Differentiation-Dependent Regulation of Secreted Phospholipases A_2 in Murine Epidermis

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The action of secreted phospholipases A_2 in skin is thought to be essential for epidermal barrier homeostasis. The incomplete knowledge of presence and functions of the novel secreted phospholipase A_2 subtypes in skin prompted us to explore their expression in epidermis and primary keratinocytes from murine neonatal skin. We detected secreted phospholipases A_2-IB, -IIA, -IIC, -IID, -IIE, -IIF, -V, -X, and -XII. To study secreted phospholipase A_2 expression during epidermal differentiation, primary keratinocytes from the basal, suprabasal, and upper differentiated layers of neonatal mouse epidermis were obtained by density gradient centrifugation. mRNA for secreted phospholipases A_2-IB, -IIE, -IIF, -V, and -XII-1 are mainly expressed in the upper differentiated layers, whereas the most prominent enzymes in the basal and suprabasal layers are secreted phospholipases A_2-IIA, -IID, and -X. The mRNA for secreted phospholipase A_2-IIC was found in all fractions. Immunohistochemical analysis in mouse skin sections reflected the mRNA distribution patterns in the different epidermal cell fractions. After *in vitro* induction of keratinocyte differentiation by increasing the calcium concentration of the medium, secreted phospholipases A_2-IB, -IIE, -IIF, -V, and -XII-1 were upregulated, whereas secreted phospholipases A_2-IIA, -IIC, -IID, and -X were mainly expressed in proliferating keratinocytes. The specific secreted phospholipase A_2 expression profile in the skin suggests a distinct function for each enzyme in the epidermis.

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During the last few years an increasing number of phospholipases A_2 has been identified in mammalian tissues (Six and Dennis, 2000; Valentin *et al*, 2000). These enzymes catalyze the hydrolysis of the sn-2 ester bond of phospholipid substrates, thereby producing free fatty acids and lysophospholipids. These products have important roles in physiologic and pathophysiologic processes. Of particular importance are eicosanoids, which are produced from released arachidonic acid and are important mediators of vascular tone and tissue homeostasis, but also of pain and inflammation. Thus, the knowledge of the expression and regulation of the different phospholipases A_2 is a prerequisite for understanding their functions in different organs and tissues and for developing specific inhibitors for therapeutic use.

The different phospholipases A_2 are classified into 12 groups (I–XII) and have been characterized in terms of their substrate specificities, Ca^{2+} sensitivity, and cellular location (for reviews see Six and Dennis, 2000; Valentin and Lambeau, 2000). Using these criteria, the phospholipases A_2 enzymes identified in mammals have been subdivided into the low molecular weight secreted phospholipases A_2 (sPLA$_2$-IB, -IIA, -IIC, -IID, -IIE, -IIF, -III, -V, -X, and -XII), the group IV cytosolic phospholipases A_2, the group VI Ca^{2+}-independent phospholipases A_2 and the group VII and VIII platelet-activating factor acetyl hydrolases.

Analysis of the expression pattern of the different sPLA$_2$, and in particular of the novel subtypes of secreted phospholipases A_2 has made clear that more than one sPLA$_2$s subtype can be expressed in a single tissue or even the same cell, where sPLA$_2$s might be localized in different subcellular compartments (Bingham *et al*, 1999; Valentin *et al*, 1999; Degousee *et al*, 2002). It is also becoming clear that the mRNA expression of a specific sPLA$_2$ subtype is not necessarily reflected by the amount of expressed protein (Schadow *et al*, 2001; Gelb *et al*, unpublished observations).

The increasing diversity of sPLA$_2$s leads to a number of questions concerning the functions of these sPLA$_2$s in physiologic and pathophysiologic processes.

sPLA$_2$s are likely to play a part in normal skin functions. Indeed, sPLA$_2$ activity is needed for the degradation of polar lipids in the upper epidermal layers for the generation of fatty acids, which, together with ceramides, are major components of the stratum corneum (Elias, 1983; Schürer and Elias, 1991). This layer of apolar lipids is called the permeability barrier, which prevents transepidermal water loss. The physiologic role of one or several sPLA$_2$s in barrier integrity became evident from the observation that inhibition of sPLA$_2$ activity in mouse skin resulted in destruction of the epidermal integrity and in transepidermal water loss (Mao-Qiang *et al*, 1995, 1996; Fluhr *et al*, 2001).

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Abbreviations: sn, stereospecific numbering; sPLA$_2$, secreted phospholipase A_2.

$sPLA_2$s are probably also involved in inflammatory processes in the skin, and the knowledge of the expression of $sPLA_2$s is important for determining among $sPLA_2$s those that are therapeutic targets in skin inflammation. In support of a pathophysiologic role of the so-called proinflammatory $sPLA_2$-IIA in skin diseases, a transgenic mouse overexpressing the human $sPLA_2$-IIA developed hyperkeratosis and hyperplasia, suggesting that human $sPLA_2$-IIA may act as a growth factor for keratinocytes (Grass *et al*, 1996); however, murine $sPLA_2$-IIA is expressed almost exclusively in small intestine, whereas human $sPLA_2$-IIA is expressed in numerous tissues, suggesting that these two enzymes are not functional orthologs of each other (Cupillard *et al*, 1999, and citations herein). These results suggest that $sPLA_2$s distinct from $sPLA_2$-IIA may contribute to murine skin functions and inflammation.

In a former study, $sPLA_2$-IB and $sPLA_2$-IIA were identified in primary mouse keratinocytes as well as in the keratinocyte carcinoma cell line HEL 30 and the possible function of these enzymes in eicosanoid biosynthesis was addressed (Li-Stiles *et al*, 1998). Meanwhile several new murine $sPLA_2$ enzymes have been found in mouse epidermis, although the knowledge of their expression pattern is still incomplete. A recent study has identified $sPLA_2$-IID mRNA in mouse skin (Valentin *et al*, 1999), and the expression of $sPLA_2$-IID, -IIE, and -IIF was recently found to be upregulated during experimental atopic dermatitis in mouse ears (Murakami *et al*, 2002a, b, c). Thus, it is not clear which $sPLA_2$ subtype is responsible for the fatty acid release described by Li-Stiles *et al*, 1998; Li-Stiles and Fischer, 1999) or involved in the formation of the permeability barrier. From studies performed in human keratinocytes (Rys-Sikora *et al*, 2000; Schadow *et al*, 2001) and in human epidermis *in vivo* (Maury *et al*, 2000; Mazereeuw-Hautier *et al*, 2000), it can be expected that these enzymes are differentially regulated at the mRNA and protein level depending on the proliferation or differentiation state of the epidermis.

This prompted us to analyze $sPLA_2$ subtypes expressed at the mRNA and protein levels in the different stages of development and in the different layers of the mouse epidermis. A suitable model system to study the expression and function of epidermal $sPLA_2$ enzymes is the neonatal mouse skin. This system represents a hyperplastic phenotype compared with adult epidermis concerning the morphology, keratin expression, and mitotic activity of the cells from the basal layer (Fürstenberger *et al*, 1985). Because of its multilayered structure, neonatal epidermis offers the advantage to separate primary keratinocytes by density centrifugation using Percoll gradients to study the pattern of $sPLA_2$ expression in the different layers. In the present study, we compared the expression levels of the full set of murine $sPLA_2$s in neoplastic skin under proliferating and differentiating conditions by using primary keratinocytes from neonatal skin cultured under low or high Ca^{2+} conditions. Moreover, we investigated the pattern of $sPLA_2$ protein expression *in vivo* by performing immunohistochemical analysis of neonatal back skin from NMRI mice.

MATERIALS AND METHODS

Preparation of mouse epidermis

Percoll density gradient fractionation of epidermal cells Adult female NMRI mice together with their neonatal 2 to 3 d old offspring were purchased from Charles River (Sulzfeld, Germany). Neonatal mouse epidermal cells were obtained by the trypsin flotation method of Yuspa and Harris (1974). All experiments were carried out according to the guidelines and with permission from the local government of Hessen, Germany. Briefly, skin of neonatal mice was incubated for 15 h in plastic Petri dishes containing 10 mL of 0.25% trypsin/phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} (Biochrom, Berlin, Germany). The epidermis was then separated from the dermis and thoroughly homogenized. The dissociated cells were loaded onto a Percoll (Fluka, Seelze, Germany) discontinuous gradient ranging from 70% to 30%. Centrifugation at 1900g for 25 min at room temperature yielded four distinct cell fractions with four different buoyant densities (Fürstenberger *et al*, 1983; Schweizer *et al*, 1984). The cell fractions corresponding to the different layers were removed and collected separately, and Percoll was removed by washing two times with PBS without Ca^{2+} (Invitrogen, Karlsruhe, Germany) with centrifugation at 600g for 18 min at room temperature. Induction of differentiation was confirmed by semiquantitative reverse transcription–polymerase chain reaction (reverse transcription–PCR) of involucrin (data not shown).

Ca^{2+} shift For cell culture experiments with primary keratinocytes, cells of the gradient fractions 3 and 4, which represent the proliferative cells of the basal and suprabasal epidermal layers of the mouse epidermis (Fürstenberger *et al*, 1986; Gross *et al*, 1987), were pooled in 4 × minimal essential medium (Fusenig and Worst, 1974) containing 10% fetal calf serum with 1.0 mM Ca^{2+}. Cells (10^7) were seeded in 100 mm cell culture dishes and incubated in high Ca^{2+} medium at 34°C. After 4 h the medium was changed to 4 × minimal essential medium plus 10% fetal calf serum with 0.05 mM Ca^{2+} (low Ca^{2+} medium). After an additional 24 h, the medium was changed to high Ca^{2+} medium. This Ca^{2+} shift procedure is a standardized method to induce differentiation of proliferating keratinocytes (Hennings *et al*, 1980, 1989; Yuspa *et al*, 1989). The cells were harvested as follows: 24 h low Ca^{2+} medium; 8, 24, 48, and 72 h high Ca^{2+} medium. Induction of differentiation was confirmed by semiquantitative reverse transcription–PCR as well as western blot analysis of involucrin (data not shown). The antibody for involucrin was a generous gift from Dr Fiona Watt, Imperial Cancer Research Fund London (UK).

For RNA isolation, the medium was removed, the cells were washed twice with ice-cold PBS, and 400 μL of guanidinium isothiocyanate (Sigma, Munich, Germany) was added to each culture dish. Proteins were extracted from parallel cell culture dishes using 500 μL of homogenization buffer (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg per mL leupeptin), was added.

Different layers of the neonatal epidermis The density gradient separated cell fractions, corresponding to the four different epidermal layers, were prepared as described above. After washing, cells were directly lyzed in guanidinium isothiocyanate for RNA isolation using the guanidinium isothiocyanate/phenol/chloroform method.

Analysis of $sPLA_2$ mRNA expression by reverse transcription–PCR

RNA extraction Total cellular RNA was extracted from the cell pellets using the guanidinium isothiocyanate/phenol/chloroform method. To exclude contamination with DNA, RNA preparations were treated with RNase-free DNase I (Roche Diagnostics, Mannheim, Germany) for 15 min at room temperature before performing reverse transcription–PCR.

Reverse transcription–PCR The expression of mRNA of the different $sPLA_2$ subtypes was investigated by reverse transcription–PCR using 5 μg total RNA. As a positive control, poly(A) RNA from various mouse tissues was used (Clontech, Heidelberg, Germany). Negative PCR controls were performed on RNA which were not reverse transcribed. First strand cDNA was transcribed with Superscript II RNase H-RT obtained from 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 (Sigma, Karlsruhe, Germany) for each $sPLA_2$ subtype and RedTaq DNA polymerase (Sigma, Munich, Germany). PCR was performed on a GeneAmp PCR System Thermal Cycler (Amersham Life Science, Freiburg, Germany) using the following primer pairs:

$sPLA_2$-IB—sense: 5′-accccagtggacgacttaga-3′; anti-sense: 5′-tcacggtcacagttgcagat-3′, amplified product 197 bp;

$sPLA_2$-IIA—sense: 5′-ggctgtgtcagtgcgataaa-3′; anti-sense: 5′-tttatcaccggggaaacttgg-3′, amplified product 201 bp;

$sPLA_2$-IIC—sense: 5′-ccgggatcctagaaaacaca-3′; anti-sense: 5′-tgtcccgaacatcctctttc-3′, amplified product 200 bp;

$sPLA_2$-IID—sense: 5′-gaaccaccggcctaattaca-3′; anti-sense: 5′-gatgaaggtaggctgggtca-3′, amplified product 200 bp;

$sPLA_2$-IIE—sense: 5′-cctgcgagtgtgacaagaga-3′; anti-sense: 5′-atgagtctgctgggagagga-3′, amplified product 206 bp;

$sPLA_2$-IIF—sense: 5′-aacactccactggacggaag-3′; anti-sense: 5′-gtagcccacaaaggacagga-3′, amplified product 203 bp;

$sPLA_2$-V—sense: 5′-ctcacactggcttggttcct-3′; anti-sense: 5′-cataacaacggtcgtgcatc-3′, amplified product 202 bp;

$sPLA_2$-X—sense: 5′-gtgacgaggagctggcttac-3′; anti-sense: 5′-cttggactgcggtgactctc-3′, amplified product 199 bp;

$sPLA_2$-XII-1—sense: 5′-gggcaggaacaggaccagacca-3′; anti-sense: 5′-ggtttatatccatagcgtgga-3′, amplified product 179 bp;

$sPLA_2$-XII-2—sense: 5′-ccacagtggtcctgggaagtactggg-3′; anti-sense: 5′-ggtttatatccatagcgtggaacaggcttcg-3′, amplified product 123 bp;

GAPDH—sense: 5′-accacagtccatgccatcac-3′; anti-sense: 5′-tccaccaccctgttgctgta-3′, amplified product 452 bp.

Semi-quantitative reverse transcription–PCR For semiquantitative reverse transcription–PCR the number of amplification steps was tested first for linearity with different dilutions of reverse transcription product and different cycle numbers. Then reverse transcription–PCR for GAPDH was performed so that equivalent amounts of cDNA could be added to individual reaction mixtures. The different cDNA probes were amplified in a prepared Mastermix containing deoxyribonucleoside triphosphates, specific primers and RedTaq-polymerase in the corresponding PCR buffer.

For the PCR reactions the cycling parameters were optimized as follows:

$sPLA_2$-IB: 95°C for 1 min (1 cycle) followed by 95°C for 50 s, 57°C for 30 s and 72°C for 20 s (39 cycles);

$sPLA_2$-IIA: 95°C for 1 min (1 cycle) followed by 95°C for 50 s, 54°C for 30 s and 72°C for 20 s (40 cycles);

$sPLA_2$-IIC: 95°C for 1 min (1 cycle) followed by 95°C for 30 s, 54°C for 45 s and 72°C for 20 s (34 cycles);

$sPLA_2$-IID: 95°C for 1 min followed by 95°C for 50 s, 55°C for 30 s and 72°C for 20 s. (32 cycles);

$sPLA_2$-IIE: 95°C for 1 min (1 cycle) followed by 95°C for 50 s, 57°C for 30 s and 72°C for 20 s (34 cycles);

$sPLA_2$-IIF: 95°C for 1 min (1 cycle) followed by 95°C for 50 s, 57°C for 30 s and 72°C for 20 s (31 cycles);

$sPLA_2$-V: 94°C for 1 min (1 cycle) followed by 94°C for 1 min, 58°C for 1 min and 72°C for 1 min, 20 s (38 cycles);

$sPLA_2$-X: 95°C for 1 min (1 cycle) followed by 95°C for 50 s, 59°C for 30 s and 72°C for 20 s (38 cycles);

$sPLA_2$-XII-1: 95°C for 1 min (1 cycle) followed by 95°C for 50 s, 57°C for 30 s and 72°C for 20 s (38 cycles);

$sPLA_2$-XII-2: 94°C for 1 min (1 cycle) followed by 94°C for 1 min, 54°C for 1 min and 72°C for 2 min (32 cycles);

GAPDH: 94°C for 1 min (1 cycle) followed by 94°C for 1 min, 60°C for 1 min and 72°C for 2 min (24 cycles).

The final extension phase for the PCR reactions of all subtypes was 10 min at 72°C. PCR products were separated on 1% agarose gels containing 0.5 μg ethidium bromide per mL and were visualized with an ultraviolet gel camera (Biorad, Munich, Germany). After purification of the PCR products with a QIAquick PCR purification kit (Qiagen, Hilden, Germany) the sequences of the different $sPLA_2$ enzymes were confirmed using a dye terminator DNA sequencing kit in combination with the automated sequence analyzer A310 (Perkin Elmer Applied Biosystems, Weiterstadt, Germany).

Western blot analysis For western blot analysis of $sPLA_2$ protein expression, cell lysates were obtained as described above. The lysates were sonified for 10 s and centrifugated at 1900g for 5 min at 4°C. The supernatants were removed and the protein concentration was measured with the Bradford method. One volume containing 50 μg of cellular protein was precipitated with 0.4 volume of 20% trichloroacetic acid. The protein pellet was diluted in 10 μL Laemmli sample buffer under reducing ($sPLA_2$-IIA, $sPLA_2$-IIF, $sPLA_2$-V) or nonreducing ($sPLA_2$-IB, $sPLA_2$-IID, $sPLA_2$-IIE, $sPLA_2$-X, $sPLA_2$-XII) conditions without boiling, and proteins were separated on a 16% 10 × 10 cm sodium dodecyl sulfate–polyacrylamide gel. Proteins were transferred to polyvinyldifluoride membranes (Millipore, Eschborn, Germany) for 40 min at 0.8 mA per cm^2. The gel was stained with Coomassie blue, to check for efficiency of blotting. Nonspecific binding was blocked with NETG–Buffer (150 mM *N*aCl, 5 mM *E*thylenediamine tetraacetic acid, 50 mM *T*ris/HCl pH 7.5, 0.05% Triton X-100, 0.25% *G*elatin) for 30 min at room temperature followed by incubation for 2 h at room temperature with a 1:150 dilution of each primary antibody specific against the corresponding mouse $sPLA_2$ subtype. The antibodies were generated by immunization of rabbits with the recombinant $sPLA_2$ proteins. The procedure of the generation of recombinant $sPLA_2$ enzymes and the antibodies will be described in a following publication by Singer *et al* and was in principle done as described for the human $sPLA_2$ enzymes (Degousee *et al*, 2002). By blotting defined amounts of each recombinant $sPLA_2$ protein and performing western blot analyses (see below), it was calculated that each anti-serum is capable of detecting 0.2 to 1 ng of each mouse $sPLA_2$ (with ECL detection, see below), and does not detect the other mouse $sPLA_2$ enzymes when 50 ng quantities are applied to the Laemmli gel (not shown). The blots were incubated at room temperature for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham) at a dilution of 1:10,000. After washing, peroxidase activity was detected by developing the blots by the ECL method (Amersham).

Immunohistochemical analysis Total skin cut from the back of neonatal mice was fixed in 4% zinc formalin (Shondon, Pittsburgh, Pennsylvania) for 16 h and then embedded in paraffin. To study $sPLA_2$ expression in adult mice, we used cross-sections from the tails, which consist of a comparable multilayered epidermis as the neonatal skin (data not shown).

To investigate the expression of the different subtypes, 4 μm full thickness biopsies were examined. The paraffin embedded samples were deparaffinized two times in xylene for 5 min. Then, rehydration of the sections was performed by sequential incubation in 100% and 96% ethanol (two times each for 10 min), distilled water and equilibration in PBS. The sections were then digested with proteinase K (1.5 mg per mL; Qiagen) for 10 min at room temperature. After washing three times with PBS, samples were blocked for 10 min with serum free blocking buffer obtained from DAKO (Hamburg, Germany) followed by an overnight incubation at 4°C with the specific rabbit anti-mouse anti-serum (dilution in 2% bovine serum albumin), which were also used for western blotting. The following dilutions were used for the different $sPLA_2$ enzymes: $sPLA_2$-IB (1:50); $sPLA_2$-IIA (1:50); $sPLA_2$-IIC (1:100); $sPLA_2$-IID (1:100); $sPLA_2$-IIE (1:50); $sPLA_2$-IIF (1:80); $sPLA_2$-V 1:80); $sPLA_2$-X (1:80); $sPLA_2$-XII (1:50).

For negative control, sections were incubated with 2% bovine serum albumin without the primary antibody. The sections were then rinsed three times with PBS and incubated at room temperature for 35 min with an alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody diluted 1:80 for all $sPLA_2$ subtypes in 2% bovine serum albumin.

Sections were rinsed as described above, and the alkaline phosphatase was stained using the DAKO fast red substrate-chromogen system, containing fast red tablets, buffered substrate, and levamisole. The staining time, depending on the dilution of the antibodies and $sPLA_2$ subtypes, was controlled by microscopic observation. Phosphatase-labeled sections were counterstained with hematoxylin.

Data presentation The preparations of primary keratinocytes and the *in vitro* experiments were performed four times with four independent series of neonatal mice (about 50 animals per series). For immunohistochemical analysis of each $sPLA_2$ subtype, skin sections from at least five different animals were used.

RESULTS

First, we investigated the expression of $sPLA_2$ mRNA in neonatal mouse epidermis, a model system for hyperproliferative skin (Fürstenberger *et al*, 1985). Neonatal epidermis can be separated into four different layers by discontinuous density gradient centrifugation (Schweizer *et al*, 1984) to give cells from stratum basale (N4), stratum spinosum (N3), stratum granulosum/corneum (N2), and upper stratum corneum (N1). The occurrence of $sPLA_2$ enzymes in keratinocytes fractionated according to their state of differentiation in the different epidermal layers was investigated.

mRNA for $sPLA_2$-IB, $sPLA_2$-IIF, $sPLA_2$-V, and $sPLA_2$-XII-2 were found to be present in keratinocytes from the upper, differentiated cell layers, i.e., in N1 and in N2, whereas these $sPLA_2$s are barely detectable or even absent in basal keratinocytes from fractions N3 and N4 (**Fig 1*A***). $sPLA_2$-IIE and $sPLA_2$-XII-1 are nearly equally well expressed in N1, N2, and N3, but weakly in N4.

In contrast, mRNA for $sPLA_2$-IIA, $sPLA_2$-IID, and $sPLA_2$-X was present at higher levels in keratinocytes from N3 and N4 compared with cells from N1 and N2 (**Fig 1*B***). $sPLA_2$-IIC mRNA did not change across the different layers of neonatal epidermis (**Fig 1*C***).

To obtain information about the *in vivo* protein localization of the different $sPLA_2$s, we performed an immunohistochemical analysis on paraffin sections of neonatal mouse back skin. Immunostaining of $sPLA_2$-IIF, $sPLA_2$-V, and $sPLA_2$-XII is detectable mainly in the suprabasal and uppermost layers of the interfollicular epidermis (**Fig 2**). The basal layer consisting of proliferating keratinocytes was free of these enzymes. The negative control without primary antibody was devoid of staining (**Fig 2**).

A clearly different expression pattern was found for $sPLA_2$-IIA, $sPLA_2$-IID, and $sPLA_2$-X (**Fig 3**). These enzymes are mainly detectable in cells of the basal layer of interfollicular and follicular epidermis. These results correspond to the mRNA expression seen in basal keratinocyte fractions (**Fig 1*A,B***). The other $sPLA_2$

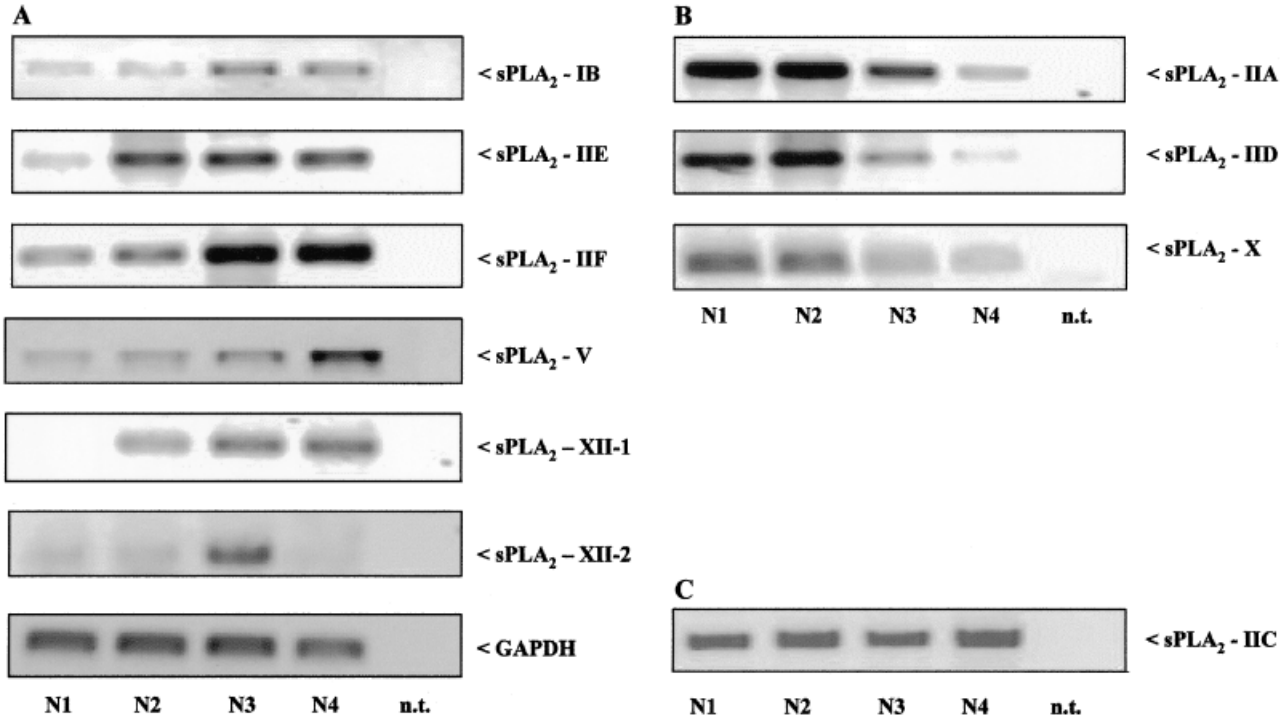


Figure 1. mRNA expression of $sPLA_2$ subtypes expressed in the four fractions of the epidermis of neonatal NMRI mice. Total RNA was extracted from cells of the four different epidermal layers N1–N4 obtained by density gradient centrifugation. Semiquantitative reverse transcription–PCR was performed with specific primers for each subtype as described in *Materials and Methods*. GAPDH was used as an internal standard. (*A*) mRNA expression of $sPLA_2$-IB, $sPLA_2$-IIE, $sPLA_2$-IIF, $sPLA_2$-V, $sPLA_2$-XII-1, and $sPLA_2$-XII-2, which are upregulated during differentiation. (*B*) mRNA expression of $sPLA_2$-IIA, $sPLA_2$-IID, and $sPLA_2$-X, which are downregulated during differentiation. (*C*) mRNA expression of $sPLA_2$-IIC, which is not regulated. Abbreviations: N4, stratum basale; N3, stratum spinosum; N2, stratum granulosum; N1, stratum corneum; n.t., no template. A representative of four independent experiments with similar results is shown.

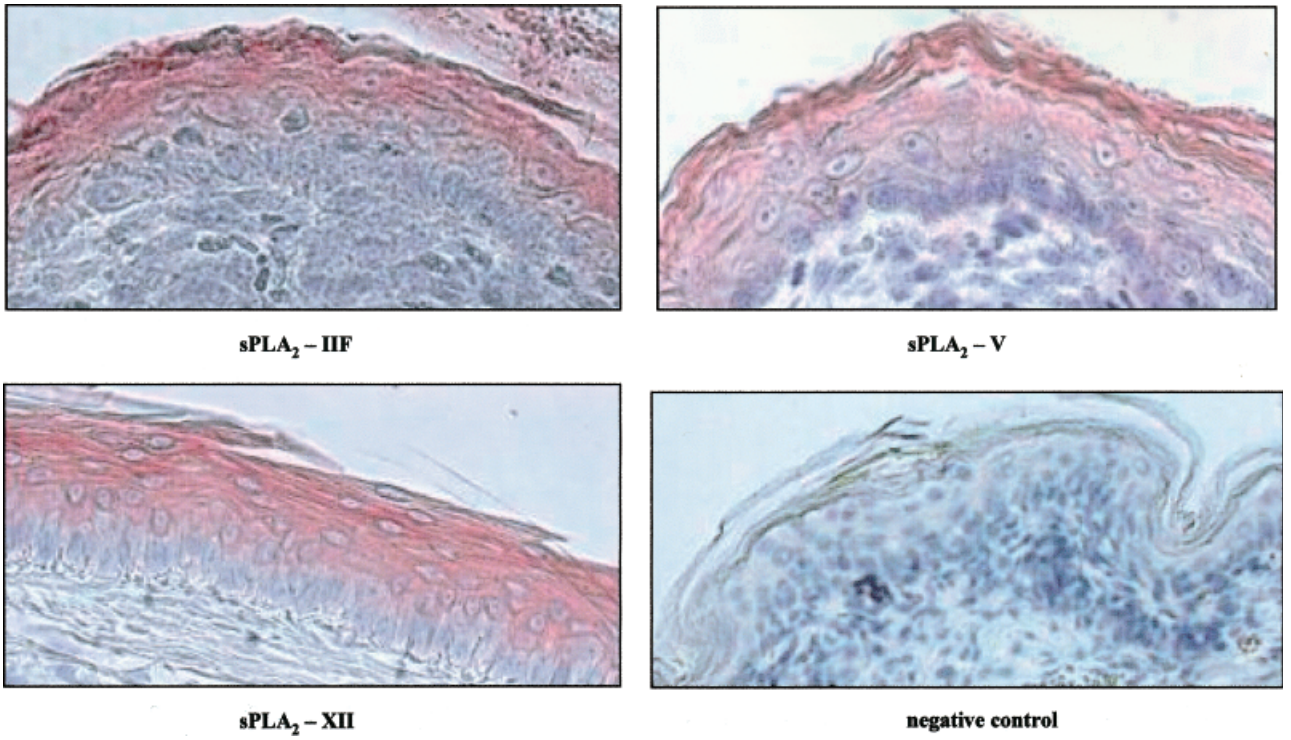
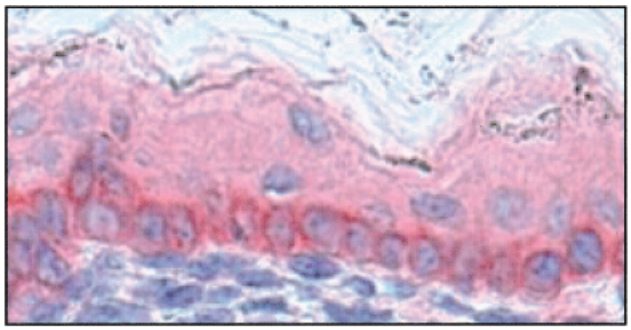
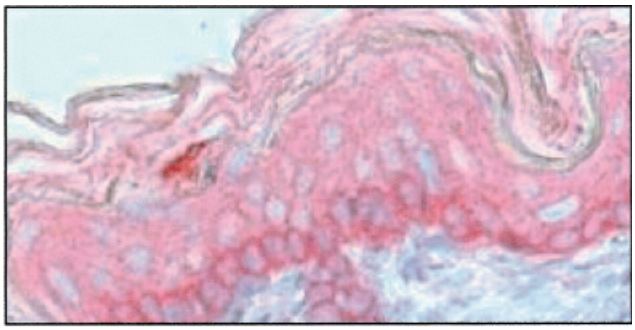


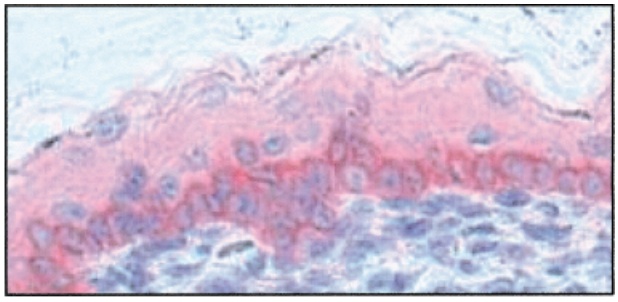
Figure 2. Immunohistochemistry of $sPLA_2$ subtypes that are mainly expressed in the differentiated layers of murine neonatal epidermis. Paraffin-embedded sections of NMRI neonatal back skin were deparaffinized and incubated with specific primary antibodies against $sPLA_2$-IIF, $sPLA_2$-V, and $sPLA_2$-X as described in *Materials and Methods* (original magnification × 40). Dilutions were used as follows: $sPLA_2$-IIF and $sPLA_2$-V 1:80; $sPLA_2$-XII 1:50. After incubation with an alkaline phosphatase-conjugated IgG secondary antibody diluted 1:80, the sections were stained with the Dako fast red substrate-chromogenic system. Negative control corresponds to a stained skin section without primary antibody.



$sPLA_2$ – IIA

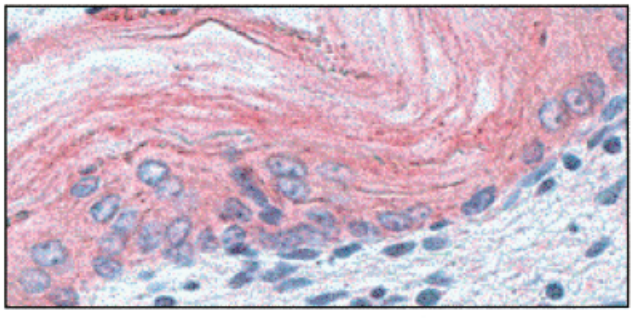


$sPLA_2$ – IID

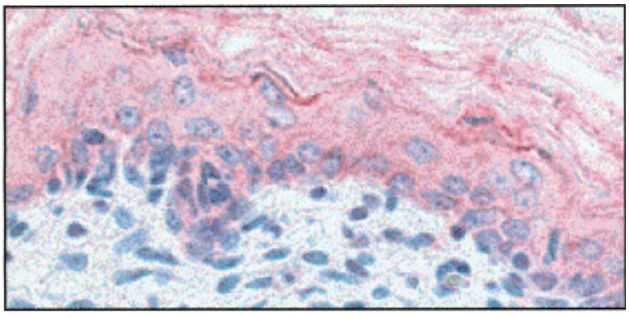


$sPLA_2$ – X

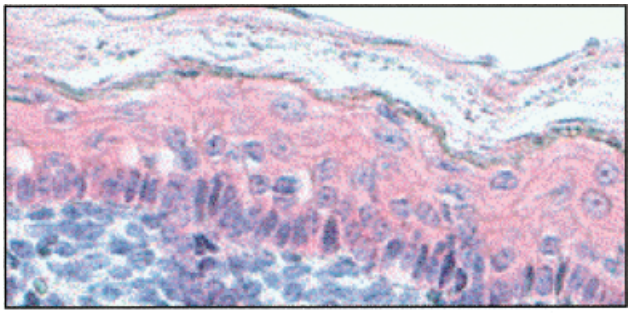
Figure 3. Immunohistochemistry of $sPLA_2$ subtypes that are mainly expressed in the basal layer in murine neonatal epidermis. The deparaffinized sections of NMRI neonatal back skin were incubated with specific primary antibodies against $sPLA_2$-IIA, $sPLA_2$-IID, and $sPLA_2$-X. Dilutions were used as follows: $sPLA_2$-IIA 1:50; $sPLA_2$-IID 1:100; and $sPLA_2$-X 1:80. After incubation with an alkaline phosphatase-conjugated IgG secondary antibody diluted 1:80 for $sPLA_2$-IIA and 1:100 for $sPLA_2$-IID and $sPLA_2$-X the sections were stained as described in **Fig 2** (original magnification × 40).



$sPLA_2$ - IB



$sPLA_2$ - IIC



$sPLA_2$ - IIE

Figure 4. Immunohistochemistry of $sPLA_2$ subtypes that are constitutively expressed in the murine epidermis. After deparaffinization, the sections of NMRI neonatal back skin were incubated with specific primary antibodies against $sPLA_2$-IB, $sPLA_2$-IIC, and $sPLA_2$-IIE with a dilution of 1:50 for $sPLA_2$-IB and $sPLA_2$-IIE and 1:100 for $sPLA_2$-IIC as described in *Materials and Methods*. After incubation with an alkaline phosphatase-conjugated IgG secondary antibody diluted 1:80, the sections were stained as described in **Fig 2** (original magnification × 40).

proteins (IB, IIC, IIE) seem to be expressed weakly throughout the interfollicular epidermis (**Fig 4**). A comparable distribution of the different epidermal $sPLA_2$s was detected in cross-sections of the tails of adult NMRI mice (data not shown).

For studying the regulation of $sPLA_2$s during keratinocyte differentiation, primary keratinocytes from N3 and N4 were cultured under low or high Ca^{2+} conditions to either maintain the proliferation of keratinocytes (0.05 mM) or to induce their differentiation (1 mM; Hennings *et al*, 1980). After different incubation periods, cells were analyzed for mRNA expression of the different $sPLA_2$ subtypes.

As shown in **Fig 5**(***A***), a shift to high Ca^{2+} concentration induced an upregulation of mRNA for $sPLA_2$-IB, $sPLA_2$-IID, $sPLA_2$-IIE, $sPLA_2$-IIF, and $sPLA_2$-XII-1 with steady-state levels at 8 h, whereas the $sPLA_2$-V mRNA level increased only at 24 h after the Ca^{2+} shift. At later time points, the mRNA levels for $sPLA_2$-IID, $sPLA_2$-IIE, and $sPLA_2$-V decreased, whereas those for $sPLA_2$-IB, $sPLA_2$-IIF, and $sPLA_2$-XII-1 remain elevated up to 72 h $sPLA_2$-IB was barely detectable after 72 h.

In contrast, mRNA for $sPLA_2$-IIA and $sPLA_2$-X were detected under low Ca^{2+} conditions and were found to be reduced after the Ca^{2+} shift (**Fig 6*A***).

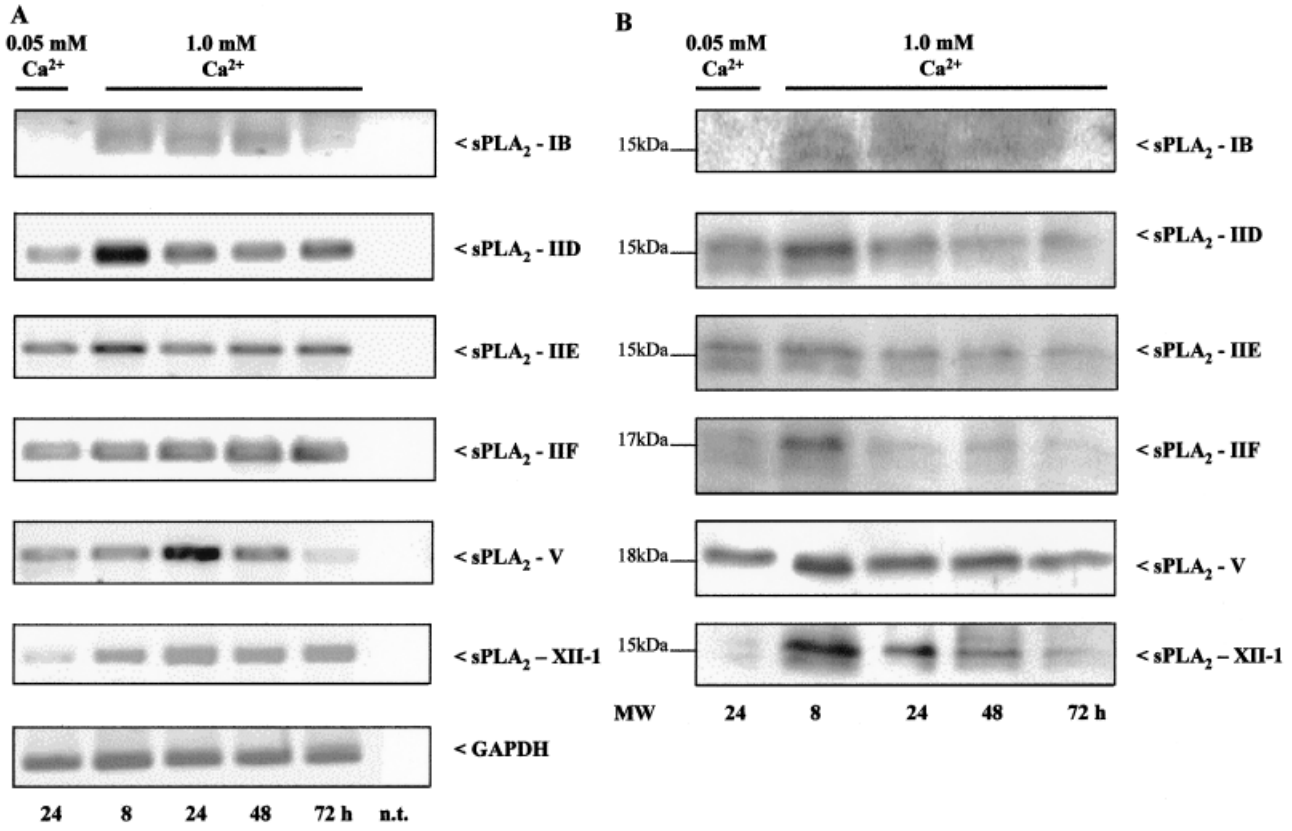


Figure 5. Reverse transcription–PCR (*A*) and western blot analysis (*B*) of $sPLA_2$ expression in murine primary keratinocytes, which are upregulated after Ca^{2+} shift. Primary keratinocytes were obtained by density gradient centrifugation and cultured under different Ca^{2+} concentrations as described in *Materials and Methods*. The cells were harvested for RNA and protein extraction after the indicated time points. (*A*) DNA-free RNA was reverse transcribed and subjected to semiquantitative PCR analysis with primer sets specific for $sPLA_2$-IB, $sPLA_2$-IID, $sPLA_2$-IIE, $sPLA_2$-IIF, $sPLA_2$-V, and $sPLA_2$-XII-1. Each of these $sPLA_2$ are upregulated after shifting the Ca^{2+} concentration in the cell culture medium to 1.0 mM. GAPDH was used as an internal standard. (*B*) Protein expression of the indicated $sPLA_2$ subtypes was analyzed by probing 50 μg of total protein on western blots, which were incubated with specific antibodies against each subtype in a dilution of 1:150. Abbreviations: n.t., no template; MW, molecular weight marker. Data are representative of four independent studies with comparable results.

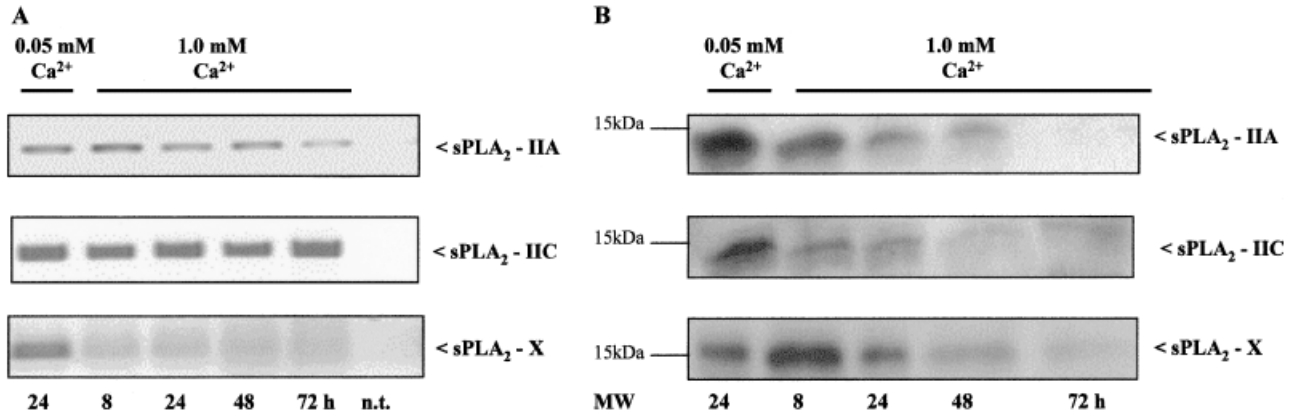


Figure 6. Semiquantitative reverse transcription–PCR and western blot analysis of $sPLA_2$ mRNA and protein expression in murine primary keratinocytes that are downregulated after Ca^{2+} shift. Primary keratinocytes were obtained and cultured as described in **Fig 5**. (*A*) DNA-free RNA was reverse transcribed and subjected to PCR analysis with primer sets specific for $sPLA_2$-IIA, $sPLA_2$-IIC, and $sPLA_2$-X. GAPDH was used as an internal standard. (*B*), Protein expression of $sPLA_2$-IIA, $sPLA_2$-IIC, and $sPLA_2$-X was obtained as described in **Fig 5**. Abbreviations: n.t., no template; MW, molecular weight marker. Data are representative of four independent studies with comparable results.

The mRNA steady-state level of $sPLA_2$-IIC remained unchanged in all conditions (**Fig 6*A***).

Despite the detection of the mRNA for $sPLA_2$-XII-2 in the cell fraction N2 after density centrifugation (**Fig 1*A***), the mRNA for this subtype was not detectable in primary keratinocytes under low or high calcium (data not shown). This suggests that *in vivo* in neonatal epidermis, $sPLA_2$-XII-2 mRNA is expressed in a cell type different from keratinocytes.

Using $sPLA_2$ subtype-specific antibodies, a similar differentiation-dependent expression pattern was found in western

blots from keratinocytes cultured under low or high Ca^{2+} conditions. For each of these anti-sera, 1 ng of the respective mouse $sPLA_2$ is readily detected (ECL detection) without giving a signal for all of the other mouse enzymes when tested at 50 ng.

Consistent with the mRNA levels (**Fig 5*A***), $sPLA_2$-IB, $sPLA_2$-IID, $sPLA_2$-IIE, $sPLA_2$-IIF, $sPLA_2$-V, and $sPLA_2$-XII proteins are weakly expressed in keratinocytes cultured in low Ca^{2+} medium and increased markedly 8 h after induction of differentiation (**Fig 5*B***). At later time points (48 and 72 h) the protein levels decreased.

In contrast, strong expression of $sPLA_2$-IIA and $sPLA_2$-X protein was detected in low Ca^{2+} medium, and that of $sPLA_2$-X even increased 8 h after the Ca^{2+} shift; however, 24 h after Ca^{2+} shift the protein levels were markedly reduced (**Fig 6*B***).

The protein expression pattern for $sPLA_2$-IIC did not appear to fit with the mRNA expression. Indeed, $sPLA_2$-IIC was mainly found in the basal layer and was decreased in high Ca^{2+} medium (**Fig 6*B***). As the mRNA expression appears constant in all conditions (**Fig 6*A***), the observed decrease in $sPLA_2$-IIC protein expression *in vivo* suggests a decrease in protein stability.

DISCUSSION

This study shows that in neonatal mouse epidermis murine group I, II, V, X, and XII $sPLA_2$ subtypes are expressed at the mRNA and protein levels. Interestingly, distinct $sPLA_2$ subsets are expressed in the basal epidermal layer and in the upper differentiated layers. *In vitro*, the regulation of $sPLA_2$ mRNA and protein expression appears to be strongly dependent on the Ca^{2+} concentration as found in cultures of primary keratinocytes isolated from neonatal NMRI mice. $sPLA_2$s expressed in the basal layer were those found to be expressed under low Ca^{2+} conditions (0.05 mM), whereas $sPLA_2$s expressed in the suprabasal layers was detected after Ca^{2+} increase (1 mM).

One proposed physiologic function of $sPLA_2$s in the normal skin is that one or more of these enzymes play a part in the formation of the epidermal permeability barrier by hydrolyzing polar phospholipids, thus generating free fatty acids at the stratum granulosum/stratum corneum border (Schürer and Elias, 1991). It is, however, still unclear, which $sPLA_2$ contribute to barrier function. In studies by Mao-Qiang *et al* (1995) and Fluhr *et al* (2001) using p-bromophenacyl bromide as a general $sPLA_2$ inhibitor and MJ33 as an active-site directed $sPLA_2$ inhibitor, it was proposed that $sPLA_2$-IB contributes to barrier formation; however, p-bromophenacyl bromide most likely inhibits several mouse $sPLA_2$ because it alkylates the active site histidine that is conserved in all $sPLA_2$s, and MJ33 has recently been shown to inhibit most of the mouse group I, II, V, and X $sPLA_2$s (Singer *et al*, 2002). Thus, the specific $sPLA_2$ involved in barrier formation remains to be established. Further investigations with more specific inhibitors as well as detailed localization studies are necessary to determine which of the $sPLA_2$s subtypes are present in lamellar bodies where they may contribute to fatty acid release for physiologic barrier functions.

In this study, we have detected mRNA expression for $sPLA_2$-IB, $sPLA_2$-IIE, $sPLA_2$-IIF, $sPLA_2$-V, and $sPLA_2$-XII-1/-2 in keratinocytes isolated from the differentiated layers (N1 and N2) of neonatal epidermis by density gradient centrifugation. Thus, one or more of these $sPLA_2$ appears to be a potential candidate for generating free fatty acids from phospholipids for formation of the permeability barrier.

The distribution of mRNA for $sPLA_2$-IB, $sPLA_2$-IIE, $sPLA_2$-IIF, $sPLA_2$-V, and $sPLA_2$-XII in the different layers was similar at the mRNA and protein levels in primary keratinocytes grown in culture and differentiated with Ca^{2+} (Hennings *et al*, 1989; Yuspa *et al*, 1989). Interestingly, $sPLA_2$-XII-2 mRNA is found in the cell fraction N2, but is not expressed in primary keratinocytes under any conditions studied. $sPLA_2$-XII-2 was so far only found in T helper type 2 cells (Ho *et al*, 2001), and thus it is possible that a cell type other than keratinocytes, being present in the N2 fraction after density gradient centrifugation, might express mRNA for this $sPLA_2$ subtype; however, it should also be mentioned that there are no reports showing the existence of mouse $sPLA_2$-XII-2 protein.

Immunohistochemical studies in paraffin sections of the back skin of neonatal mice support a suprabasal expression of $sPLA_2$-IIF, $sPLA_2$-V, and $sPLA_2$-XII protein *in vivo*. $sPLA_2$-IB and $sPLA_2$-IIE, which are also upregulated after the Ca^{2+} shift, appeared to be weakly expressed throughout the interfollicular epidermis. From these results, we suggest that not only $sPLA_2$-IB, but also $sPLA_2$ subtypes IIF, V, and XII, which are expressed in the uppermost layers, may be involved in barrier function. A more detailed localization study and the use of group specific $sPLA_2$ inhibitors are necessary to unravel which $sPLA_2$ subtypes are present in lamellar bodies and contribute to fatty acid release for physiologic barrier functions.

A different expression pattern of $sPLA_2$ subtypes was obvious in keratinocytes from the basal layer. By performing immunohistochemical staining, we have observed a specific basal expression of $sPLA_2$-IIA, $sPLA_2$-IID, and $sPLA_2$-X *in vivo*. Interestingly, in the Ca^{2+} experiments *in vitro*, $sPLA_2$-IID and $sPLA_2$-X show an additional upregulation 8 h after calcium shift. As the Ca^{2+}-mediated increase in $sPLA_2$-IID and $sPLA_2$-X protein expression occurs during a relatively early time period after the start of differentiation, we suggested that the cells expressing $sPLA_2$-IID and $sPLA_2$-X still reside in the basal layer and have not yet moved upwards to suprabasal cell layers. Similarly, a subpopulation of basal keratinocytes was shown to express the "suprabasal" keratin 10, a marker protein of terminal differentiation (Schweizer *et al*, 1984). Interestingly, $sPLA_2$-IIC, which was found at high level in testis and as a pseudogene in humans (Tischfield *et al*, 1996; Chen *et al*, 1997), is expressed at the protein level in proliferating primary keratinocytes *in vitro*, whereas it is found *in vivo* in the entire epidermis by immunochemical staining. The decrease in protein levels during terminal differentiation might be due to a more rapid degradation *in vitro* as compared with the *in vivo* situation.

The strong expression of $sPLA_2$-IIA, $sPLA_2$-IID, and $sPLA_2$-X proteins in the basal layer raises questions regarding their functions in this layer. Interestingly, mouse $sPLA_2$-IIA, which was first identified as enhancing factor (Mulherkar *et al*, 1993), was also found in proliferating cells in the skin and in Paneth cells of the small intestine (Desai *et al*, 1991). The enhancing factor had the property to enhance the binding of epidermal growth factor to its receptor in the epidermoid carcinoma cell line A431 (Mulherkar *et al*, 1993), suggesting a role of $sPLA_2$-IIA, and possibly of other $sPLA_2$ proteins, in hyperproliferation of keratinocytes. Moreover, the murine $sPLA_2$ enzymes are potentially able to release arachidonic acid (Singer *et al*, 2002), and thus, they might be involved in eicosanoid homeostasis. Mouse $sPLA_2$-X can release free fatty acids, including arachidonic acid from adherent cells (Hanasaki *et al*, 1999; Murakami *et al*, 1999; Bezzine *et al*, 2000; Morioka *et al*, 2000a, b; Saiga *et al*, 2001), suggesting that this enzyme may significantly contribute to eicosanoid formation. $sPLA_2$-IIA as well as $sPLA_2$-IID may also release arachidonic acid from stimulated cells via heparanoid binding (Murakami *et al*, 1999, 2002a; for review, see Kudo and Murakami, 2002). Thus, regarding potential functions of basally expressed $sPLA_2$, it is tempting to speculate that they might be involved in keratinocyte hyperproliferation during inflammatory processes. On the other hand, because of their broad substrate specificity, $sPLA_2$s may also produce fatty acids, such as linoleic acid and some of its metabolites, which in turn promote anti-inflammatory functions and oppose their effects to those of $cPLA_2$s and its lipid mediator products (Buckley *et al*, 1992; Fischer *et al*, 1996).

We have observed that the expression of some $sPLA_2$s is modulated *in vitro* after changing the Ca^{2+} concentrations of the medium ($sPLA_2$-IB, $sPLA_2$-IIC, and $sPLA_2$-IIE), whereas there was no difference in protein expression detectable in

immunohistochemical staining in epidermis *in vivo*. The reason for this discrepancy is currently unknown, but there might be some additional regulatory factors or differences in mRNA or protein stability *in vivo*, which cannot be mimicked in keratinocyte cultures by Ca^{2+} shift.

Furthermore, it cannot be excluded that other $sPLA_2$ subtypes, which are expressed at low level in the basal layer of normal neonatal skin, may be upregulated under inflammatory conditions. In this respect, a recent study showed an increased mRNA expression of $sPLA_2$-IIF in atopic dermatitis induced in the mouse ear, whereas in normal murine skin, the mRNA for this $sPLA_2$ was not detected (Murakami *et al*, 2002b). In this study, we could clearly show that in normal murine skin, $sPLA_2$-IIF mRNA and protein expression is found mainly in differentiated keratinocytes. Further studies focusing on the protein expression of $sPLA_2$-IIF in inflamed mouse skin are mandatory. In other studies, mRNA expression of $sPLA_2$-IID and $sPLA_2$-IIE was found to be upregulated in mice with experimental atopic dermatitis, but again protein studies are lacking (Murakami *et al*, 2002a, c).

The functional role of certain $sPLA_2$ subtypes for eicosanoid biosynthesis in the skin under inflammatory conditions *in vivo* and *in vitro* remains to be determined. Interestingly, the differentiation-dependent expression of $sPLA_2$ subtypes correlated with the expression pattern of eicosanoid-generating enzymes. The expression of the individual cyclooxygenase-1/2 and lipoxygenase enzymes in mouse epidermis was also found to be dependent on the terminal differentiation of keratinocytes (Müller-Decker *et al*, 1998; Heidt *et al*, 2000). Thus, a concerted action of distinct $sPLA_2$ subtypes with individual cyclooxygenase or lipoxygenase isozymes may generate distinct patterns of eicosanoids being involved in autocrine or paracrine effects of the autacoids. Moreover, it has to be considered that murine $sPLA_2$s might be secreted and may act in a paracrine fashion on keratinocytes of other epidermal layers by mediating signal transduction via the M-type $sPLA_2$ receptor (Cupillard *et al*, 1999; Yokota *et al*, 2000).

In summary, we have described a specific expression pattern for group I, II, V, X, and XII $sPLA_2$s in murine skin. This pattern is related to the regulation of growth and differentiation, and suggest distinct functions for each $sPLA_2$ subtype in epidermal homeostasis under both normal and pathologic conditions.

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