25 Hydroxyvitamin D 1 α-Hydroxylase Is Required for Optimal Epidermal Differentiation and Permeability Barrier Homeostasis

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Keratinocytes express high levels of 25OHD 1α-hydroxylase (1OHase). The product of this enzyme, 1,25-dihydroxyvitamin D (1,25(OH)$_2$D), promotes the differentiation of keratinocytes *in vitro* suggesting an important role for this enzyme in epidermal differentiation. To test whether 1OHase activity is essential for keratinocyte differentiation *in vivo* we examined the differentiation process in mice null for the expression of the *1αOHase* gene (*1αOHase*$^{-/-}$). Heterozygotes for the null allele were bred, and the progeny genotyped by PCR. The epidermis of the *1αOHase*$^{-/-}$ animals and their wild-type littermates (*1αOHase*$^{+/+}$) were examined by histology at the light and electron microscopic level, by immunocytochemistry for markers of differentiation, and by function examining the permeability barrier using transepidermal water loss (TEWL). No gross epidermal phenotype was observed; however, immunocytochemical assessment of the epidermis revealed a reduction in involucrin, filaggrin, and loricrin—markers of differentiation in the keratinocyte and critical for the formation of the cornified envelope. These observations were confirmed at the electron microscopic level, which showed a reduction in the F (containing filaggrin) and L (containing loricrin) granules and a reduced calcium gradient. The functional significance of these observations was tested using TEWL to evaluate the permeability barrier function of the epidermis. Although TEWL was normal in the basal state, following disruption of the barrier using tape stripping, the *1αOHase*$^{-/-}$ animals displayed a markedly delayed recovery of normal barrier function. This delay was associated with a reduction in lamellar body secretion and a failure to reform the epidermal calcium gradient. Thus, the 25OHD 1OHase is essential for normal epidermal differentiation, most likely by producing the vitamin D metabolite, 1,25(OH)$_2$D, responsible for inducing the proteins regulating calcium levels in the epidermis that are critical for the generation and maintenance of the barrier.

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Keratinocytes are not only capable of producing vitamin D_3 from endogenous sources of 7-dehydro-cholesterol (7-DHC) in a regulated fashion but of metabolizing vitamin D via 25 hydroxylation and 1α-hydroxylation to 1,25(OH)$_2$D (Bikle *et al*, 1986a, b; Matsumoto *et al*, 1991; Lehmann *et al*, 2001). Thus, keratinocytes are the only cells in the body with the whole pathway from 7-DHC to 1,25(OH)$_2$D. The vitamin D-25 hydroxylase in keratinocytes is the same mitochondrial enzyme (CYP27) that converts vitamin D to 25OHD in the liver (Lehmann *et al*, 1999). Similarly, the 25OHD-1α hydroxylase (1OHase) in the epidermis, which is responsible for 1,25(OH)$_2$D production, is the same enzyme (CYP27B1) as in the kidney (Fu *et al*, 1997). The expression and enzymatic activity of 1OHase in keratinocytes are tightly regulated and coupled to the differentiation of these cells (Pillai *et al*, 1988a).

Abbreviations: 1,25(OH)$_2$D, 1,25-dihydroxyvitamin D; 25OHD, 25 hydroxyvitamin D; 1OHase, 25OHD 1α-hydroxylase; TEWL, transepidermal water loss; VDR, vitamin D receptor

The observation that 1,25(OH)$_2$D induces keratinocyte differentiation *in vitro* was made by Hosomi *et al* (1983) and provided a rationale for the previous and unexpected finding of 1,25(OH)$_2$D receptors in the epidermis *in vivo* (Stumpf *et al*, 1979). 1,25(OH)$_2$D is likely to be an autocrine or paracrine factor for epidermal differentiation since it is produced by the keratinocyte, but under normal circumstances keratinocyte production of 1,25(OH)$_2$D contributes minimally to circulating levels (Bikle *et al*, 1986a, b). The receptors (vitamin D receptor (VDR)) for and the production of 1,25(OH)$_2$D vary with differentiation (Horiuchi *et al*, 1985, Merke *et al*, 1985, Pillai *et al*, 1988a) in a manner that suggests feedback regulation; i.e., VDR and 1OHase are found in highest levels in the stratum basale *in vivo* and both are reduced with differentiation of keratinocytes *in vitro*. *In vitro* 1,25(OH)$_2$D induces involucrin and transglutaminase, and stimulates cornified envelope formation at subnanomolar concentrations in preconfluent keratinocytes (Hosomi *et al*, 1983; Smith *et al*, 1986; McLane *et al*, 1990; Pillai and Bikle, 1991; Su *et al*, 1994). Where examined, these effects of 1,25(OH)$_2$D could be reproduced by 25OHD (Bikle *et al*, 1991; Matsumoto *et al*, 1991), presumably because of

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endogenous conversion of 25OHD to 1,25$(OH)_2$D, but are not observed with the biologically inactive β isomer of 1,25$(OH)_2$D (Smith *et al*, 1986) (the natural isomer is lα,25$(OH)_2$D) indicating specificity for the natural metabolite of 1OHase. Thus, 1,25$(OH)_2$D and the enzyme that produces it are likely to play an important role in epidermal differentiation *in vivo* as well as *in vitro*, the hypothesis being tested in this study.

Calcium is the best-studied prodifferentiating agent for keratinocytes. As for 1,25$(OH)_2$D, most of these studies have been performed *in vitro*. But a calcium gradient exists in the epidermis with low levels of calcium in the basal and spinous layers but with substantially higher levels of calcium in the upper granular layers, accumulating in the cytoplasm and the intercellular matrix (Menon *et al*, 1985). This gradient of calcium appears to provide the driving force for differentiation in intact epidermis (Elias *et al*, 2002).

Calcium and 1,25$(OH)_2$D interact in their ability to stimulate differentiation (Su *et al*, 1994). Both calcium (in the absence of 1,25$(OH)_2$D) and 1,25$(OH)_2$D (at 0.03 mM Ca^{2+}) raise the mRNA levels for involucrin and transglutaminase in a concentration-dependent fashion. The stimulation is synergistic at intermediate concentrations of calcium (0.1 mM) and 1,25$(OH)_2$D (10^{-10} M), but inhibition is observed in combination at higher concentrations because mRNA stability decreases (Su *et al*, 1994).

The recent availability of mice lacking the 1OHase (*1αOHase*$^{-/-}$) (Dardenne *et al*, 2001; Panda *et al*, 2001) provides an opportunity to evaluate the degree to which 1,25$(OH)_2$D and its epidermal production are required for epidermal differentiation *in vivo*. We report here that these mice show a reduction in levels of proteins forming the corneocyte associated with a marked reduction in intraepidermal calcium. Furthermore, *1αOHase*$^{-/-}$ animals have a retarded recovery of permeability barrier function after acute disruption of the stratum corneum, associated with an impaired reestablishment of the calcium gradient in the epidermis. Unlike *VDR*$^{-/-}$ mice, *1αOHase*$^{-/-}$ mice do not have a defect in hair follicle cycling. These observations indicate that endogenously produced 1,25$(OH)_2$D and/or 1OHase are critical for normal epidermal differentiation, perhaps by facilitating the formation and maintenance of the calcium gradient.

Results

General appearance is little affected The *1αOHase*$^{-/-}$ mice demonstrated little difference in gross phenotype over the first 6 wk of life. Body size and weight gain were not significantly different from their wild-type littermates during this period regardless of whether the mice were maintained on the 1.3% calcium diet or the 2.4% calcium rescue diet. Only after 6 wk were differences in body size observed in the *1αOHase*$^{-/-}$ animals on the 1.3% diet, although the 2% calcium rescue diet prevented any decline in growth rate. At no time was alopecia observed.

Reduced expression of markers of differentiation in the epidermis of *1αOHase*$^{-/-}$ mice *In vitro*, 1,25$(OH)_2$D reduces keratinocyte proliferation, while inducing the expression of differentiation markers and promoting cornified envelope formation. In the *1αOHase*$^{-/-}$ mouse epidermis, hyperplasia was not observed, nor were differences in the epidermal expression of the PCNA found at any age between wild-type and *1αOHase*$^{-/-}$ mice (data not shown). But when the expression of differentiation markers in the epidermis was evaluated by immunocytochemistry, a decrease in the levels of filaggrin, loricrin, and involucrin was observed in *1αOHase*$^{-/-}$ mice (Fig 1). These differences were apparent by 1 wk of age and persisted throughout the 6 wk study. The reduction in expression of these differentiation markers was accompanied by a reduction in the number of keratohyalin granules in the upper layers (stratum granulosum) of the epidermis (Fig 1). Electron microscopic examination of the epidermis of the *1αOHase*$^{-/-}$ mice confirmed the marked reduction in both the F-type (filaggrin-containing) and L-type (loricrin-containing) granules (Fig 2).

Barrier function is abnormal in *1αOHase*$^{-/-}$ mice The barrier to water loss is a critical feature of the terminal differentiation of the epidermis. In the resting (basal) state, TEWL rates were comparable in the *1αOHase*$^{-/-}$ mice and their wild-type littermates. But the *1αOHase*$^{-/-}$ mice showed a marked retardation in the recovery of the barrier following acute disruption (Fig 3). In particular, 3 h after barrier disruption the wild-type mice had recovered 55% of the baseline value, with a further recovery to 65% of the basal value by 6 h, whereas the *1αOHase*$^{-/-}$ mice had recovered only 27% of the baseline value by 3 h, with little or no further improvement by 6 h.

Lamellar body secretion is abnormal in the epidermis of *1αOHase*$^{-/-}$ mice following barrier disruption Following barrier disruption, the cells in the stratum granulosum normally accelerate the secretion of their contents of lamellar bodies into the intercellular space between the stratum granulosum and stratum corneum, a process that leads to rapid restoration of normal barrier function. To ascertain whether reduced lamellar body secretion contributed to the delay in barrier recovery in *1αOHase*$^{-/-}$ mice, we examined the ultrastructure of the outer layers of the epidermis from *1αOHase*$^{-/-}$ mice and their wild-type littermates 3 h after barrier disruption (Fig 4). Reduced lamellar body contents were present at the stratum corneum–stratum granulosum interface of the epidermis of *1αOHase*$^{-/-}$ mice compared with that of wild-type mice (compare Fig 4*A* and *B* to Fig 4*C* and *D*). Note again the reduction in both F and L granules in the epidermis of the *1αOHase*$^{-/-}$ mice compared with that in wild-type mice. The reduction in lamellar body secretion correlates with and explains the delay in barrier recovery in the *1αOHase*$^{-/-}$ mice.

Epidermal calcium gradient is reduced in *1αOHase*$^{-/-}$ mice 1,25$(OH)_2$D increases the calcium levels of keratinocytes *in vitro*, an effect thought to be critical for its ability to stimulate differentiation. Similarly, the epidermis displays a calcium gradient, with low levels of calcium in the basal and spinous layers, but increased levels in the granular layer, peaking just under the stratum corneum. The calcium

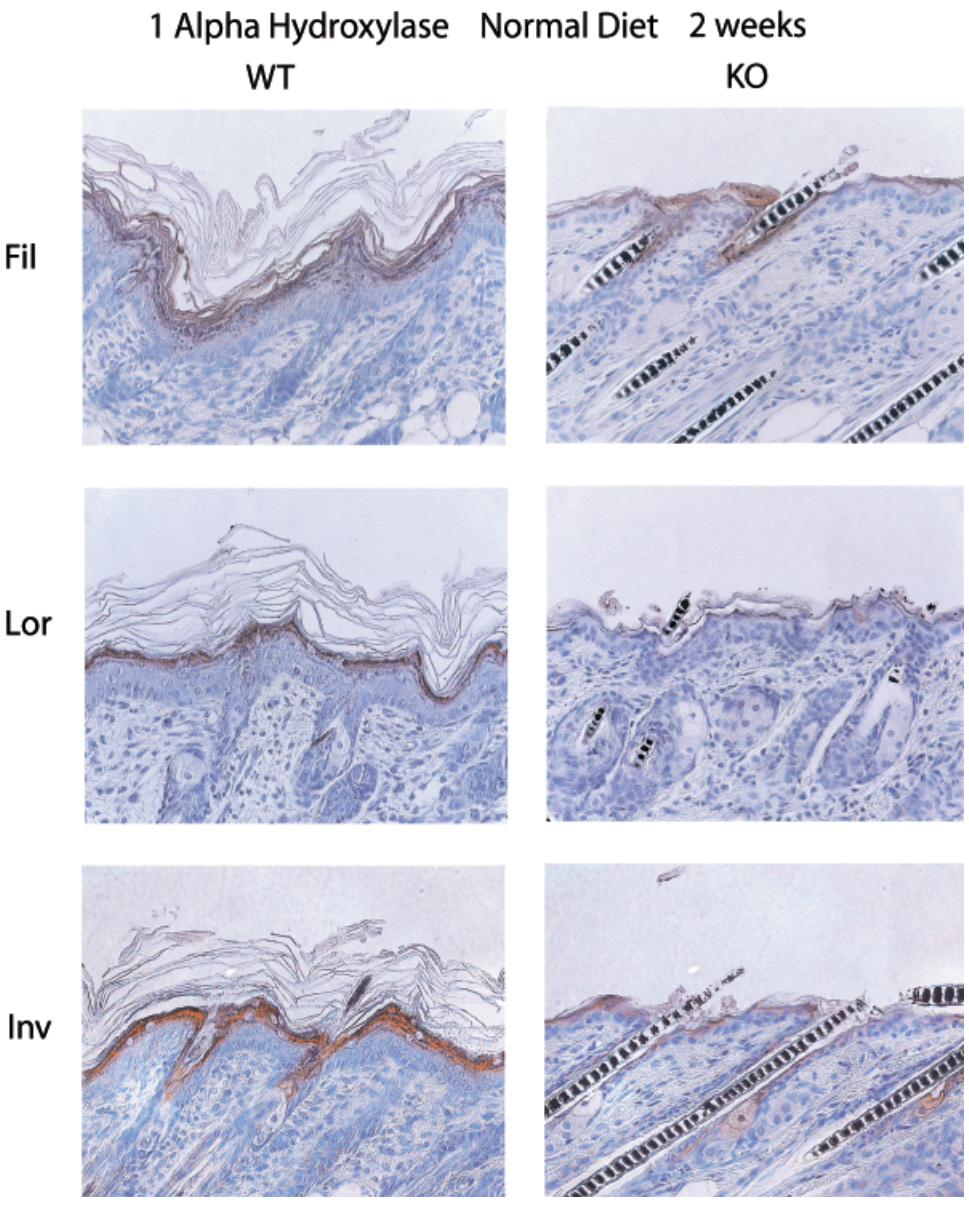

Figure 1
Decreased levels of filaggrin, loricrin, and involucrin in the *1αOHase*$^{-/-}$ mouse. Representative sections of the skin from the backs of 2-wk-old wild-type and *1αOHase*$^{-/-}$ mice (*n = 3 per group*) were assessed by immunocytochemistry for the expression of filaggrin (Fil), loricrin (Lor), and involucrin (Inv). As expected these proteins were expressed primarily in the outer layers of the epidermis. Expression was substantially lower in the epidermis of the *1αOHase*$^{-/-}$ mice. Note also the marked reduction in granules in the outer epidermis of the *1αOHase*$^{-/-}$ mice.

gradient in the epidermis *in vivo* is associated with the maintenance of barrier function by two mechanisms. First, it regulates lamellar body secretion, and, second, it appears to play a critical role in the induction of proteins required for cornified envelope formation. As shown in Figs 2 and 4 both lamellar body secretion and levels of corneocyte proteins are reduced in the epidermis of *1αOHase*$^{-/-}$ mice. Therefore, we next examined the calcium gradient in the epidermis of the *1αOHase*$^{-/-}$ mice compared with that of their wild-type littermates (Fig 5). In the basal state, the levels of calcium in the outer epidermis of *1αOHase*$^{-/-}$ mice were reduced overall, with little evidence of a gradient from the stratum basale to the stratum granulosum as was found in wild-type mice (compare Fig 5*A* to Fig 5*C*). Moreover, some sections of the *1αOHase*$^{-/-}$ epidermis showed increased calcium in the stratum corneum (Fig 5*B*) consistent with an abnormality in barrier function.

To determine whether the delay in permeability barrier recovery in the *1αOHase*$^{-/-}$ mouse correlated with an abnormal recovery of the calcium gradient following barrier disruption, we examined the calcium gradient at different times after acute barrier disruption (Fig 6). Immediately after barrier disruption, calcium levels in the epidermis of both wild-type and *1αOHase*$^{-/-}$ mice became negligible, with loss of the gradient (compare Fig 6*A* to Fig 6*C*); however, by 6 h the calcium gradient was *well on its way to restoration* in wild-type animals, but was not in *1αOHase*$^{-/-}$ mice (compare Fig 6*B* to Fig 6*D*). These results suggest that endogenous 1,25$(OH)_2$D production influences the formation of the epidermal calcium gradient, and in the absence of this gradient the rate of recovery of barrier function following perturbation is impaired.

Discussion

The discovery of high levels of 1OHase activity in the keratinocyte was a well-documented early example of extrarenal 1,25$(OH)_2$D production (Bikle *et al*, 1986a, b). But the actual requirement for this enzyme or its product,

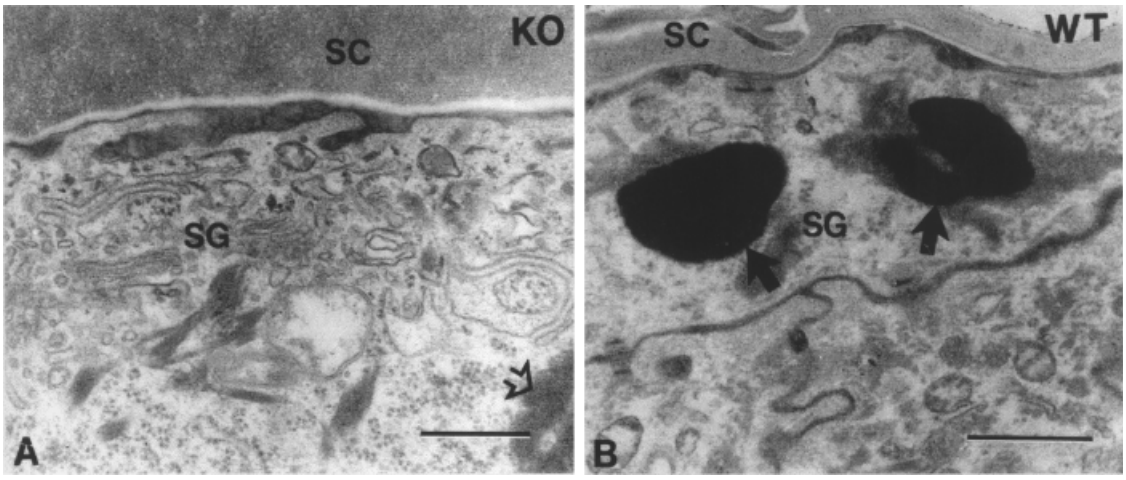

Figure 2
Decreased granules in the outer epidermis in *1αOHase*$^{-/-}$ mice. Electron micrographs of representative sections of the epidermis from skin samples taken from the backs of 6-wk-old *1αOHase*$^{-/-}$ mice and their wild-type littermates (*n = 3 per group*) demonstrate a marked reduction in number and size of loricrin and keratohyalin granules in the outer epidermis of the *1αOHase*$^{-/-}$ mice. These findings are consistent with the reduction in loricrin and filaggrin demonstrated in Fig 1.

1,25$(OH)_2$D, in epidermal function has not been clarified. *In vitro* studies performed in the absence of serum, and so in the absence of vitamin D and its metabolites, have indicated that 1,25$(OH)_2$D is not required for calcium-induced differentiation (Pillai *et al*, 1988b), although 1,25$(OH)_2$D clearly influences the differentiation process by itself and in coordination with calcium (Hosomi *et al*, 1983; Smith *et al*, 1986; McLane *et al*, 1990; Pillai and Bikle, 1991; Su *et al*, 1994). Furthermore, in humans with mutations in *1αOHase* no striking epidermal phenotype has been described, although this has not been examined in detail. Conceivably, calcium and other regulators of differentiation can compensate to a large degree for the lack of 1,25$(OH)_2$D. The lack of apparent epidermal phenotype in these subjects contrasts with the alopecia and prominent skin changes seen in humans and mice with mutations in the vitamin D receptor gene (*Vdr*) (Marx *et al*, 1986; Li *et al*, 1997; Xie *et al*, 2002). Thus, one might conclude that 1,25$(OH)_2$D has at best a modulating influence on calcium-regulated epidermal differentiation, and in its absence other factors sufficiently compensate so as to preserve a normal phenotype. These studies with the *1αOHase*$^{-/-}$ mouse call this conclusion into question.

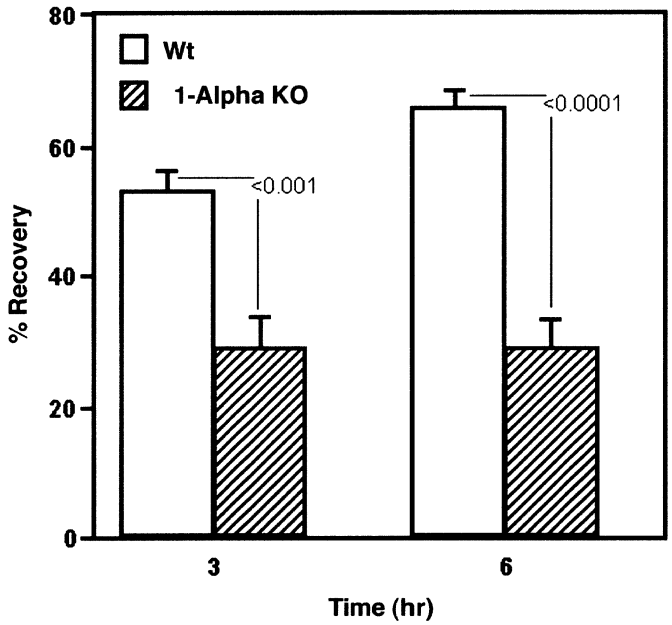

Figure 3
Barrier recovery is delayed in *1αOHase*$^{-/-}$ mice. The permeability barrier of the epidermis was disrupted by tape stripping. Transepidermal water loss (TEWL) was measured prior to and 3 and 6 h after tape stripping. The degree to which recovery of the barrier after tape stripping is achieved at these time points is recorded. Eleven *1αOHase*$^{-/-}$ mice and 12 wild-type mice, ages 6–7 wk, were used in this experiment. At both the 3 and 6 h time points, recovery of barrier function was significantly delayed in *1αOHase*$^{-/-}$ mice. The error bars enclose mean ± SD.

The role of 1,25$(OH)_2$D in regulating calcium transport is well established in calcium-transporting cells such as the intestinal epithelial cell (Bikle 1990). 1,25$(OH)_2$D also regulates calcium signaling by non-calcium-transporting cells such as the keratinocyte by inducing the calcium receptor (Ratnam *et al*, 1999; Tu *et al*, 2001) and the phospholipase C *and D* families (Pillai *et al*, 1995; Griner *et al*, 1999), proteins essential for calcium-regulated differentiation. 1,25$(OH)_2$D increases intracellular calcium *in vitro* and accelerates differentiation as assessed by increased expression of involucrin and transglutaminase and stimulated cornified envelope formation (McLane *et al*, 1990; Pillai and Bikle, 1991). Our results *in vivo* suggest a similar role for 1,25$(OH)_2$D in that the gradient of calcium seen in normal epidermis does not form in the epidermis of mice lacking 1OHase. Disruption of the barrier *in vivo* by tape stripping or acetone results in loss of calcium from the outer epidermis (Mao-Qiang *et al*, 1997) and increased secretion of a preformed pool of lamellar bodies into the stratum corneum/stratum granulosum junction. This abrupt loss of calcium stimulates lamellar body secretion, leading to restoration of the permeability barrier and the calcium gradient (Menon *et al*, 1992, 1994). Furthermore, the loss of calcium from the stratum granulosum immediately after

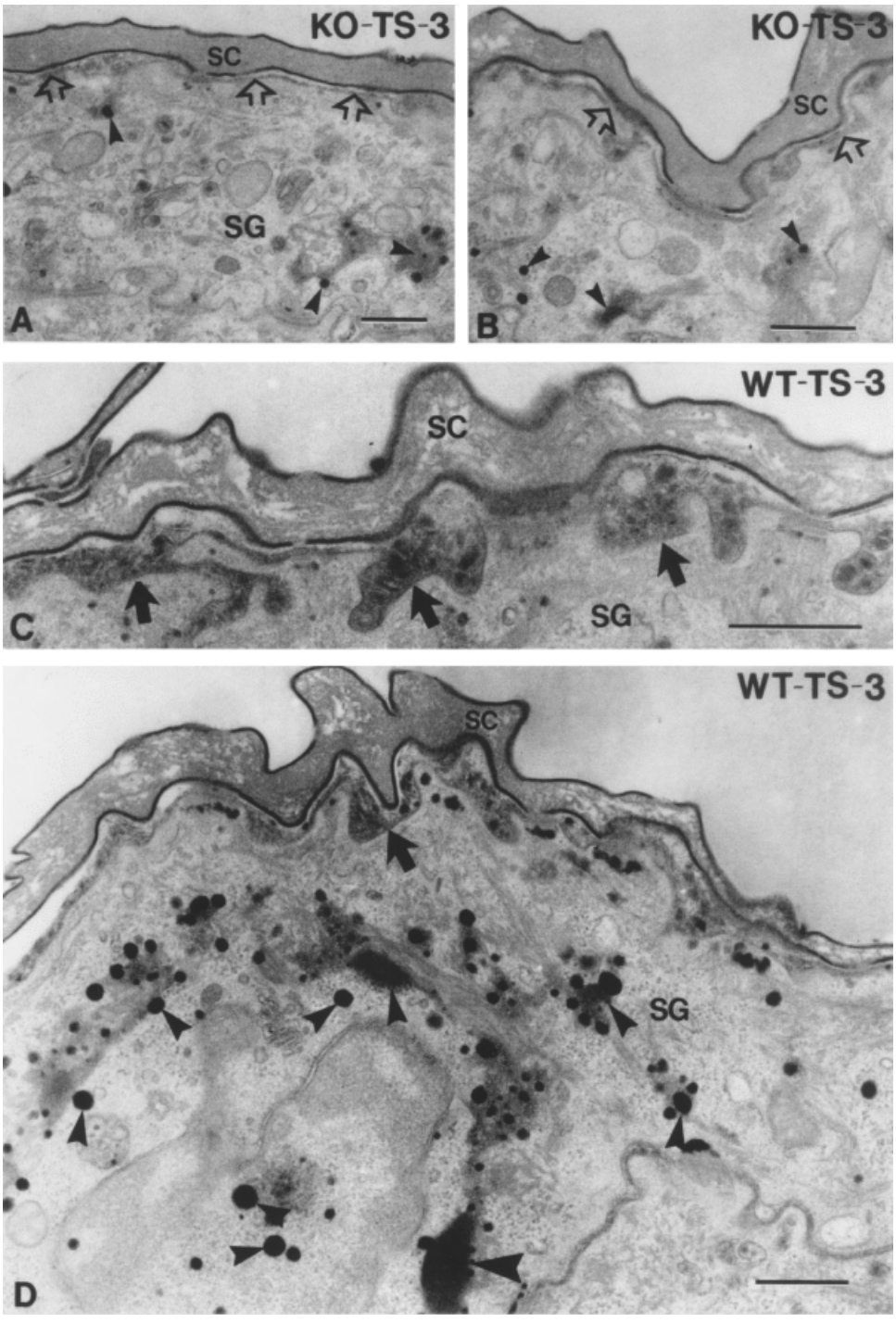

Figure 4
***1αOHase*$^{-/-}$ mice have a reduced secretion of lamellar body content into the stratum corneum/stratum granulosum junction following barrier disruption.** Skin samples from the backs of *1αOHase*$^{-/-}$ mice and their wild-type littermates (*n = 3 per group*) were processed for electron microscopy 3 h after tape stripping to break the permeability barrier. The epidermis from *1αOHase*$^{-/-}$ mice (*A*, *B*) showed a marked reduction in the amount of osmiphilic material in the intercellular space between the cells of the stratum corneum and stratum granulosum (open arrowheads) following tape stripping compared with their wild-type littermates (*C*, *D*) (large closed arrowheads). Note also the reduced number of keratohyalin granules in the epidermis of the *1αOHase*$^{-/-}$ mice (*A*, *B* compared with *D*) (small arrowheads).

barrier disruption leads to a transient decrease in expression of the differentiation markers loricrin, profilaggrin, and involucrin (Elias *et al*, 2002) suggesting that calcium regulates their expression *in vivo* as well as *in vitro*.

In this study, we compared *1αOHase*$^{-/-}$ mice compared with their wild-type littermates to determine whether 1OHase activity was required for normal differentiation of keratinocytes *in vivo*. Although there was no striking gross phenotype in *1αOHase*$^{-/-}$ mice, the levels of the differentiation markers involucrin, profilaggrin, and loricrin were reduced. This reduction was associated with a reduction of calcium in the outer layers of the epidermis, consistent with the observations *in vitro* that increased intracellular calcium regulates the expression of these genes. Although we did not find a difference in barrier function in the basal state between *1αOHase*$^{-/-}$ mice and their wild-type littermates, we observed a pronounced retardation in the ability of *1αOHase*$^{-/-}$ mice to recover normal barrier function following acute perturbation. This barrier abnormality identified functionally as increased TEWL was shown morphologically to correlate with decreased lamellar body secretion and failure of the calcium gradient to form after barrier disruption in *1αOHase*$^{-/-}$ mice. Placing the animals on a high-calcium diet previously shown to normalize serum

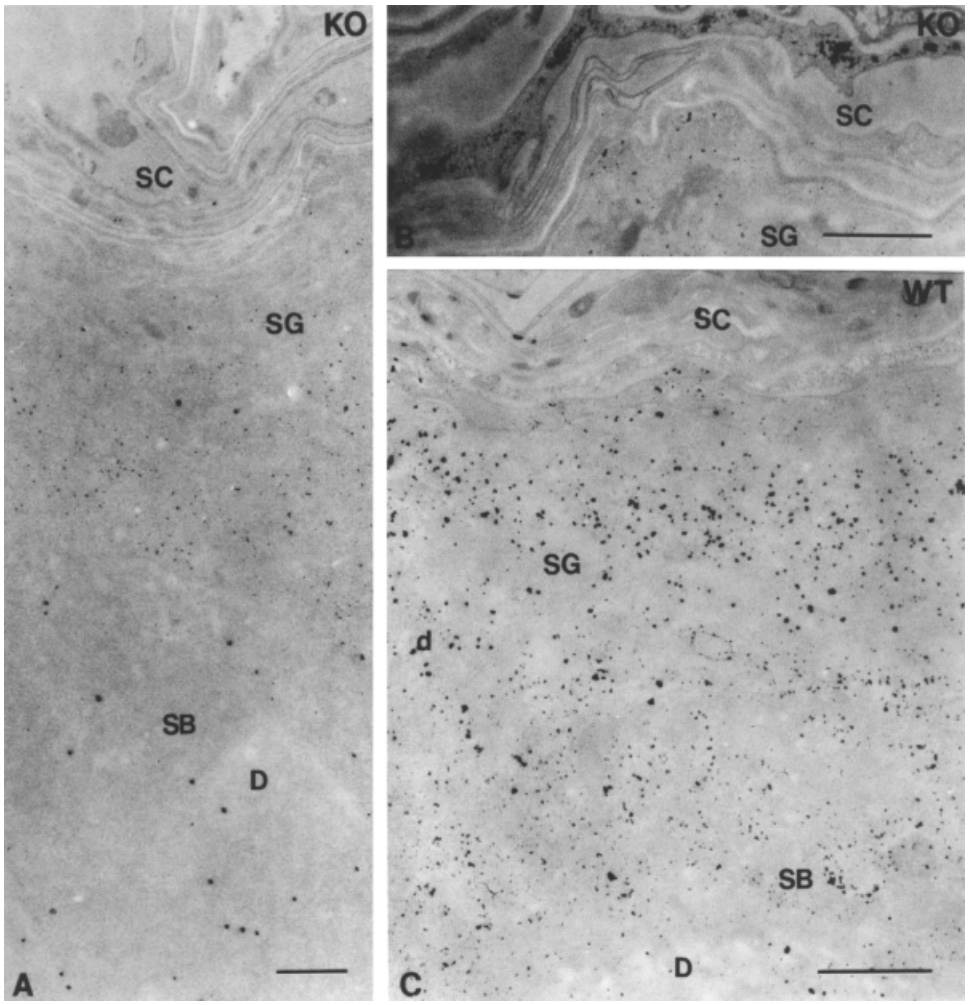

Figure 5
The calcium gradient is reduced in the epidermis of *1αOHase*$^{-/-}$ mice. Skin samples from the backs of *1αOHase*$^{-/-}$ mice and their wild-type littermates (*n = 3 per group*) were processed by ion capture cytochemistry to detect calcium in the epidermis. The *1αOHase*$^{-/-}$ mice (*A*, *B*) have substantially less calcium in their epidermis than do wild-type mice (*C*), and no obvious gradient of calcium is seen from the stratum basale to the stratum granulosum in *1αOHase*$^{-/-}$ mice unlike their wild-type littermates. In some sections of skin from the *1αOHas*$^{-/-}$ mice, increased calcium in the stratum corneum was observed (*B*).

calcium and correct the rickets in these mice (Dardenne *et al*, 2003) did not normalize these findings (data not shown). Similarly, we were not able to correct the abnormality in the calcium gradient or the delay in permeability barrier recovery by exogenous 1,25$(OH)_2$D application (data not shown). As previously shown by von Brenken *et al* (1997), topical application of 1,25$(OH)_2$D to mouse skin increased TEWL (i.e., decreased permeability barrier function), whereas systemic administration had little effect (data not shown), suggesting either that the site and mode of delivery of 1,25$(OH)_2$D to the epidermis (autocrine/paracrine *versus* exogenous) is critical for its actions or that 1OHase has a function other than 1,25$(OH)_2$D production that influences epidermal differentiation and permeability barrier formation.

Although the reduction in levels of corneocyte proteins is consistent with the lack of 1,25$(OH)_2$D and calcium in the epidermis, the reduction in lamellar body secretion in the *1αOHase*$^{-/-}$ mice is somewhat paradoxical in that the loss of the calcium gradient in normal epidermis following barrier disruption results in increased lamellar body secretion. Thus, one might anticipate that lamellar body secretion would be increased in the *1αOHase*$^{-/-}$ mice lacking a normal calcium gradient. Conceivably, however, it is the rate of calcium flux out of the keratinocyte at the time of barrier disruption that stimulates lamellar body secretion, and this rate would be reduced in keratinocytes from the *1αOHase*$^{-/-}$ mouse that have little calcium in their stratum granulosum. Alternatively, 1,25$(OH)_2$D may regulate lamellar body secretion and/or formation independent of its effects on the calcium gradient.

We conclude from these observations that the 1OHase produces a product, presumably 1,25$(OH)_2$D, that plays an important role in calcium handling by the keratinocyte both *in vivo* and *in vitro*. *In vivo*, the lack of this activity leads to a reduction in calcium in the stratum granulosum, a failure to respond appropriately to barrier disruption with restoration of the barrier, and decreased expression of markers of differentiation. In the basal state, compensatory mechanisms appear sufficient to maintain a normal barrier despite a reduced calcium gradient and reduced levels of the differentiation markers; however; one might predict from these observations that disruption of the barrier would have greater pathologic consequences in the human or mouse with *1αOHase* deficiency than in normal animals.

Materials and Methods

Animals Mice heterozygous for the *1αOHase* null mutation, *1αOHase*$^{-/+}$, outbred to C57BL/6 as previously described (Dardenne *et al*, 2001), were bred to provide wild-type (*1αOHase*$^{+/+}$) and homozygous mutant *1αOHase*$^{-/-}$ littermates used for these studies. Genotyping was performed by PCR.

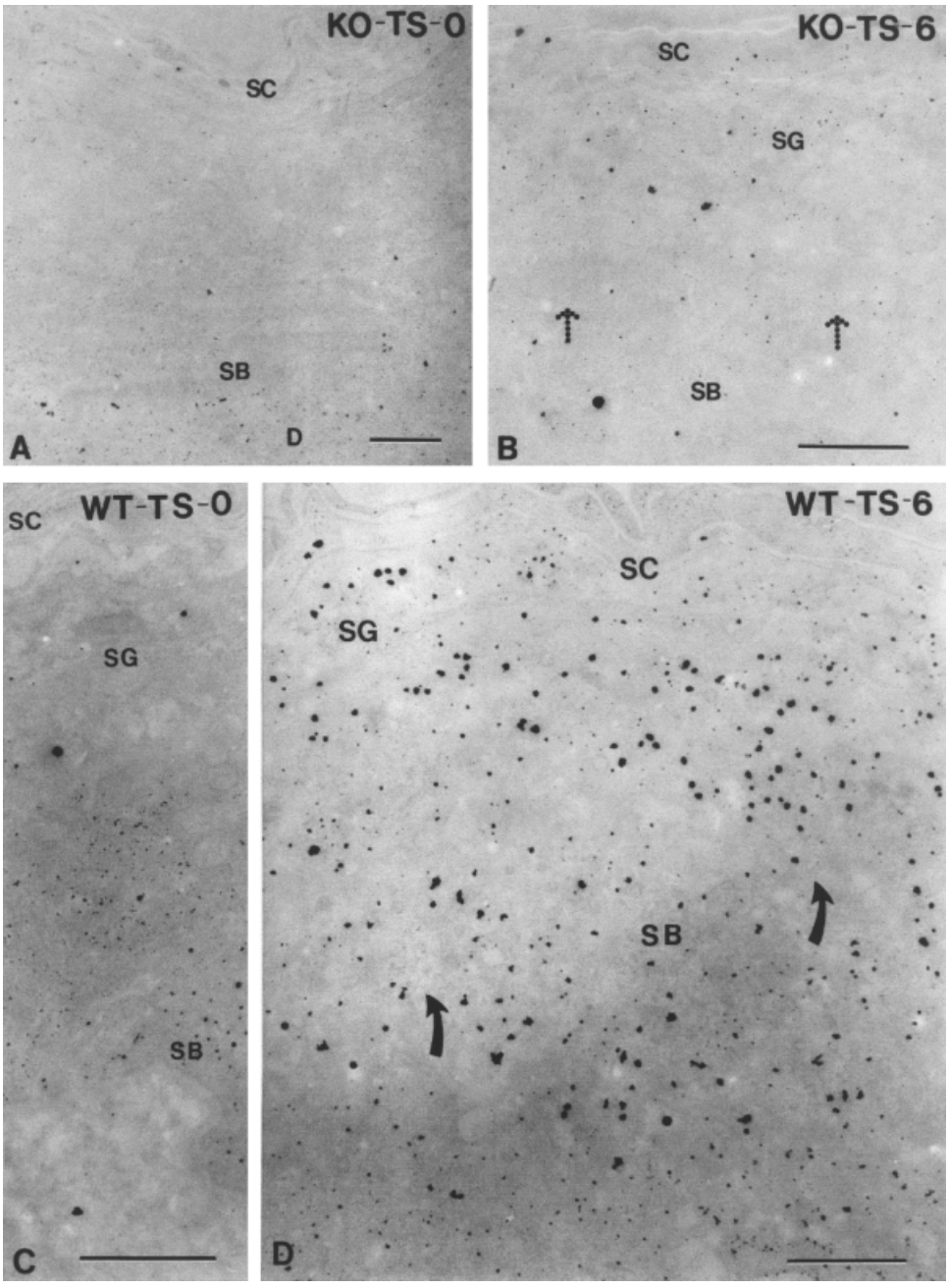

Figure 6
Response of the calcium gradient to barrier disruption. The stratum corneum was removed by tape stripping to disrupt the barrier. Immediately following barrier disruption, calcium was lost from the outer epidermis in all mice (*A*, *C*); however, *1αOHase*$^{-/-}$ mice (*B*) failed to regain calcium by 6 h, whereas wild-type mice did (*D*). *These sections are representative of samples taken from three wild-type and three 1αOHase*$^{-/-}$ *mice at each time point*.

Primers used for PCR include an upper primer (5′-CCCATCCCGAGAACTCTA-3′) and a lower primer (5′-GTGCCGTGATAAATGCTT-3′) that encompass the deleted exon 8 in the mutant *1αOHase* allele. After weaning, the mice were raised on a 1.3% calcium, 1.03% phosphorus diet (Teklad diet 8656, Harlan Teklad, Madison, Wisconsin) or on a 2% calcium, 1.25% phosphorus, 20% lactose diet (rescue diet) (TD96348, Teklad) shown previously to normalize mineral ion homeostasis and prevent rickets in these mice (Dardenne *et al*, 2003). Results were comparable between the two diets, so the data shown will be from mice on the lower calcium diet. These studies were approved by the Animal use Committee of the San Francisco Veterans Affairs Medical Center.

Assessment of barrier function and its recovery after perturbation For studies of barrier function, the backs of mice were shaved 24 h prior to the determination of transepidermal water loss (TEWL) as previously described (Grubauer *et al*, 1989). The barrier was disrupted by cellophane tape stripping until TEWL levels were 4 mg per cm^2 per h, and measurements of TEWL repeated at 3 and 6 h following tape stripping. Samples of skin were also obtained for electron microscopy and calcium gradient determinations prior to and immediately following barrier disruption as well as 3 and 6 h later to evaluate recovery. All studies were approved by the Animal Use Committee at the Veterans Affairs Medical Center, San Francisco.

Immunohistochemistry The skin from the upper portion of the back was excised and fixed with 4% formaldehyde (freshly prepared from paraformaldehyde) in phosphate-buffered saline at 4°C for 12 h, then embedded in paraffin. The paraffin-embedded tissues were cut into 5 μm sections. After deparaffinization and rehydration, the sections were boiled in 10 mM citrate buffer for 20 min. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol for 15 min. The sections were then incubated with 4% bovine serum albumin in Tris-buffered saline for 30 min to block non-specific binding. To assess proliferation, a monoclonal biotinylated proliferating cell nuclear antigen (PCNA) antibody (to detect proliferating cells) (CalTag,

South San Francisco, California) was used. To assess differentiation, affinity-purified rabbit antipeptide antibodies (BabCo, Berkeley, California) specific for involucrin, loricrin, and filaggrin were used. The primary antibodies were used at a concentration of 4 μg per mL. All the immunoreagents were diluted in 10 mM Tris buffer, pH 7.6, containing 4% bovine serum albumin, 1% teleostean skin gelatin, 0.1% Tween 20, and 500 mM NaCl. The binding of the primary antibodies to the sections was detected by affinity-purified, biotinylated goat anti-rabbit IgG, followed by ABC-peroxidase reagent, both purchased from Vector (Burlingame, California). Peroxidase activity was revealed with DAB substrate (QualTek Laboratories, Santa Barbara, California) followed by counterstaining with methyl green or hematoxylin. Omitting the first antibodies resulted in no signal, indicating the specificity of immunodetection. Sections were then counterstained with hematoxylin. The level of expression of the differentiation markers in the sections was then scored by the three senior authors (D. D. B., K. F., P. M. E.), who were blinded as to genotype.

Electron microscopy Skin samples from 6-wk-old wild-type (*1αOHase*$^{+/+}$) and homozygous mutant *1αOHase*$^{-/-}$ littermates were prepared according to previously reported procedures (Yu *et al*, 1996). Briefly, they were fixed in 2.5% glutaraldehyde, post-fixed with 1% reduced osmium tetroxide, dehydrated in a graded ethanol series, and embedded in an Epon mixture. Ultrathin sections were cut with a diamond knife, double stained with uranyl acetate and lead citrate, and examined in a Zeiss 10A electron microscope (Carl Zeiss Microimaging, Thornwood, NY) operated at 60 kV. In each section, the entire area of the granular layer was examined, and representative images were photographed for analysis. Micrographs were then coded and examined by two observers blinded with respect to treatment or genotype.

Assessment of epidermal calcium gradient Skin samples were removed and processed for ion-capture cytochemistry, as previously described (Menon *et al*, 1985). Samples were minced and immediately immersed in an ice-cold fixative containing 2% glutaraldehyde, 2% formaldehyde, 90 mM potassium oxalate, and 1.4% sucrose, pH 7.4. After overnight fixation at 4°C in the dark, samples were post-fixed in 1% osmium tetroxide containing 2% potassium pyroantimonate at 4°C in the dark for 2 h, rinsed in cold distilled water (adjusted to pH 10 with KOH), and routinely processed, embedded, and sectioned as above. Ultrathin sections were examined without further processing in a Zeiss electron microscope operating at 60 kV. Micrographs were coded and evaluated as above.

Statistical analysis Statistical comparisons were made using Student's *t* test. Where more than two groups were compared, ANOVA was also employed.

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