Characterization and Differentiation-dependent Regulation of Secreted Phospholipases A_2 in Human Keratinocytes and in Healthy and Psoriatic Human Skin

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Secreted phospholipases A_2 ($sPLA_2$) expressed in the skin are thought to be involved in epidermal barrier homeostasis as well as in inflammation. We investigated the expression of the novel $sPLA_2$ subtypes in human skin at mRNA and protein levels in the epidermis and primary keratinocytes from healthy human skin, and in skin sections from patients with psoriasis, where the integrity of the epidermis is drastically affected. Immunofluorescence studies using specific antibodies for the different $sPLA_2$ enzymes show that $sPLA_2$-IB, -IIF, and -X are predominantly expressed in suprabasal layers, whereas $sPLA_2$-V and -IID are detected in the basal and spinous layers. $sPLA_2$-IIA is weakly expressed, and $sPLA_2$-IIE and XIIA are not detectable. Accordingly, in differentiated human primary keratinocyte cultures, the expression of $sPLA_2$-IB, -IIF and -X was increased, whereas that of $sPLA_2$-V and -IID was markedly decreased. In psoriatic skin, $sPLA_2$-X was dramatically downregulated in the epidermis, whereas increased amounts of this enzyme together with $sPLA_2$-IIA, -IID, and -IB appeared in the dermis. An enhanced release of these enzymes with the exception of $sPLA_2$-IID was also observed after treatment of HaCaT keratinocytes with tumor necrosis factor-α/interferon-γ. Treatment of HaCaT cells with $sPLA_2$-X and -IB resulted in an increase in prostaglandin E_2 formation, suggesting a proinflammatory role of these enzymes during psoriasis. $sPLA_2$-V completely disappeared. The differential locations of the $sPLA_2$ enzymes propose distinct roles of individual enzymes in skin.

Key words: calcium gradient/differentiation/epidermal barrier/inflammation/psoriasis
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Mammalian phospholipases A_2 (PLA_2) are now classified into 12 groups (I–XII). They are further subdivided in terms of their substrate specificities, calcium sensitivity, and cellular location into low-molecular-weight secreted phospholipases A_2 ($sPLA_2$-IB, -IIA, -IIC, -IID, -IIE, -IIF, -III, -V, -X, XIIA, and XIIB), group IV cytosolic phospholipases A_2, group VI calcium-independent phospholipases A_2, and group VII and VIII platelet-activating factor acetyl hydrolases (for reviews, see Valentin and Lambeau, 2000; Kudo and Murakami, 2002; Granata *et al*, 2003). Several of these studies have clearly demonstrated that the intracellular PLA_2 play roles in lipid mediator release and remodeling of phospholipids. On the other hand, the biological functions of $sPLA_2$ are much less understood, despite studies suggesting that $sPLA_2$ play a role in host defense and contribute to lipid mediator release during inflammation and its associated diseases, and also in various types of cancer (see reviews above and Granata *et al*, 2003).

Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco's modified essential medium; IFN-γ, interferon-γ; PGE_2, prostaglandin E_2; $sPLA_2$, secreted phospholipase A_2, HaCaT cells; TNF-α, tumor necrosis factor-α

$sPLA_2$ are thought to play a role in normal skin functions as well as in inflammatory skin diseases. In healthy skin, $sPLA_2$ activity is likely to play a role in the conversion of polar lipids of the upper epidermal layers into fatty acids, which, together with ceramides, are major components of the stratum corneum that constitutes the permeability barrier against transepidermal water loss (Schürer and Elias, 1991). From the studies performed in humans (Mazereeuw-Hautier *et al*, 2000), and murine (Gurrieri *et al*, 2003) epidermis *in vivo,* it is likely that these enzymes are differentially regulated at the mRNA and protein levels depending on the proliferation or differentiation states of the epidermis.

But, the current data on the expression and regulation of the different phospholipases A_2 and in particular for the newly identified $sPLA_2$ enzymes in human skin are incomplete. Mazereeuw-Hautier *et al* (2000) showed the presence of the $sPLA_2$-IB protein at the stratum corneum–stratum granulosum junction in healthy human skin, whereas the $sPLA_2$-IIA protein was undetectable. Andersen *et al* (1994) described an overexpression of a non-pancreatic $sPLA_2$ in human psoriatic skin without further identification of the enzyme. In cultures of human primary keratinocytes, an upregulation of $sPLA_2$-IIA and -V proteins was found after

serum stimulation, suggesting a role of these enzymes in hyperproliferation (Rys-Sikora *et al*, 2000).

Moreover, $sPLA_2$ activity is involved in keratinocyte motility and migration (Rys-Sikora *et al*, 2003). In addition, our studies showed that $sPLA_2$-X is the major subtype constitutively expressed in human keratinocytes and in human skin biopsies (Schadow *et al,* 2001), suggesting a function of $sPLA_2$-X in physiological processes in the epidermis.

Together, it is not clear which $sPLA_2$ subtype may be involved in the formation of the permeability barrier or may be responsible for the eicosanoid formation in human skin, because the *in vivo* expression levels and locations of the different $sPLA_2$ subtypes are largely unknown.

This prompted us to analyze the $sPLA_2$ protein expression in sections of healthy and psoriatic human skin biopsies as well as in human primary keratinocytes and HaCaT keratinocytes under proliferating and differentiating conditions by culturing the cells with low or high calcium concentrations.

Results

Expression of $sPLA_2$ enzymes in healthy human skin To depict the expression pattern of the human group I, II, V, X, and XII $sPLA_2$ enzymes, immunofluorescence studies have been performed on paraffin sections of healthy human skin biopsies using specific antibodies, which were previously shown to be specific for the $sPLA_2$ antigen with no cross-reactivity on the other human $sPLA_2$ (Degousee *et al*, 2002).

By performing confocal microscopy of the sections, $sPLA_2$-IB, -IIF and -X were essentially detected in the suprabasal layers of the epidermis (Fig 1*A–C*). $sPLA_2$-IB and -X appeared equally distributed in all suprabasal layers. For $sPLA_2$-IIF, a higher level was observed in the upper, terminally differentiated layers (stratum granulosum/-corneum).

On the other hand, $sPLA_2$-IID and -V proteins were mainly found in the basal layer with lower expression in the suprabasal layers (Fig 1*D*, *E*). In contrast to all other $sPLA_2$ enzymes, which have a diffuse location within the keratinocytes, $sPLA_2$-IID appeared to be located exclusively around the nucleus. $sPLA_2$-IIA is weakly detectable in cells of the basal and spinous layers (Fig 1*F*). A positive staining is also seen in the uppermost cornified layer of the epidermis, and this staining seems to be specific, because the negative controls without primary antibody or with preimmune serum (not shown) did not give similar signals.

$sPLA_2$-IIE and XIIA were not detectable (data not shown). No staining of any of the investigated $sPLA_2$ enzymes was detectable in the dermis of healthy human skin.

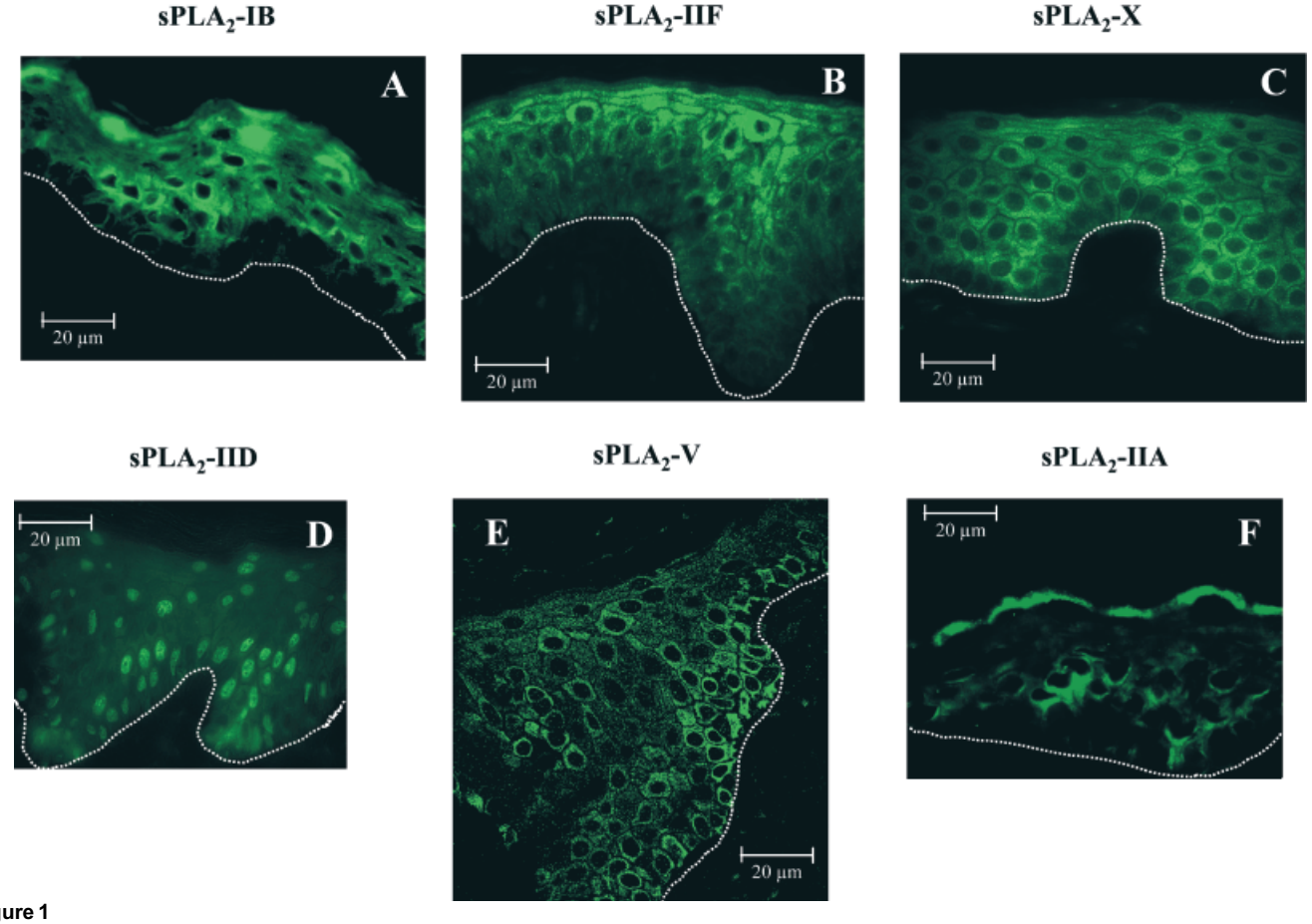

Figure 1
Immunofluorescence studies of secreted phospholipases ($sPLA_2$) subtypes in healthy human skin. Paraffin-embedded sections of human healthy skin were incubated with specific primary antibodies against the indicated $sPLA_2$ enzymes and a secondary antibody with an Alexa Fluor 488-conjugated goat anti-rabbit IgG as described in Materials and Methods. Pictures were taken using a Confocal laser scanning microscope (original magnification: × 630). The dermo-epidermal junction is indicated (dotted line).

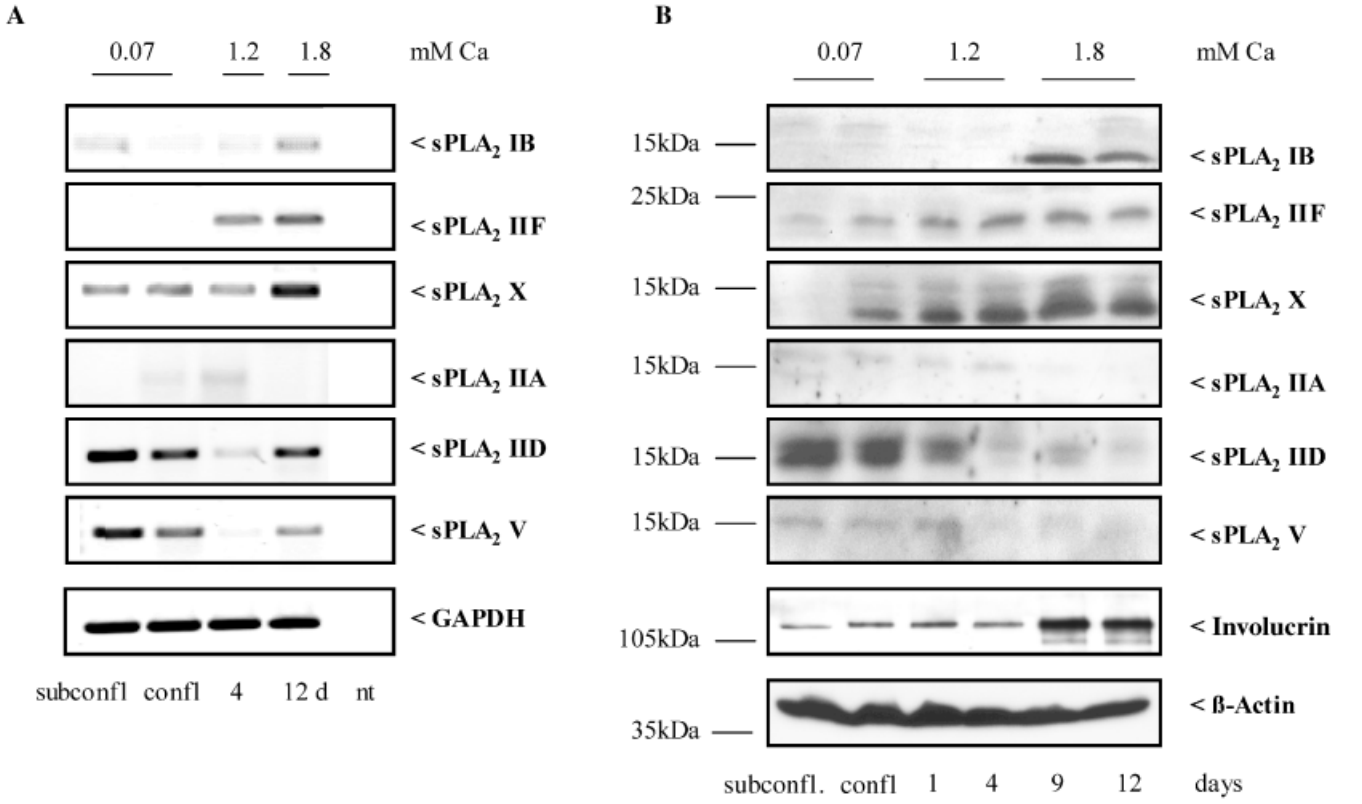

Figure 2
mRNA and protein expression of secreted phospholipases ($sPLA_2$) in human primary keratinocytes. Human primary keratinocytes were isolated from human foreskin epidermis and cultured under the indicated Ca^{2+} concentrations in the cell culture medium as described in Materials and Methods. The cells were harvested for RNA and protein extraction after the indicated time points. Semi-quantitative RT-PCR (*A*) and western blot analysis with specific antibodies against each subtype (*B*) were performed as described in Materials and Methods. Involucrin was used as a differentiation marker. β-actin western blot was performed to check for equal loading. n.t., no template; pc, positive control, MW, molecular weight markers. Data are representative of three independent studies with comparable results.

Together, these studies show that six $sPLA_2$ enzymes are expressed in distinct compartments of healthy human epidermis.

Differentiation-dependent regulation of $sPLA_2$ in human primary keratinocytes We next performed calcium-shift experiments on human primary keratinocyte cultures to confirm a differentiation-dependent regulation of these enzymes. The induction of differentiation by increasing the calcium concentration from 0.07 to 1.2 mM (Gurrieri *et al*, 2003) was addressed by monitoring the expression of the keratinocyte differentiation marker involucrin (Fig 2).

According to their *in vivo* expression in the suprabasal layer (Fig 1), the mRNA (Fig 2*A*) and protein (Fig 2*B*) for $sPLA_2$-IB, -IIF, and -X were detected at low levels under low calcium conditions and high levels in the presence of high calcium medium.

$sPLA_2$-IID and -V proteins were mostly found in proliferating keratinocytes in the presence of low calcium medium and during the early phases of differentiation, i.e., 1 day after the shift to 1.2 mM calcium (Fig 2). After 4 days in high calcium medium, the mRNA (Fig 2*A*) and protein levels (Fig 2*B*) were significantly decreased. After 12 days, an upregulation of the mRNA was observed (Fig 2*A*), but this was not reflected by an increase at the protein level (Fig 2*B*; equal loading was checked by β-actin).

$sPLA_2$-IIA protein was only weakly detectable in proliferating human primary keratinocytes and the expression was decreased under high calcium conditions. A further increase in $sPLA_2$-IIA, which may reflect the *in vivo* staining found in the stratum corneum (Fig 1), was not detectable in the keratinocyte cultures because of the incomplete terminal differentiation of keratinocytes under *in vitro* conditions (Breitkreutz *et al*, 1984).

$sPLA_2$-XIIA was expressed at the mRNA level, but was not regulated in response to calcium increase. This enzyme was not detectable by western blot analysis, possibly because of the low sensitivity of the antibodies (the detection limit for the recombinant protein is about 10 ng, not shown). $sPLA_2$-IIE was not detectable at mRNA and protein levels (data not shown).

Expression of $sPLA_2$ enzymes in psoriatic skin The expression of $sPLA_2$-IB, -IIF, and -X in the suprabasal layers of healthy human skin suggests physiological functions in relation with epidermal homeostasis and permeability barrier. It was thus of interest to examine the possible changes in expression of these $sPLA_2$ in psoriatic skin, where the integrity of the epidermis is profoundly affected.

The data in Fig 3*C* show that $sPLA_2$-X, which is the major $sPLA_2$ subtype in the healthy epidermis, completely disappears from the keratinocytes of the interfollicular psoriatic

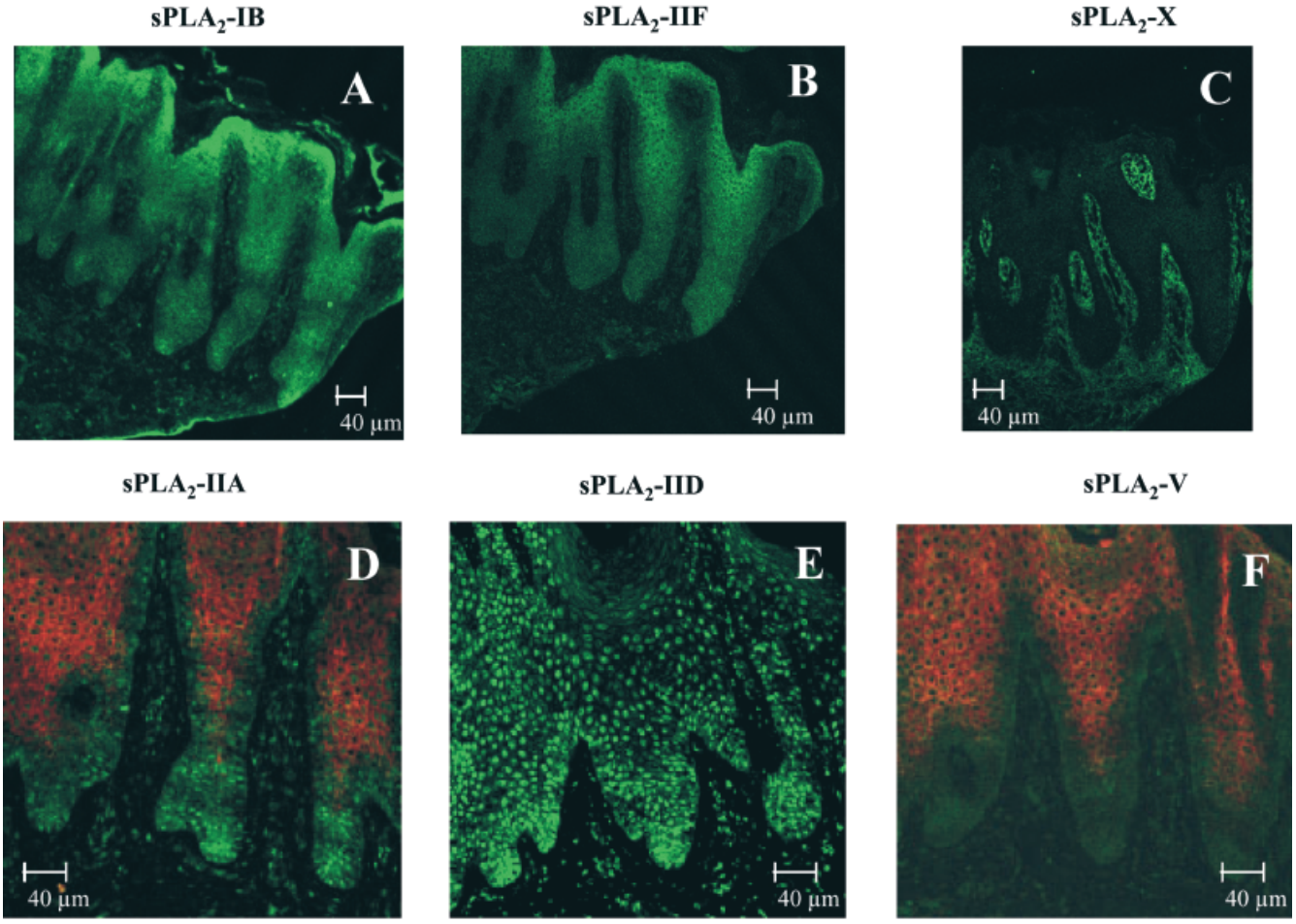

Figure 3
Expression of secreted phospholipases ($sPLA_2$) enzymes in psoriatic epidermis. Sections of human psoriatic skin were incubated with specific primary antibodies against the indicated $sPLA_2$ (green) as described in Materials and Methods (red staining: involucrin). Original magnification: $\times$ 100 *A–C*, $\times$ 160 *D–F*.

epidermis, whereas it appears at fairly high levels in the dermis.

The expression of $sPLA_2$-IB and -IIF was not changed in the psoriatic epidermis, as compared with the healthy epithelium (Fig 3*A*, *B*). However, a staining of dermal structures of the psoriatic skin was observed for both enzymes, which was not detected in healthy skin.

A marked upregulation of $sPLA_2$-IIA was observed in keratinocytes of the hyperproliferative psoriatic epidermis (Fig 3*D*). For comparison, sections were double stained with involucrin as a specific differentiation marker (Fig 3*D*, red staining). In addition, increased levels of $sPLA_2$-IIA protein were detectable in cells of psoriatic dermis.

Most remarkably, $sPLA_2$-IID was massively upregulated throughout the whole psoriatic epidermis except the uppermost layers and additionally, in dermal cells (Fig 3*E*). Interestingly, $sPLA_2$-V was completely absent in the total psoriatic skin (Fig 3*F*).

As a preliminary attempt to investigate whether $sPLA_2$ are secreted by keratinocytes during psoriasis, we treated proliferating HaCaT cells under low calcium conditions with a combination of tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), which are Th-1 cytokines that are considered to play an important role in psoriasis (Asadullah *et al*, 2002). A marked release into the cell culture supernatants was observed for $sPLA_2$-IB, -IIA, and -X (Fig 4*A*), whereas the other $sPLA_2$ were not detectable (data not shown).

We next analyzed whether these $sPLA_2$, when added as exogenous enzymes, have an effect on the formation of prostaglandin E_2 (PGE_2), which is one of the most prominent eicosanoids in inflammed skin (Reilly *et al*, 2000). An 8 h incubation with $sPLA_2$-X and a 48 h incubation with $sPLA_2$-IB significantly increased PGE_2 biosynthesis, whereas $sPLA_2$-IIA had no effect (Fig 4*B*). This strongly suggests that once released by inflammatory cytokines, these two $sPLA_2$ may contribute to the enhanced production of PGE_2 in inflammatory skin diseases such as psoriasis.

Discussion

$sPLA_2$ enzymes in healthy human skin In this study, we have analyzed the expression of the human $sPLA_2$ enzymes in paraffin sections of healthy human skin biopsies, and also their expression in human primary keratinocytes during *in vitro* cell differentiation. As in murine epidermis (Gurrieri *et al*, 2003), we observed a differentiation-dependent up- or

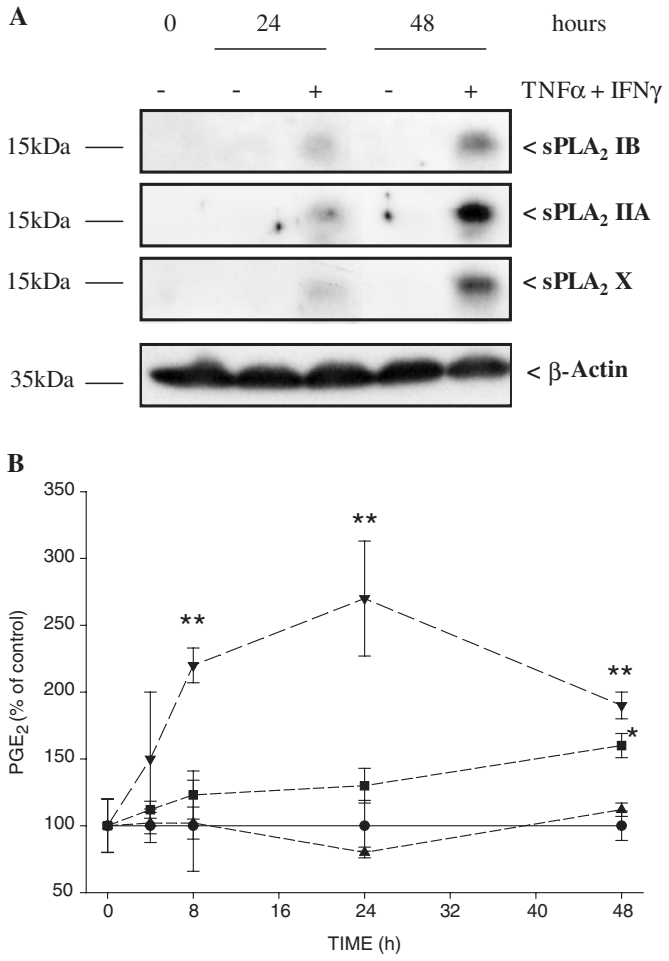

Figure 4
Secretion of secreted phospholipases ($sPLA_2$) enzymes from HaCaT cells after tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) stimulation. (*A*) HaCaT cells were cultured and stimulated with cytokines (TNF-α 1 nM, IFN-γ 100 U per mL) as described in Materials and Methods. Supernatants were precipitated and western blot analysis with specific antibodies against each subtype were performed as described in Materials and Methods. (*B*) HaCaT cells were treated with vehicle (●), exogenous $sPLA_2$-IB (100 nM; ■), $sPLA_2$-IIA (100 nM; ▲) and $sPLA_2$-X (10 nM, ▼), and prostaglandin E_2 (PGE_2) formation was measured as described in Materials and Methods. The experiment was performed three times with comparable results. Data are shown as PGE_2 formation as percent of control, which is calculated as 100%. $^*p<0.05$; $^{**}p<0.01$.

downregulation of the distinct $sPLA_2$ subtypes in the epidermis as well as in keratinocytes cultivated under low or high calcium conditions. In healthy human skin, we observed substantial differences compared with the neonatal murine epidermis with respect to $sPLA_2$-IIA, -V, and -X expression. The different expression pattern observed for the most abundant mouse and human $sPLA_2$ enzymes in skin makes it clear that despite sequence similarities of the respective subtypes, these three $sPLA_2$ may not be necessarily the orthologs in terms of physiological and pathophysiological functions (Cupillard *et al*, 1999; Valentin *et al*, 1999a, b).

Marked differences are obvious between the murine and human $sPLA_2$-X. Whereas the murine $sPLA_2$-X is mainly found in the basal layer of the mouse epidermis, this $sPLA_2$ subtype is the major enzyme expressed in the suprabasal layer of human healthy skin. $sPLA_2$-X is unique because it can release fatty acids from the outside of the plasma membrane of intact cells (Hanasaki *et al*, 1999; Bezzine *et al*, 2000). We have shown earlier that $sPLA_2$-X is constitutively secreted by confluent keratinocytes cultured under high calcium conditions. This finding is in accordance with the constitutive release of free fatty acids such as linoleic and oleic acids (Schadow *et al*, 2001), suggesting that $sPLA_2$-X is involved in epidermal lipid homeostasis.

Besides $sPLA_2$-X, $sPLA_2$-IB and -IIF were also found in these layers. A strong upregulation of $sPLA_2$-IB and -IIF mRNA and protein was also observed during terminal differentiation of the keratinocytes in our calcium shift experiments. The $sPLA_2$-IB protein was already detected by Mazereeuw-Hautier *et al* (2000) at the border between the stratum granulosum–stratum corneum junction in human skin. This location points to a role in the hydrolysis of phospholipids from lamellar bodies to form fatty acids as precursors for barrier formation. A putative role for $sPLA_2$-IB in this respect was also proposed by Mao-Qiang *et al* (1995) in murine epidermis.

In accordance with the studies by Andersen *et al* (1994) and Mazereeuw-Hautier *et al* (2000), $sPLA_2$-IIA was detected at low levels in the basal layers in healthy human skin. The protein was also detected in the uppermost epidermal layer. The location of $sPLA_2$-IIA in the cornified layers of healthy skin may contribute to its well-defined role in bacterial defense (Beers *et al*, 2002). An *in vivo* antibacterial role of $sPLA_2$-IIA in human skin, however, remains to be definitively demonstrated.

$sPLA_2$-IID, one of the most abundant enzymes of human skin, shows a scattered expression in some cells throughout the healthy epidermis, with no expression in terminally differentiated cells *in vivo* and *in vitro,* but a strong expression in proliferating and early differentiated keratinocytes. $sPLA_2$-IID belongs to the heparin-binding $sPLA_2$ and is closely related to $sPLA_2$-IIA with respect to its potential to release arachidonic acid from stimulated cells (Kudo and Murakami, 2002). Thus, this enzyme may exert a putative function in the proliferation of keratinocytes of the basal and suprabasal epidermal layers.

$sPLA_2$ enzymes in psoriatic skin In psoriatic skin, which is characterized by epidermal hyperproliferation, altered maturation of skin cells, inflammation, and changes in biochemical markers such as keratins, involucrin, filaggrin, and cytokines (for a review, see Peters *et al*, 2000), there is a dramatic change in the location of $sPLA_2$-X as the enzyme disappears from the epidermis and increases in the dermal papillae and in lower parts of dermis. In accordance with this finding, proliferating HaCaT cells release this enzyme after stimulation with TNF-α and IFN-γ, which are important factors in psoriasis. In addition, immune cells may also release this enzyme (Andersen *et al*, 1994; Cupillard *et al*, 1997; Degousee *et al*, 2002), further promoting the production of proinflammatory lipid mediator in concert with $cPLA_2$ and cyclooxygenase-2. Indeed, treatment of proliferating HaCaT cells with exogenous $sPLA_2$-X resulted in a significant PGE_2 production.

In addition, an increased secretion of $sPLA_2$-IB was observed in cytokine-stimulated HaCaT cells, and treatment of

these cells with exogenous sPLA$_2$-IB also resulted in an enhanced PGE$_2$ formation after 48 h, suggesting a role of this enzyme in the delayed formation of proinflammatory mediators.

In psoriatic skin, we observed an increased expression of sPLA$_2$-IIA in the basal epidermal layer as well as in cells in the dermis. Our observations correlate with those published by Andersen *et al* (1994). Rys-Sikora *et al* (2000) showed that sPLA$_2$-IIA and -V are upregulated after serum stimulation of human primary keratinocytes *in vitro*, suggesting a function of these enzymes during hyperproliferation. In our studies, we observed a release of sPLA$_2$-IIA, but not sPLA$_2$-V, from cytokine-stimulated HaCaT cells. An increased sPLA$_2$-IIA level in psoriatic skin may contribute to the sustained activation of mitogen-activated protein (MAP) kinase, which is present in basal and suprabasal keratinocytes, resulting in a delayed onset of terminal differentiation (Haase *et al*, 2001), thereby leading to epidermal hyperproliferation. An activation of the raf-MAP kinase cascade by extracellular sPLA$_2$-IIA with subsequent activation of cPLA$_2$ and stimulation of proliferation was described in rat mesangial cells (Huwiler *et al*, 1997). In our study, however, we did not observe an enhanced PGE$_2$ formation by treating HaCaT cells with exogenous sPLA$_2$-IIA, which is in accordance with a recent observation by Mounier *et al* (2004), who showed that exogenously added human sPLA$_2$-IIA is poorly able to liberate arachidonic acid from mammalian cells. This suggests that this enzyme is not primarily involved in eicosanoid formation but exerts distinct but yet unknown functions. An increase in epidermal sPLA$_2$ activity seems to play an additional role in stimulating motility of human keratinocytes independent of its functional linkage to eicosanoid formation, proposing a potential role in keratinocyte migration during wound repair (Rys-Sikora *et al*, 2003).

Interestingly, sPLA$_2$-V was found to be completely downregulated in psoriatic skin, and it was also not released by TNF-α/IFN-γ-stimulated HaCaT cells, indicating that this enzyme is not involved in this inflammatory skin disease.

sPLA$_2$-IID is dramatically upregulated throughout the whole epidermis and also in the dermis of psoriatic skin, suggesting that it contributes to the inflammatory processes during this disease. sPLA$_2$-IID is not released by cytokine-stimulated HaCaT cells. But this enzyme may still play a role in eicosanoid formation based on the fact that sPLA$_2$-IID appears to be located in the perinuclear region of keratinocytes, where cyclooxygenases and lipoxygenases are also found.

The role of sPLA$_2$-IIF in human skin is completely unclear. Recent studies showed an increased expression of the mRNA of this sPLA$_2$ in a mouse model of atopic dermatitis (Murakami *et al*, 2002). In our study, we found no changes in the expression pattern of sPLA$_2$-IIF in psoriatic skin compared to healthy skin.

In summary, the various expression patterns for the different sPLA$_2$ in the human skin suggest that each enzyme has non-redundant functions in both physiological or pathophysiological conditions. The respective biological roles of the different sPLA$_2$ will be determined using specific siRNA strategies and specific inhibitors for each enzyme when available (Smart *et al,* 2004).

Materials and Methods

Cell culture Human epidermis was isolated from newborn foreskin as previously described (Uchida *et al*, 2001). Second passages of keratinocytes were cultured in serum-free keratinocyte growth medium (Cascade Biologics, Portland, Oregon), containing 0.07 mM calcium, and grown to 90%–100% confluence. Then, the medium was switched to Dulbecco's modified eagle's medium (DMEM) and Ham F-12 (2:1, vol/vol), containing 1.2 mM calcium, supplemented with 10% fetal bovine serum, 10 μg insulin per mL, and 0.4 μg hydrocortisone per mL to induce differentiation. Half of the cells were cultured with this medium for 1–4 d; the other half was cultured with DMEM/Ham F-12 (2:1 vol/vol), containing 1.8 mM calcium and vitamin C (50 μg per mL; Ponec *et al,* 1997) for 9–12 d.

HaCaT cells were cultured in Hank's medium (components Invitrogen, Karlsruhe, Germany) supplemented with 5% FCS. For experiments, cells were kept in serum-free Hank's medium (high Ca conditions). To mimic psoriatic conditions (Asadullah *et al*, 2002), HaCaT cells were treated with 1 nM TNF-α and 100 U per mL IFN-γ (Bio Trend, Cologne, Germany) for 24 h and 48 h.

Moreover, cells were treated with 100 nM porcine sPLA$_2$-IB (Sigma, Munich, Germany), 100 nM human sPLA$_2$-IIA (a gift from Prof. Tibes, Roche, Penzberg), or 10 nM sPLA$_2$-X (Bezzine *et al*, 2000) for the indicated time points.

PGE$_2$ formation was measured using the PGE$_2$ correlate-EIA kit from Bio Trend (Cologne, Germany) according to the manufacturer's instructions.

RNA extraction and RT-PCR Total cellular RNA was extracted from the cells using the GSCN/phenol/chloroform method. The mRNA expression of the different sPLA$_2$ subtypes was investigated by RT-PCR using 5 μg total RNA. As a positive control, poly(A) RNAs from human lung and placenta were used (Clontech, Heidelberg, Germany). Negative PCR controls were performed on RNA that was not reverse transcribed. First-strand cDNA was produced with Superscript II RNase H-RT (Gibco BRL, Karlsruhe, Germany) and Oligo(dT)15 Primer (Promega, Mannheim, Germany). Aliquots of the first-strand cDNA were taken to perform PCR with the specific primers (Invitrogen) for each sPLA$_2$ subtype and RedTaq DNA polymerase (Sigma). PCR was performed on a GeneAmp PCR System Thermal Cycler (Amersham Life Science, Freiburg, Germany). RT-PCR for GAPDH was performed as an internal standard. Primer pairs and conditions for the semi-quantitative RT-PCR are available from the authors upon request.

Western blot analysis For protein isolation, cells were lyzed in 500 μL of homogenization buffer (20 mM Tris/HCl pH 7.5, 1 mM EDTA pH 8, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg per mL Leupeptin) per 10 cm dish. For western blot analysis, protein pellets were diluted in 15 μL Laemmli sample buffer under reducing conditions with boiling (IB, IID, V, XII) or without boiling (IIA, IIE, IIF, X), and proteins were separated on a 16% sodium dodecyl sulfate-polyacrylamide gel. Proteins were transferred to polyvinyldifluoride membranes (Millipore, Eschborn, Germany) for 45 min at 0.8 mA per cm^2, and blots were incubated with a dilution of each primary antibody specific against the corresponding human sPLA$_2$ subtype as follows: sPLA$_2$-IB (1:200), sPLA$_2$-IIA (1:200), sPLA$_2$-IID (1:200), sPLA$_2$-IIF (1:400), sPLA$_2$-V (1:400), sPLA$_2$-X (1:400), and sPLA$_2$-XII (1:400). The antibodies were generated as described earlier (Degousee *et al*, 2002; Singer *et al*, 2002). The blots were incubated at room temperature for 1 h with horseradish peroxidase-conjugated donkey anti-rabbit Ig (Amersham Life Science) at a dilution of 1:10,000. After washing, peroxidase activity was detected by developing the blots with the ECL method (Amersham Life Science).

To assess differentiation of the primary keratinocytes, western blot was performed to detect involucrin as a differentiation marker. Non-specific binding was blocked with 2% BSA/PBS-T (1 $\times$ PBS, 0.05% Tween) for 30 min at room temperature followed by

incubation for 2 h at room temperature with a 1:200 dilution of a mouse anti-human involucrin antibody (Sigma) in 0.01% milk powder/PBS and developed as described above.

β-Actin western blot was performed as loading control. Blots were incubated with a 1:10,000 dilution of a mouse anti β-actin antibody (ICN, Eschwege, Germany) in 0.01% milk powder/PBS and developed as described above.

For western blot analysis of $sPLA_2$ released into HaCaT cell culture supernatants, 1.5 mL was precipitated, and $sPLA_2$ proteins were detected as described above.

Immunofluorescence analysis Four micron sections of paraffin-embedded tissue samples from psoriatic (four males, two females, 48 ± 11 y) or normal skin (age matched) were used. The use of the skin biopsies for this study has been approved by the local ethics committee (Johann Wolfgang Goethe-University of Frankfurt am Main, Germany). The study was conducted according to the Declaration of Helsinki Principles. All participants gave their written informed consent. All psoriatic skin samples used in this study were from patients who had not received any systemic treatment or phototherapy 4 wk and no topical treatment for at least 2 wk before taking the biopsy.

The samples were deparaffinized and incubated with the specific rabbit anti-human anti-serum (dilution in 15% goat serum/PBS). The following dilutions were used for the different $sPLA_2$ enzymes: $sPLA_2$-IB (1:500); $sPLA_2$-IIA (1:300); $sPLA_2$-IID (1:500); $sPLA_2$-IIF (1:700); $sPLA_2$-V (1:1000); $sPLA_2$-X (1:700). Involucrin was detected with a specific mouse anti-human antibody (dilution 1:500 in 15% goat serum/PBS).

As a negative control, sections were incubated with 15% goat serum/PBS without primary antibody or with the respective preimmune sera. The sections were incubated for 35 min with an Alexa 488-conjugated goat anti-rabbit IgG (MoBiTec, Heidelberg, Germany) at a 1:400 dilution in 15% goat serum/PBS for all $sPLA_2$ subtypes, and with an Alexa 594-conjugated goat anti-mouse IgG at a 1:400 dilution for the detection of involucrin. After three washes with PBS, samples were embedded in Mounting Medium (Dako, Hamburg, Germany).

Sections were analyzed using a Zeiss confocal laser scanning microscope (LSM 510, Zeiss, Göttingen, Germany) equipped with a Zeiss Axiovert 100 M. Confocal images were obtained in multitracking mode at the indicated magnifications. In order to visualize $sPLA_2$ subtypes, monochromatic light at 488 nm with a dichroic beam splitter (FT 488/543) and an emission bandpass filter of 505–530 nm were used. For detection of involucrin, monochromatic light at 543 nm and an emission bandpass filter of 585–615 nm were used.

Data presentation The experiments with human primary keratinocytes and the *in vitro* experiments were performed three times with three independent series of foreskin primary keratinocytes.

For immunofluorescence analysis of each $sPLA_2$ subtype, skin sections from at least five different patients were used.

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