter phosphatidic acid substrate for performing the phosphatase reaction. PAP1 enzymes are Mg2+-dependent, NEM sensitive and are responsible for most of PAP activity involved in the glycerol phosphate biosynthetic pathway.
pathway. Of note PAP2 has lipid phosphate phosphatase activity that is Mg2+-independent, NEM resistant, localized to the plasma membrane and is not involved in triglyceride synthesis through glycerol phosphate pathway. The PAP catalytic motif is present in the carboxyl-terminal lipin domain (C-LIP) of lipin proteins. This motif is critical for PAP function, as conversion of aspartate residue to glutamate in this motif completely abolishes PAP activity. In addition, a naturally occurring glycine to arginine mutation in amino-terminal lipin domain dramatically reduces PAP activity in mouse, indicating the importance of N-LIP domain as well. Finally, the PAPI family is composed of three members, Lipin-1, -2 and -3. In addition to its enzyme activity Lipin-1 serves as a transcriptional co-activator interacting with PPARα, PPARδ, PPARγ, hepatocyte nuclear factor 4α, and the glucocorticoid receptor to enhance transcriptional activity. Human lipin-1 and mouse lipin-1, lipin-2 and lipin-3 all have PAP activity, which is specific for phosphatidic acid and no activity for other related lipid phosphate substrates, including LPA, sphingosine phosphate, ceramide-1-phosphate. Furthermore, while lipin genes are conserved across a broad range of eukaryotes, including vertebrates, fly, nematodes and yeast, lipin orthologs in all species have conserved regions of both C-LIP and N-LIP. This evolutionary conservation of lipin proteins indicates the important role of lipin proteins in cellular function.

There is a distinct tissue distribution of the three lipin family members. Lipin-1 is mainly found in adipose tissue and muscle, whereas lipin-2 is expressed at high levels in liver, brain, and kidney, and lipin-3 is detected at low levels in several visceral tissues, including small intestine and liver. In human keratinocytes under proliferating conditions (maintained in low calcium medium), lipin-1 expression is relatively high (Ct: 23–24), followed by lipin-3 (Ct: 25–27) and lipin-2 (Ct: 27–28) (Table 1). In mouse epidermis, however, lipin-3 expression is relatively abundant (Ct: 25–26), while lipin-1 and -2 expression levels are in similar ranges (Ct: 28–29). All three lipin mRNAs are localized to the upper epidermis, while lipin-2 mRNA is also found in the lower epidermis.

Acute permeability barrier disruption by tape stripping results in an increase in Lipin 1 mRNA levels as early as 1 hour after barrier disruption by tape stripping with an ∼5.5-fold increase at 3 hours, returning towards baseline levels at later times (Fig. 2). Similar changes occur in lipin-2 mRNA levels after permeability barrier disruption, albeit the magnitude of the increase is smaller (Fig. 2). In contrast, no alteration occurs in lipin-3 mRNA levels (Fig. 2). To confirm these observations, acetone treatment was employed to disrupt permeability barrier function. As shown in Figure 3, 3 hours after acetone treatment, both lipin-1 and -2 mRNA levels increase, with no change in lipin-3 mRNA levels. However, occlusion following permeability barrier disruption does not block the increases in lipin-1 and -2 expression, suggesting that the upregulation of lipin gene expression may be due to an injury reaction rather than being mediated by the loss of permeability barrier function.

Calcium induces differentiation, and treatment of keratinocytes with either high calcium, or PPAR/LXR activators has a limited effect on the expression of all three lipin isoforms in keratinocytes.

When skin structure and function were examined in the lipin-1 deficient mice, neither epidermal morphology, surface pH, SC hydration, basal trans-epidermal water loss (a sensitive marker of permeability barrier function), or the recovery of permeability barrier function were altered in the lipin-1 deficient mice compared to wild type controls (data not shown). Thus, epidermal structure and function is not abnormal in lipin-1 deficient mice, presumably due to compensation by lipin-2 and -3.

DGAT. In the final step of triglyceride biosynthesis, diacylglycerol is converted to triglyceride, a reaction catalyzed by diacylglycerol acyltransferase (DGAT). In mammals, there are two DGAT isoforms, DGAT-1 and DGAT-2. DGAT-1 has 6–12 putative trans-membrane domains and is a member of the mammalian acyl-CoA: cholesterol acyltransferase gene family, whereas DGAT-2 has two trans-membrane domains and is a member of a gene family that includes DGAT-2 and three monoacylglycerol acyltransferase isoforms. Both DGAT-1 and DGAT-2 recognize DAG and monoacylglycerol as substrates for triglyceride

![Figure 2](Image)
Both DGAT-1 and -2 are widely expressed in mammalian tissues, including small intestine, liver, adipose tissue, mammary gland, skeletal muscle, heart, spleen and pancreas. In neonatal mouse skin, DGAT-2 is expressed at higher levels in the epidermis than dermis. DGAT-1 is barely detectable in both epidermis and dermis of neonatal mice skin. Using RT-PCR, DGAT-1 and 2 mRNA can be detected in adult mouse epidermis but DGAT-2 expression is much more robust than DGAT-1 (Table 1). The lower epidermis (basal layer) accounts for the majority of DGAT-2 expression (Fig. 4). While DGAT-1 is not highly expressed in adult mouse epidermis it is very abundantly expressed in sebaceous glands.

In cultured human keratinocytes the expression levels of DGAT-1 is as expected modest (Table 1). However, very surprisingly, in cultured human keratinocytes the expression level of DGAT-2 is very low (C_T = 34–35) compared with that in mouse epidermis (C_T = 23–25) (Table 1). That the low abundance of DGAT-2 mRNA in human keratinocytes is not a technical problem is shown by the ability to detect significant amounts (C_T = 29–30) of DGAT-2 mRNA in Hep3G cells, a human liver cell line. Why the level of expression of DGAT-2 is different between human keratinocytes and mouse epidermis is unclear. It could be a species difference or perhaps due to the procedures used to grow human keratinocytes. It would be of interest to measure DGAT-2 mRNA in human epidermis.

Treatment of keratinocytes with either high calcium, or PPAR/LXR activators did not alter the expression of DGAT-1 or -2 in keratinocytes. Additionally, neither DGAT-1 nor DGAT-2 expression changed following acute barrier disruption by tape-stripping in hairless mouse epidermis, indicating that basal DGAT expression suffices to meet the permeability barrier requirement in adult mice.

The skin of Dgat2-deficient mice lacks elasticity and is shining, dry and cracked shortly after birth. Dgat2-deficient mice rapidly lose weight from dehydration owing to impaired permeability barrier function and increased transepidermal water loss. Structurally, light microscopy of Dgat2-/- skin reveals compact hyperkeratosis of affected stratum corneum, thinning of the epidermis and flattened of the dermo-epidermal interface owing to the effacement of the epidermal rete ridges/papillary projections. Electron microscopy of Dgat2-/- skin further reveals a reduced number of lamellar membranes and lamellar bodies with decreased lipid content, reflecting abnormalities in the lamellar body secretory system. A 96% reduction in triglyceride content is found in Dgat2-/- skin, with normal levels of other lipids. Further analysis reveals a drastically reduction (>90%) of linoleic acid content in triglycerides and free fatty acids. Most interestingly, there is a marked reduction (>60%) in the content of

![Figure 3. Lipin gene expression in mouse epidermis after acetone treatment.](image)

![Figure 4. Localization of DGAT-2 in mouse epidermis.](image)
acylceramide, a skin lipid containing linoleic acid that is critical for skin barrier maintenance. Thus, the lack of DGAT2 results in a decrease in triglyceride synthesis leading to abnormal lamellar body formation and secretion, and ultimately impaired skin permeability barrier function.

Furthermore, there are striking differences in the phenotype of DGAT-1 and DGAT-2 knock-out mice: Dgat2-deficient mice die shortly after birth, owing to severe abnormalities in both energy metabolism and skin permeability barrier function. In contrast, Dgat1-deficient mice are viable and healthy, but have significant changes in lipid metabolism in several tissues including white adipose tissue and skin, and the sebaceous glands. Dgat1-deficient mice developed dry fur and hair loss after puberty and exhibited impaired water repulsion and hypothermia due to the defective production of fur lipids, in particular type II wax diesters. These changes are accounted for by abnormalities in sebaceous gland function. Dgat1-deficient mice do not have epidermal abnormalities and permeability barrier function is normal.

**Conclusion**

The biosynthesis of triglycerides and phospholipids requires multiple steps each of which are catalyzed by several different enzyme isoforms. Studies have shown that the epidermis expresses three GPAT isoforms, at least five AGPAT isoforms, three lipin isoforms, and two DGATs (in the mouse DGAT-2 is predominant with relatively low expression of DGAT-1). Why the epidermis expresses multiple isoforms is unclear but there are a number of possible explanations. First, having multiple different enzymes ensures that a mutation in a single enzyme is not likely to significantly compromise triglyceride or phospholipid synthesis, which is essential to form a functional permeability barrier. The exception to this is DGAT, where mice deficient in DGAT-2 die shortly after birth, in part due to an abnormality in permeability barrier function. Second, the epidermis is structurally very complex with undifferentiated proliferating cells in the basal layer and markedly differentiated cells in the outer layers. It is possible that expression of different isoforms differs for each cell type. Third, each isoform may localize to a different site within the cell. For example GPAT-1 is located in the mitochondria whereas GPAT-3 is located in the endoplasmic reticulum, which could result in different functional roles. Fourth, keratinocyte have a wide variety of fatty acids, including very long chain fatty acids, and it is possible that different isoforms have specificity for certain fatty acids. To utilize the diverse variety of fatty acids in keratinocytes may require different isoforms. Finally, the factors that regulate the expression or catalytic activity of different isoforms could differ allowing for a more precise regulation of triglyceride and phospholipid synthesis in keratinocytes. Future studies will need to address the roles of these various isoenzymes in epidermal metabolism.

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