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Expression and Potential Function of β-Amyloid Precursor Proteins during Cutaneous Wound Repair

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sAPP, the secretory domain of the β-amyloid precursor protein (APP), exerts a growth promoting and motogenic activity on keratinocytes. Here we report on the expression of APP and its homologue, the amyloid precursor like protein 2 (APLP2), during cutaneous wound repair using a full-thickness excisional wound healing model in mice. In unwounded skin APP was predominantly expressed in the basal cell layer. During wound healing increased suprabasal expression of APP was observed in all cell layers of the hyperproliferative epithelium at the wound margin. APP mRNA was increased up to 2.3-fold, whereas the APLP2 mRNA was decreased. Immunocytochemically, all proliferation competent keratinocytes of the normal as well as the wound site epidermis showed increased expression of APP but not of APLP2. Using culture models of keratinocyte differentiation the release of sAPP was found to be significantly higher in proliferating cells, i.e., when cultured at subconfluency or at low [Ca^{2+}], than in quiescent, partially differentiated keratinocytes cultured at confluency or at high [Ca^{2+}]. Our results suggest that sAPP secretion is presumably also increased in proliferation competent keratinocytes of the wound margin and that sAPP due to its growth promoting and motogenic function might participate in the control of epidermal wound repair.
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***Key Words:* β-amyloid precursor protein; cutaneous wound repair; reepithelialization.**

INTRODUCTION

Localized disruption of skin architecture in the adult organism of higher vertebrates implies a break of the protective barrier and initiates collaborative and complex cellular processes, which finally result in cutaneous wound repair. These events include sequentially the phases of inflammation, new tissue formation, and tissue remodeling [1]. An important stage of new tissue formation is reepithelialization, which involves a variety of epidermal activities such as keratinocyte migration from the wound edges, keratinocyte proliferation which replenishes the advancing epidermal sheet, epidermal differentiation and stratification of the newly established epithelium, the new formation of the basement membrane, and, eventually, the incorporation of a new population of cells involved in sensory functions, pigmentation, or immune response [2]. These processes are controlled by a variety of cytokines and growth factors released at the wound site [2]. Key regulators of reepithelialization are the platelet-derived epidermal growth factor (EGF) [3], transforming growth factor-α (TGF-α) from basal keratinocytes [3, 4], keratinocyte growth factor (KGF) from dermal fibroblasts [5, 6], and activin [7]. However, inhibition of these growth factors at the wound site or blockade of receptor signaling revealed that none of these factors is absolutely essential for the reepithelialization process [8]. These results imply functional redundancy among different mitogens as well as the existence of other as yet unknown growth factors involved in reepithelialization.

The β-amyloid precursor protein (APP) is an integral membrane protein largely known for its role as the precursor of Aβ peptides and, therefore, for its involvement in the pathogenesis of Alzheimer's disease [9–13]. The biological role of APP is unclear; its overall structure suggests, however, that APP might function as a receptor protein [11, 14] or that its soluble N-terminal portion might operate as a growth factor [15, 16]. Indeed, sAPP has been observed to reestablish cell growth in APP-deficient fibroblasts [17] and to promote neurite outgrowth [18, 19]. We have recently shown that sAPP exerts a growth factor-like function on epithelial cells by stimulating the proliferation of thyrocytes [20] and of keratinocytes [21] and by enhancing keratinocyte migration [Kirfel *et al.*, unpublished]. As keratinocyte proliferation and migration are key processes of reepithelialization, the question arose whether sAPP might also be involved in cutaneous wound repair. In this study we report on the expression of APP during the healing process using full-thickness excisional wounds in mice [6] as an *in vivo* model. *In*

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vitro keratinocytes cultured at subconfluency or at low Ca^{2+} concentrations showed strongly increased sAPP release compared to cells cultured at confluency or at high Ca^{2+} concentrations. The results support the view of sAPP as a new member of the family of growth factors that regulate reepithelialization after skin wounding.

MATERIALS AND METHODS

Cells and antibodies. HaCaT cells [22] were kindly provided by Dr. N. E. Fusenig (DKFZ, Heidelberg). Normal human keratinocytes (NHK cells) were kindly provided by Dr. H. W. Kaiser (Klinik für Dermatologie, Bonn) after the local ethics committee of the University of Bonn had approved the study. Polyclonal antiserum CT15 [26] directed against the carboxy terminus (C-terminus) of APP, monoclonal antibodies (IG7 and 5A3) directed against the amino terminus (N-terminus) of APP, and polyclonal antiserum (D2II) directed against APLP2 were kindly provided by Dr. E. Koo (UCSD, La Jolla, CA). The polyclonal antibody directed against the cell proliferating protein Ki-67 (Tec-3) was purchased from Dianova (Hamburg, Germany), the monoclonal antibody directed against keratins 1/10/11 was from Sigma (Deisenhofen, Germany), and monoclonal antibody 1560 recognizing the N-terminus of APP was purchased from Chemicon (Hofheim, Germany).

Wounding and preparation of wound tissues. Four full-thickness excisional wounds of 0.4 cm diameter were generated on the back of mice (10–12 weeks old) by excising skin and panniculus carnosus as described by Werner *et al.* [42]. Wounds were left uncovered without a dressing. For expression studies, the complete wounds including 2 mm of the epithelial margins were excised at different time points after injury, immediately frozen in liquid nitrogen, and stored at −80°C until used for RNA isolation. Nonwounded back skin served as a control. For histological analysis, the complete wounds with 3 mm of adjacent normal tissue were isolated, dissected, and frozen in tissue freezing medium (Jung, Nussloch, Germany). Sections (6 μm) from the middle of the wound were used for immunofluorescence studies. All experiments with animals were carried out with permission from the local authorities (Kantonales Veterinäramt Zürich).

In vitro culture models. HaCaT keratinocytes [22] were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). NHK cells were grown in keratinocyte basal medium (KBM; BioWhittaker, Apen, Germany) containing 0.4% bovine pituitary extract (BPE), 0.5 mg/ml insulin, 0.1% gentamicin sulfate/amphotericin B, 100 pg/ml hEGF, 500 ng/ml hydrocortisone, and 20 μM $CaCl_2$. DMEM and FCS were purchased from Gibco/BRL (Eggenstein, Germany). Two culture models of keratinocyte differentiation were used: (1) NHK cells were grown in KBM medium to 70% confluency. Cells were washed with KBM without BPE. Culture medium was replaced with BPE-free KBM containing 180 μM Ca^{2+}; control cells were cultured in BPE-free KBM containing 20 μM Ca^{2+}. After 72 h culture media were concentrated by centrifugation using Centricon centrifugal filter devices (cutoff 30 kDa), at 3000*g* for 30 min. The concentrated supernatants were adjusted to the same volume and boiled with SDS sample buffer for 5 min. NHK cells were lysed in RIPA buffer and centrifuged and the protein concentration was determined by the Bradford assay [23]. The supernatants each corresponding to 5 μg cell lysate were analyzed by SDS–PAGE and immunoblot using the antibody 1560 (Chemicon) recognizing the N-terminus of APP. Quantitation of the sAPP protein bands was performed using Optiquant software 2. HaCaT cells were cultivated at different stages of confluency (50, 80, and 100% and postconfluent) and the stages of confluency were determined morphometrically by determining the area of the culture dish covered with HaCaT cells. Cells were subjected to biosynthetic radiolabeling.

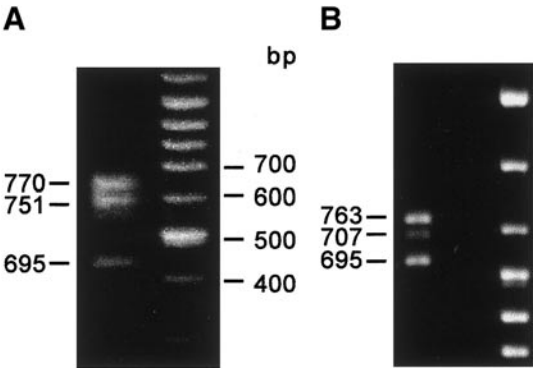

FIG. 1. Detection of APP (A) and APLP2 (B) isoforms in the back skin of Balb/c mice by PCR analyses using primers flanking the differentially spliced exons 7 and 8 (A, APP) or exons 7 and 14 (B, APLP2). Three major isoforms of APP (695, 751, 770) and three APLP2 isoforms (695, 707, 763) were detectable.

Biosynthetic radiolabeling of HaCaT cells. HaCaT cells were grown to different stages of confluency (50, 80, and 100% and postconfluent), for 1 h in DMEM (with glutamine) without FCS, cysteine, and methionine (Sigma), followed by labeling for 1 h with 50 μCi [^{35}S]cysteine/methionine Promix (Amersham; Freiburg, Germany)/0.5 ml DMEM (+ glutamine) without FCS, cysteine, and methionine (Sigma).

Immunoprecipitation. Immunoprecipitation was performed after biosynthetic radiolabeling of the cells. Media were collected, and cells were washed twice with sodium phosphate buffer pH 7.4 (PBS) and lysed for 30 min at 4°C in 500 μl lysis buffer (10 mM Tris–HCl (pH 8.5), 1 mM EDTA, 1 mM DTT, 0.5% Triton X-100, 150 mM NaCl). Protein concentration was determined using the Bradford assay [23] and equivalent protein amounts of each sample were used for precipitation. The amount of supernatant used for immunoprecipitation corresponded to 5 μg cell lysate. sAPP was immunoprecipitated from culture supernatants with antibodies IG7 and 5A3 recognizing the N-terminus of the protein and protein G–agarose (Santa Cruz; Santa Cruz, CA). APP in cell lysates was precipitated with antibody CT15 recognizing the C-terminus of the protein and Pansorbin (Calbiochem, Darmstadt, Germany). Precipitation was performed overnight at 4°C at continuous rotation. Subsequently, precipitates were washed with Neufeld buffer (10 mM Tris–HCl, pH 8.5, 0.6 M NaCl, 0.1% sodium dodecyl sulfate (SDS), 0.05% NP-40), IMM (1% Triton X-100, 0.5% sodium deoxycholate), IMM/2 M KCl (1% Triton X-100, 0.5% sodium deoxycholate, 2 M KCl), 10-fold diluted PBS, separated by 10% SDS–PAGE, and detected by autoradiography. Densitometric analysis of signals was performed using a Cyclone Storage Phosphor Scanner (Packard, Meriden, CT), which integrates areas and corrects for background. Each experiment was performed at least three times.

RNA isolation. For generation of cRNA standards isolation of total RNA was performed using the RNeasy kit (Qiagen; Hilden, Germany) according to the manufacturer's instructions. RNA isolation from normal and wounded skin was carried out as described [24].

Reverse transcription polymerase chain reaction (RT-PCR). Total RNA was reverse transcribed using 200 U Superscript II (Gibco BRL). PCR was performed in a 50-μl reaction volume containing 500 ng total RNA and 300 nM APP_{iso}f (5′-AATGTGGATTCTGCTGATGCGGAG-3′) and APP_{iso}r (5′-CCCATTCTCTCATGACCTGGGA-3′) primers flanking exons 7 and 8 or APLP2f (5′-CAGATCTGGAAGACTTCACAGAA-3′) and APLP2r (5′-TCCAACTCTCTCGGCATTGAAA-3′) with 30 cycles at 94°C for 30 s, 57 or 54°C, respectively, for 1 min, and 72°C for 1 min. PCR products were resolved by electro-

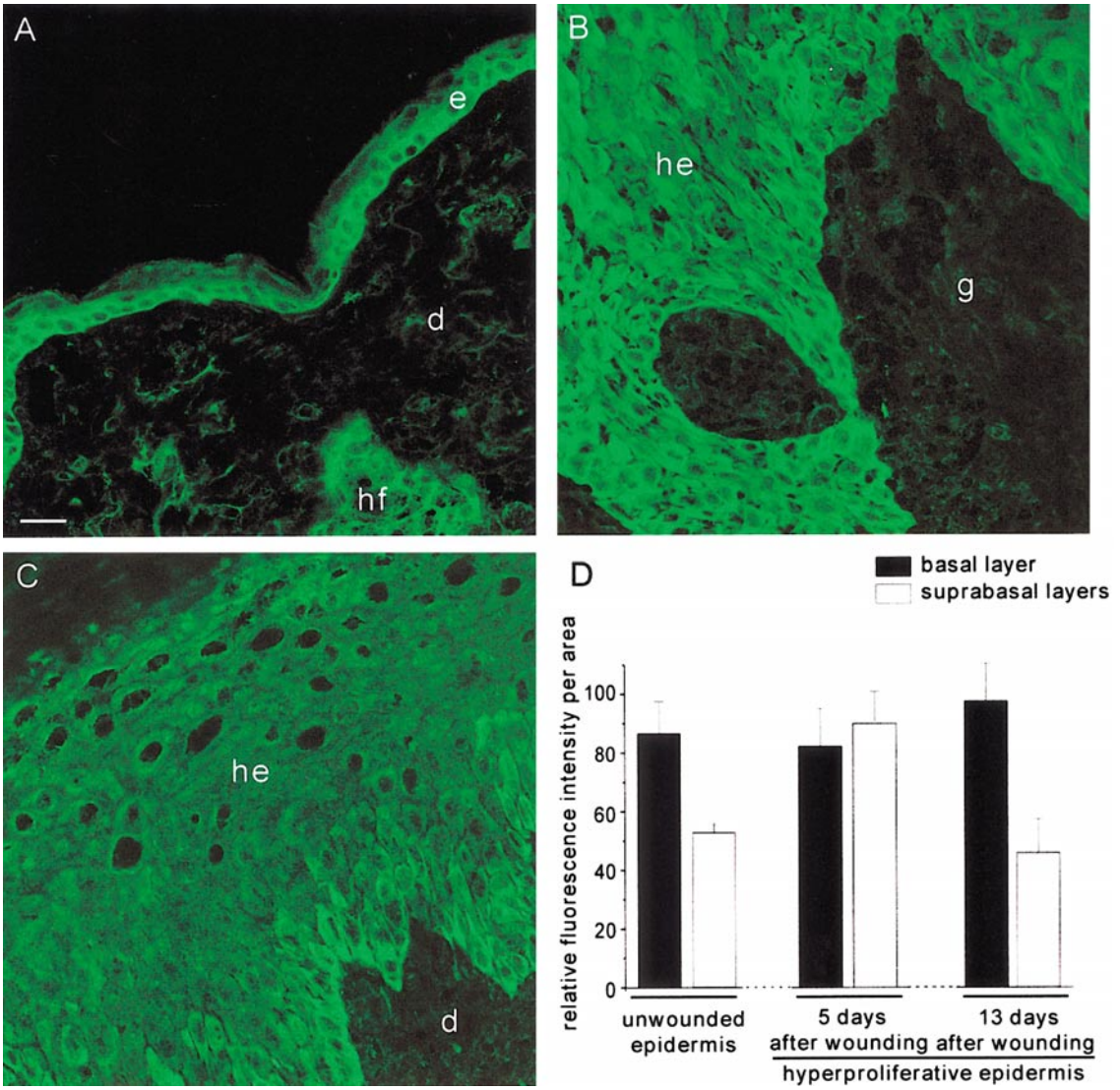

FIG. 2. Immunohistochemical localization of APP in the epidermis of the unwounded skin (A) or in the skin at Day 5 (B) or 13 (C) after wounding. APP was predominantly expressed in the basal layer of unwounded epidermis and in epithelial cells of the hair follicles. In wounded skin (5 days after injury) APP expression of suprabasal cells of the hyperthickened epithelium at the wound margin was elevated. Thirteen days after wounding, when the wound was already closed, APP expression again was restricted to the basal layer of the still slightly hyperthickened epidermis. (D) Quantitation of the relative fluorescence intensity per area (d, dermis; hf, hair follicle; g, granulation tissue; he, hyperproliferative epidermis; bar, 20 μm).

phoresis on 1% agarose gels and visualized by ethidium bromide staining.

Quantitative determination of APP and APLP2 mRNA. For quantitation of APP and APLP2 mRNA during wound healing competitive PCR was performed as described [21]. The following primers were used for the generation of cRNA standards: $APP_{total}f\Delta$ (5′-GAATTCCGACATGAGAAGTTCATCATCAAAAATTG-3′); $APP_{total}r\Delta$ (5′-TGATGATGAACTTCTCATGTCGGAATTCTGCATCCATC-3′); $APP_{total}f$ (5′-CTGTGGAGCTCCTTCCCGTGA-3′); $APP_{total}r$ (5′-ATGATGAATGGATGTGTACTG-3′); APLP2fΔ (5′-CGGACTCGACTATGTGGAGGAAGAGGAAGAGGATG-3′); APLP2rΔ (5′-TCTTCCTCTTCCTCCACATAGTCGAGTCCGAGTCA-3′); APLP2f (5′-GACCTTATATAGCTATGGCAT-3′); and APLP2r (5′-TCCTCCCCTTCCTCCTCAT-3′). The optimal amount of cRNA standard was determined and PCR reactions were adjusted to the exponential range. Quantitation of mRNA was performed using the primers APPr-FAM (5′-TTTGTTCGAACCCACATCTT-3′); $APP_{ges}f$ (5′-CTGTGGAGCTCCTTCCCGTGA-3′); APLP2r-FAM (5′-CCAAATCTGCTTCAGTAGGAA-3′); and APLP2f (5′-GACCTTATATAGCTATGGCAT-3′). PCR products were separated on an ABI Prism 310 genetic analyzer (Applied Biosystems, Weiterstadt, Germany) using performance-optimized Polymer 4 (POP4, Applied Biosystems). Two independent wound series were examined by three quantitative PCR reactions, each.

Immunolabeling. Localization of APP and APLP2 in wounded skin occurred with antibodies CT15 and D2II, respectively. Double immunofluorescence was performed with the following antibody combinations: (i) CT15/anti-Tec-3 (Dianova) and (ii) CT15/anti-keratin 1/10/11. Unfixed frozen sections (6 μm) were blocked for 1 h at

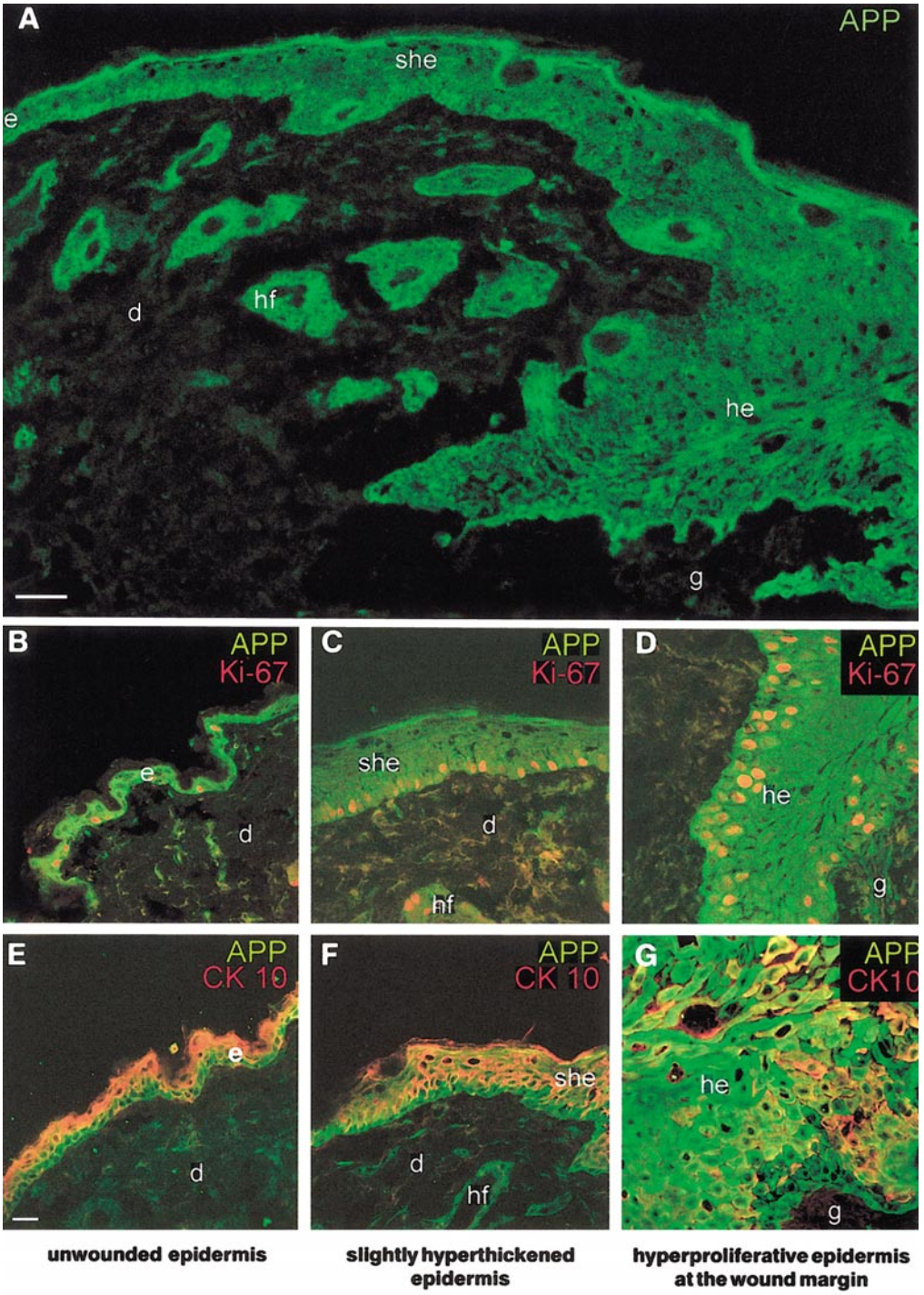

FIG. 3. Immunohistochemical localization of APP and Ki-67 (B–D) or APP and CK1/10/11 (E–G) in wounded skin. Frozen sections were taken from the middle of a 5-day wound and incubated with antibodies directed against APP (green) and Ki-67 (red) (B–D) or against APP (green) and CK1/10/11 (red) (E–G). (B and E: unwounded skin; C and F: slightly hyperthickened epithelium; D and G: hyperproliferative epidermis at the wound margin.) (A) A survey of the wound margin. Note the correlation of upregulated suprabasal APP expression and of Ki-67 as a marker of cell proliferation. The majority of suprabasal keratinocytes which were in an undifferentiated stage expressed enhanced amounts of APP (d, dermis; e, epidermis; hf, hair follicle; she, slightly hyperproliferative epidermis; g, granulation tissue; he, hyperproliferative epidermis; bar in (A), 50 μm; bar in (B–G); 20μm).

37°C with 3% bovine serum albumin (BSA) in PBS and incubated with the primary antibody for 1 h at 37°C. The following dilutions (in 0.3% BSA in PBS) were used: CT15, 1:100; D2II, 1:100; anti-Tec-3, 1:50; anti-keratin 1/10/11, 1:20. After three 5-min washes with 0.3% BSA in PBS sections were incubated for 1 h with the secondary DTAF- or Cy-3-conjugated antibodies (Dianova) as described by the manufacturer. When using secondary mouse antibodies sections were preblocked with human immunoglobulins (IgG) for 1 h at 37°C to minimize cross reactions. The slides were rinsed three times with PBS and once with water and mounted with anti-fade reagent. Sections were analyzed using a Zeiss 510 laser scanning confocal microscope (LSM) (Jena, Germany). Quantitation of the fluorescence intensity per area was performed using the standard software of the laser scanning microscope.

Statistical analyses. Data were expressed as means ± SD. Statistical significance was defined at a value of $P < 0.05$ using one-way ANOVA. Significantly different measurements are depicted by asterisks (*).

RESULTS

Expression of APP and APLP2 Isoforms in the Skin of Balb/c Mice

Wounded back skin of Balb/c mice was used as an *in vivo* model to study the expression of APP or APLP2 during cutaneous wound repair [6]. Detection of APP and APLP2 isoforms expressed in the skin of Balb/c mice was based on PCR using primers flanking the differentially spliced exons. RNA of unwounded skin was isolated and reversely transcribed. Three major isoforms of APP (i.e., APP_{770}, APP_{751}, and APP_{695}) were detected (Fig. 1A), displaying the expression pattern characteristic for APP isoforms in peripherial tissues [25] with predominant expression of the isoforms APP_{770} and APP_{751}. Three of the four possible splice products of the APLP2 gene were also detected in the skin of Balb/c mice ($APLP2_{763}$, $APLP2_{707}$, and L-$APLP2_{695}$) (Fig. 1B).

Localization of APP and APLP2 in the Wounded Skin

Immunofluorescence localization of APP in wounded skin was performed on frozen sections (6 μm) of wounded skin 5 and 13 days after wounding using the antibody CT15 directed against the C-terminus of APP. This antibody had been shown before to recognize APP without interacting with the APP-related proteins APLP1 or APLP2 [26]. APP was mainly localized in the epidermis, whereas the cellular constituents of the dermal layer including the dermal fibroblasts showed only a weak immunocytochemical signal (Fig. 2A). Whereas the subcellular distribution of APP has been previously described [21], in this study we were interested in the differential expression of APP in various epidermal layers. In unwounded skin APP was mainly expressed in keratinocytes of the basal layer and the hair follicles, while keratinocytes of suprabasal layers showed only a weak signal. In keratinocytes of 5-day wounds APP expression was increased 1.6-fold in suprabasally located cells at the wound margin. There was no difference in APP expression between basal and suprabasal keratinocytes of the hyperthickened wound epidermis, and the expression levels corresponded to those of the basal layer of unwounded epidermis. Thirteen days after wounding when wound reepithelialization was completed suprabasal expression of APP declined to the level of unwounded epidermis (Figs. 2B, 2C, 2D).

The question arose whether a correlation existed between APP expression and cell proliferation. For this purpose, we performed double staining with antibodies CT15 and Tec-3. The latter detects the Ki-67 protein present in proliferation competent cells which were predominantly seen within the basal layer of the normal epidermis and in the lower suprabasal layers of the hyperthickened wound epithelium (including the hair follicles adjacent to the wound). An increased number of proliferating cells was detected in keratinocytes at the wound margin. Coexpression of Ki-67 and APP was restricted to basal keratinocytes of the normal epithelium and of the slightly hyperthickened epidermis adjacent to the wound, as well as to basal and some suprabasal keratinocytes of the hyperthickened epidermis above the granulation tissue (Figs. 3B, 3C, 3D). Taken together, increased APP expression appears to be a characteristic feature of all proliferation competent suprabasal keratinocytes of the hyperthickened epidermis at the wound margin. To determine the stage of differentiation of APP expressing keratinocytes double labeling with the anti-APP (CT15) and anti-keratin 1/10/11 antibodies was performed. The latter detects the differentiation specific keratins 1, 10, and 11, which were present in all suprabasal cells of the normal epidermis (Figs. 3E, 3F, 3G) [27]. The expression of the keratins 1/10/11 declined in suprabasal keratinocytes of the hyperproliferative epidermis, in which the expression of APP was upregulated. Although a few suprabasal keratinocytes of the hyperthickened epithelium coexpressed APP and keratins 1/10/11, APP was mainly expressed by nondifferentiated, proliferation competent cells.

The localization of APLP2 in the healing wound was studied immunocytochemically in frozen sections (6 μm) from 5- and 13-day wounds using the antibody D2II. In contrast to APP, APLP2 expression of the unwounded epidermis was not restricted to the basal layer. Furthermore, there was no significant difference in the expression and localization of APLP2 in unwounded and wounded skin (Fig. 4).

Differential Regulation of APP and APLP2 during Cutaneous Wound Repair

For quantification of mRNA levels of APP or APLP2 during wound healing, RNA was isolated from excisional wounds at different time points after wounding. We chose time points characteristic of the different phases of wound repair: the early inflammatory phase, which is characterized by influx and activation of neutrophils and macrophages (Days 1–3), the phase of new tissue formation (Days 3–7), and the phase of tissue remodeling (starting around Day 7) [1]. Two independent wound series were examined by three quantitative PCR reactions, each, revealing a biphasic expression of APP mRNA. Within 12 h after skin injury a 1.3-fold induction of APP expression was detected. At Day 3 after wounding the mRNA level declined again. A 2.3-fold induction of APP mRNA was detected again at Days 5 to 13 after injury. At Day 13, when wound reepithelialization was completed, the mRNA level was still elevated (Fig. 5A). In contrast to APP, APLP2 mRNA levels declined significantly within 12 h after wounding and remained low until Day 13 (Fig. 5B).

Differentiation Inhibited sAPP Secretion in in Vitro Models of Keratinocyte Growth

We have shown that the suprabasally located wound margin keratinocytes expressing enhanced amounts of APP are less differentiated and proliferation competent. However, no reliable assay is available to selectively detect the secreted form of APP within tissues. To investigate the sAPP secretion of these nondifferentiated, proliferating keratinocytes two *in vitro* models were applied:

(1) *In vitro model of subconfluent (50 and 80%) and proliferating cultures of HaCaT cells compared to confluent (100% and postconfluent), nonproliferating HaCaT cells.* The stage of confluency was estimated as described under Materials and Methods. After biosynthetic radiolabeling the expression of APP in lysates was determined by immunoprecipitation with antibody CT15 recognizing the C-terminus of the protein, whereas the secreted form was immunoprecipitated from the medium using antibodies IG7 and 5A3 recognizing the N-terminus of APP. The autoradiography of three independent experiments showed that APP expression was about 3-fold higher in subconfluent cells than in confluent cells (Fig. 6). The ratio of mature to immature APP was independent of the cell density. Subconfluent cultures of HaCaT cells secreted about 10-fold more sAPP than confluent cultures (Fig. 7).

(2) *In vitro model of undifferentiated NHK cells cultured at low or high Ca^{2+}.* Culture of NHK cells at low Ca^{2+} concentration increased the secretion of sAPP into the culture medium as compared to culture at high Ca^{2+} concentrations (Fig. 8). Quantitation of three independent experiments revealed a threefold increase in the secretion of sAPP at low Ca^{2+} medium.

Both *in vitro* models showed that sAPP secretion is strongly increased in undifferentiated and proliferation competent cells as compared to differentiated cells.

DISCUSSION

Recent reports have presented evidence that sAPP operates in the stimulation of keratinocyte proliferation [21] and migration [Kirfel *et al.*, unpublished]. On the basis of these observations we postulated a possible role of sAPP in cutaneous wound repair. Here we report on the expression of APP and APLP2 in a full-thickness excisional wound healing model, which had previously been used for the identification and characterization of other factors involved in wound reepithelialization, such as KGF and other members of the fibroblast growth factor (FGF) family [6], transforming growth factors β (TGF-βs) [28], and activin [29].

APP and APLP2 Expression during Epidermal Wound Healing

The expression of APP in the unwounded epidermis is predominantly observed in keratinocytes of the basal layer whereas in the hyperthickened epidermis at the wound margin the expression of APP is increased and no longer restricted to basal cells but observed also in all suprabasally localized keratinocytes. The upregulated APP expression coincides with an increased basal expression of proliferation markers such as Ki-67. Similar changes in cells located at the wound margin have been reported for keratin 14 [30] and for β-integrins [31] which are characteristically expressed in proliferation competent, nondifferentiated keratinocytes. Hence, the majority of suprabasal keratinocytes of the hyperthickened epithelium at the wound margin with increased expression of APP seem to belong to a population of undifferentiated keratinocytes. This is supported by the reduced expression of keratin 1/10/11 in suprabasally located keratinocytes in the hyperthickened epithelium of the wound margin. Keratins 1/10/11 are markers of early differentiation as they are observed in the unwounded epidermis in keratinocytes of the suprabasal cell layers [32]. As sAPP can be released proteolytically from APP and APLP2, the expression of APLP2 during wound healing was of particular interest. However, in contrast to our observations with APP we did not detect a specific localization or any changes in the expression of APLP2 in the wounded as compared to the unwounded epidermis.

Consistent with our immunohistochemical data, APP mRNA levels increased after wounding. Interestingly, there is a biphasic expression with a decline of mRNA levels at 3 days after wounding, followed by an

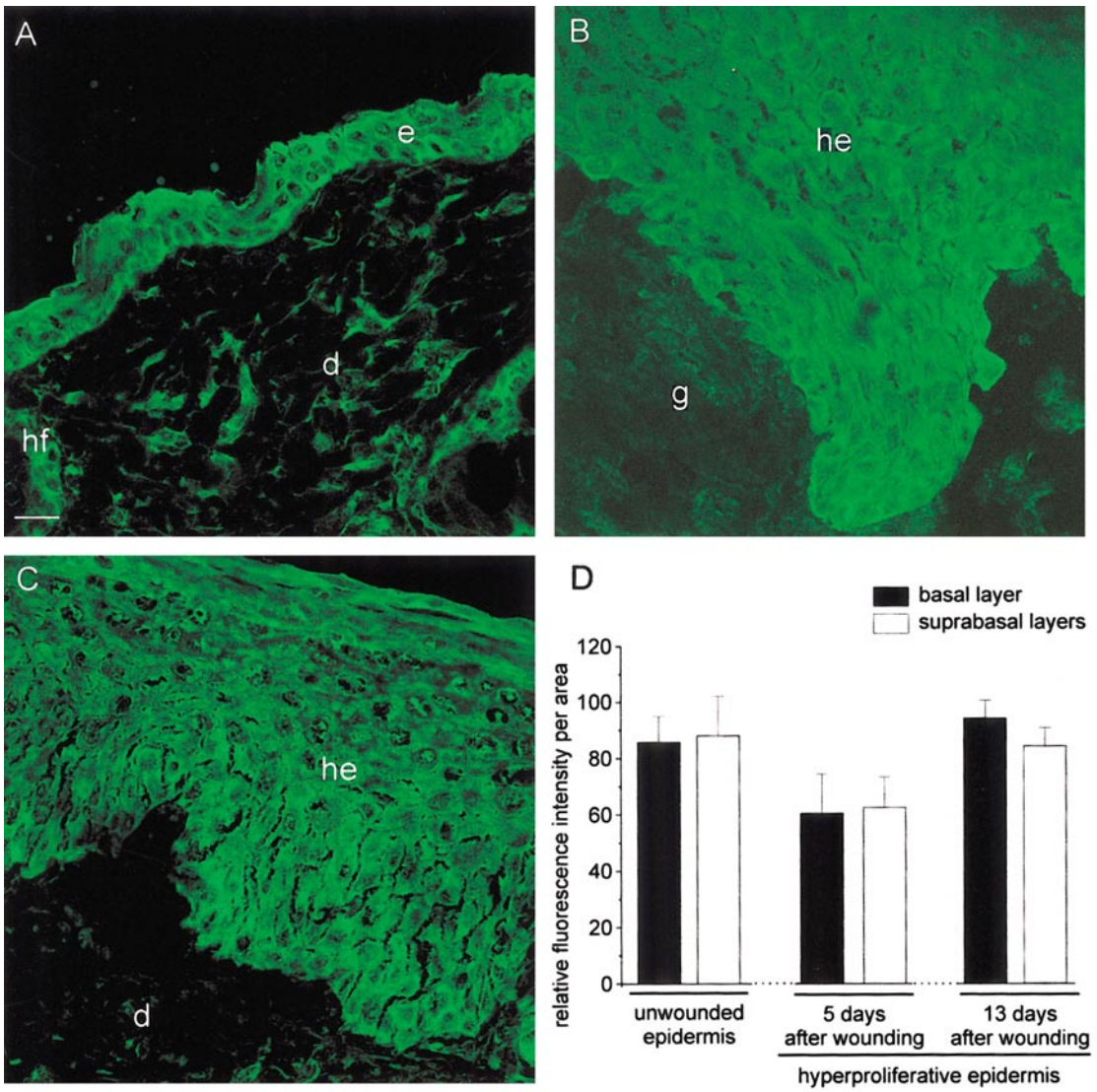

FIG. 4. Immunohistochemical localization of APLP2 in the epidermis of the unwounded skin (A) or 5 (B) and 13 days (C) after wounding. In contrast to the expression pattern of APP no significant differences between the expression of APLP2 in the basal layer and suprabasal cells of unwounded epidermis were detectable. Keratinocytes of the hyperproliferative epidermis at the wound margin showed no principle changes in the localization of APLP2 compared to unwounded skin. However, there is a decrease in the expression of APLP2 in the wounded epidermis. (D) Quantitation of the relative fluorescence intensity per area (d, dermis; hf, hair follicle; g, granulation tissue; he, hyperproliferative epidermis; bar, 20 μm).

increase at Day 13 after injury when wound closure is achieved. This biphasic lapse has also been observed in studies on the expression of the proliferation marker keratin 14 during the healing process [30]. The reason for this biphasic course is unknown. The first peak occurred during the early inflammatory phase. This period is characterized by invasion of neutrophils and macrophages, which secrete various proinflammatory cytokines, including interleukin-1α and -1β (IL-1α and -1β) [33]. The latter have been shown to induce the expression of APP in different cell types, such as astrocytes [34], PC12 cells [35], or human endothelial cells [36, 37]. Therefore it seems likely that IL-1 is also responsible for the increased expression of APP in early phases after wounding.

The second peak of APP expression was observed between Days 5 and 13 after wounding, thus coinciding with enhanced epidermal growth. By contrast, the levels of APLP2 mRNA declined early after wounding and remained low until reepithelialization was completed. This finding suggests that factors present in wounded skin diminish expression and processing of APLP2. Indeed, preliminary data from our laboratory revealed that KGF suppresses the expression of APLP2 in cul-

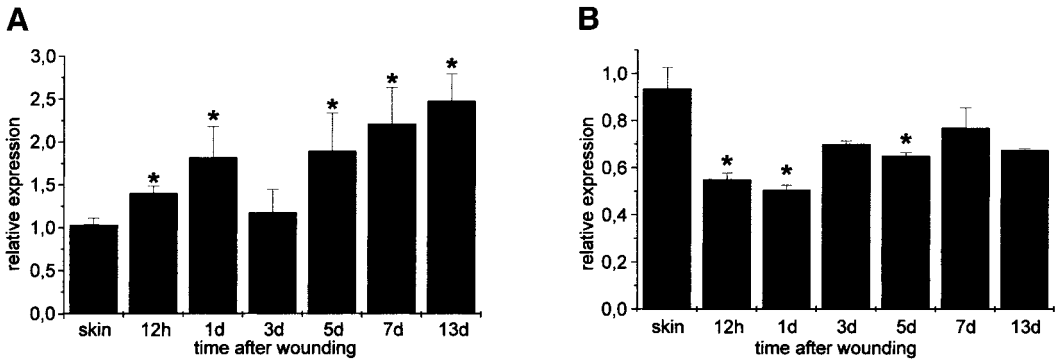

FIG. 5. Quantitative determination of APP (A) and APLP2 (B) mRNA levels in cutaneous wounds of Balb/c mice by competitive PCR. (A) Within 12 h after injury a 1.3-fold induction of APP mRNA was observed. At Day 3 after injury the expression level declined. A 2.3-fold induction of APP mRNA was detected again at Days 5 to 13 after injury when wound closure was achieved. (B) A 1.8-fold decline of the APLP2 mRNA level was seen 1 day after injury with increasing levels at Days 3 to 13 after injury. Two independent wound series were examined by three quantitative PCR reactions, each (*significant to unwounded skin).

tured keratinocytes, suggesting that KGF which is highly expressed between Day 1 and Day 7 after injury [6] might participate in the downregulation of APLP2 in the epidermis of the wounded skin.

The downregulation of APLP2 after skin injury differs from results obtained with a corneal wound healing model, where upregulation of APLP2 after wounding has been described [38]. Apparently, the cornea and the back skin differ in their APLP2 expression during wound repair. This is not unusual as different epidermal regions of the organism are characterized by their distinct protein expression. Our observations suggest that APLP2 might foster corneal but not epidermal wound healing.

Increased sAPP Secretion in Proliferating Keratinocytes in Vitro

Increased expression of APP in proliferation competent, less differentiated keratinocytes presumably results in increased proteolytic release of sAPP. However, no techniques are currently available to determine the rate of sAPP secretion from tissue *in situ*.

Therefore, we employed two *in vitro* culture models of keratinocyte differentiation to compare the release of sAPP into the supernatants of proliferating cells grown to subconfluency or at low Ca^{2+} concentrations and of quiescent, partially differentiated cells grown at confluency or at high Ca^{2+} concentrations. Keratinocytes grown at confluency or at high Ca^{2+} concentrations are indeed differentiated as shown before by others [39, 40]. Both *in vitro* models showed that the release of sAPP was increased up to 10-fold in proliferation competent, nondifferentiated keratinocytes as compared to differentiated cells.

We conclude from these observations that sAPP release is presumably also increased in keratinocytes *in situ*, i.e., in proliferation competent, less differentiated

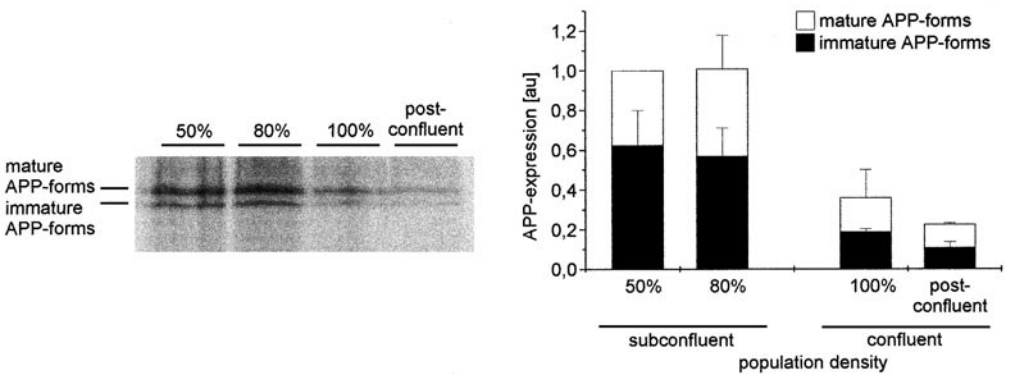

FIG. 6. Autoradiograph of three independent immunoprecipitations showing the expression of APP in proliferating compared to nonproliferating cells. HaCaT cells were grown to different stages of confluency (50, 80, and 100% and postconfluent). Cells were lysed and equal amounts of protein were subjected to immunoprecipitation of APP using an antibody directed against the C-terminus of the protein. APP expression of subconfluent, proliferating keratinocytes was three-fold higher than that of confluent, nonproliferating cells whereas the ratio of mature to immature APP forms was independent of the population density and approximately the same.

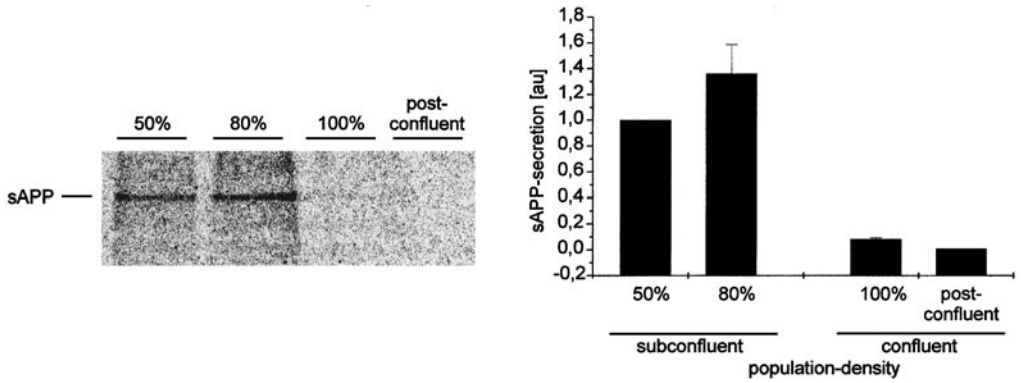

FIG. 7. Autoradiograph of three independent immunoprecipitations showing the secretion of sAPP by subconfluent, proliferating HaCaT cells compared to confluent, nonproliferating cells. HaCaT cells were grown to different stages of confluency (50, 80, and 100% and postconfluent) followed by immunoprecipitation of sAPP from the medium using an antibody recognizing the N-terminus of the protein. The amount of the medium which was subjected to immunoprecipitation was normalized to the number of secreting cells. Subconfluent keratinocytes secreted about 10-fold more sAPP than confluent cells.

keratinocytes of the hyperproliferative epidermis at the wound site. Reepithelialization during wound repair is a complex process which requires cell migration and proliferation. Keratinocyte migration and proliferation are regulated by a large number of ions, cytokines, and growth factors [8, 41]. Since sAPP has been shown to exert a mitogenic [21] and motogenic [Kirfel *et al.*, unpublished] effect on keratinocytes, sAPP may be released also from proliferation competent, less differentiated keratinocytes of the wound margin and, thus, be able to act as a novel regulator of wound reepithelialization.

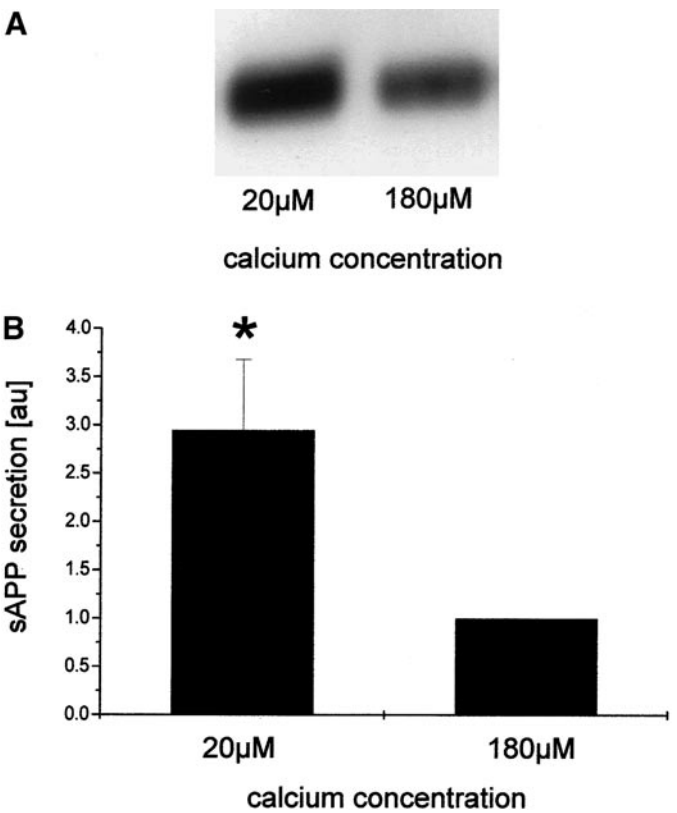

FIG. 8. Western blot of Ca^{2+}-dependent sAPP secretion of NHK cells (A). NHK cells were grown with 20 and 180 μM $CaCl_2$ for 72 h in KBM medium. Supernatants were separated by SDS–PAGE and immunoblotted with antibodies IG7 and 5A3 against the N-terminus of sAPP. (B) Quantitation of sAPP secretion band. Three independent experiments were quantitated using Tina software (*significant).

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