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Sortilin Is Expressed in Cultured Human Keratinocytes and Is Regulated by Cutaneous Neuropeptides

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Sortilin, a member of the family of Vps10p domain receptors, has been shown to be able to bind the precursor peptide of nerve growth factor (proNGF). ProNGF interacts with sortilin and the p75^{NTR} receptor on the cell surface to form a molecular complex capable of activating an apoptotic cascade. Keratinocytes can secrete proNGF and they have p75^{NTR} on their surface. The expression of sortilin in normal human keratinocytes has not yet been clearly shown. In this study, we show that keratinocytes express sortilin mRNA, and the presence of sortilin protein is shown in cultured keratinocytes and in normal human skin. We have also shown that the cutaneous neuropeptides substance P, calcitonin gene-related peptide, vasoactive intestinal polypeptide, and galanin are able to reduce the expression of sortilin mRNA and sortilin protein in cultured human keratinocytes. In addition, each of the analyzed neuropeptides has the ability to arrest the proNGF-induced apoptosis of human keratinocytes. These results suggest that all the participants in the NGF/proNGF pathway are present in the keratinocytes, and cutaneous neuropeptides can modulate their expressions and actions. The NGF/proNGF balance and its regulation by neuropeptides may have an important role in skin homeostasis.

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INTRODUCTION

Sortilin, a 95 kDa glycoprotein, is a member of the recently discovered mammalian Vps10p domain-containing transmembrane receptor family. This domain was named after the yeast-sorting protein Vps10p (Marcusson *et al.*, 1994). In sortilin, the domain comprises the entire N-terminal luminal/extracellular part of the receptor and the receptor contains a short cytoplasmic domain that furnishes typical motifs for interactions with cytosolic adapter molecules. Sortilin is mainly expressed in the neurons of the central nervous system (Hermans-Borgmeyer *et al.*, 1999; Domeniconi *et al.*, 2007). Besides being present in the brain, sortilin is abundant in diverse cell types, including the spinal cord, skeletal muscle, testis, heart, adipocytes, and melanocytes (Morris *et al.*, 1998; Hassan *et al.*, 2004; Truzzi *et al.*, 2008; Hermey, 2009). The experimental data indicate that sortilin, acting as an endocytic and intracellular sorting receptor, contributes to the targeting of ligands to lysosomes and the sorting between the Golgi apparatus and endosomes (Lefrancois *et al.*, 2003; Canuel *et al.*, 2008; Mari *et al.*, 2008). However, sortilin has also been shown to serve additional cell- and tissue-specific functions. There is appreciable evidence of its expression on the cell surface (Nielsen *et al.*, 1999; Navarro *et al.*, 2001). Accordingly, sortilin not only serves as a general intracellular sorting receptor, but also mediates specialized functions, for instance as a co-receptor determining the neurotrophin signaling pathway (Nykjaer *et al.*, 2004; Hermey, 2009).

The nerve growth factor (NGF) is a polypeptide that belongs in the neurotrophin family. NGF binds to two classes of transmembrane receptors: a tyrosine kinase receptor of 140 kDa (TrkA) (Kaplan *et al.*, 1991) and a glycoprotein receptor of 75 kDa (p75^{NTR}) (Dechant and Barde, 1997). In neuronal cells, NGF binds to TrkA, and dimerization and autophosphorylation of the receptor occur, promoting survival and differentiation (Segal, 2003). By contrast, p75^{NTR} can induce apoptosis through its own signal transduction (Wang *et al.*, 2001). NGF is synthesized as precursor NGF (proNGF) and processed posttranslationally into mature NGF by an enzymatic cleavage (Kliemannel *et al.*, 2004). Sortilin has been shown to be able to bind proNGF (Nykjaer *et al.*, 2004) and proNGF is a high-affinity ligand for p75^{NTR}. There is experimental and computational evidence that proNGF interacts through its pro-domain with sortilin, while the mature domain of NGF can interact with p75^{NTR} (Paiardini and Caputo, 2008). Thus, sortilin can itself bind both precursors, and the formation of a complex with

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Abbreviations: CGRP, calcitonin gene-related peptide; GAL, galanin; NGF, nerve growth factor; proNGF, precursor nerve growth factor; SP, substance P; VIP, vasoactive intestinal polypeptide

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p75^{NTR} leads to extended affinity for proNGF. The p75^{NTR}-sortilin complex couples with proNGF to induce apoptosis (Kaplan and Miller, 2004; Schweigreiter, 2006).

NGF has been shown to have a major role in skin homeostasis and inflammatory skin diseases. It has several regulatory functions in cutaneous nociception, nerve development, and regeneration (Pincelli and Yaar, 1997; Stander *et al.*, 2009). In the skin, NGF is expressed mostly by keratinocytes (Tron *et al.*, 1990). NGF in cutaneous keratinocytes has been suggested to be a key player in an autocrine loop, acting as mitogen, and survival factor through its high-affinity receptor TrkA (Pincelli and Marconi, 2000). In keratinocytes, NGF production is influenced by neuropeptide release.

The human skin is abundantly supplied with sensory nerves, which form part of the peripheral nervous system (Reilly *et al.*, 1997). This rich network of free sensory nerve endings surrounds and penetrates hair follicles, eccrine, and apocrine glands, the papillary dermis and the epidermis (Hilliges *et al.*, 1995). Both under normal conditions and under pathological conditions, various neuropeptides are released from these free nerve endings (Scholzen *et al.*, 1998). Moreover, keratinocytes possess several receptors for these neuropeptides (Takahashi *et al.*, 1993; Kemeny *et al.*, 1994; Lotti *et al.*, 1995; Dallos *et al.*, 2006b; Chang *et al.*, 2007). We earlier showed that the cutaneous neuropeptides substance P (SP), calcitonin gene-related peptide (CGRP), vasoactive intestinal polypeptide (VIP), and galanin (GAL) are able to increase the mRNA expression of NGF in cultured human keratinocytes and to enhance NGF secretion (Dallos *et al.*, 2006a). The secretion of proNGF by keratinocytes into the intercellular space has also been shown to occur (Dallos *et al.*, 2006a). In the epidermal milieu, all the participants in the NGF/proNGF signaling pathway are present except sortilin. To date, the presence of sortilin in the human keratinocytes or in normal human skin has not been clearly shown. However, there is significant evidence of the presence of p75^{NTR} in these cells (Dechant and Barde, 1997; Okumura *et al.*, 2003; Peters *et al.*, 2007) and its role in the apoptosis of the keratinocytes can be hypothesized.

This study was performed to determine the presence of sortilin in cultured human keratinocytes and in human skin, with regard to mRNA and protein levels. In addition, we studied the effects of SP, CGRP, VIP, and GAL on the expression of sortilin mRNA and protein and on proNGF-induced apoptosis in cultured human keratinocytes.

RESULTS

Identification of sortilin mRNA and sortilin protein in cultured human keratinocytes

In the first step, we analyzed the presence of sortilin at the level of mRNA. Using the Human Sortilin TaqMan Gene Expression Assay and the human keratinocyte template, we succeeded in detecting an amplified product with the expected molecular size. With human brain complementary DNA as a positive control instead of the keratinocyte template, we detected the amplified product with the same molecular size (Figure 1a). Next, we performed western blotting assays on cultured human keratinocyte extracts to detect sortilin protein, with human brain protein extract as a positive control. With both the human keratinocyte and the human brain extracts, we were able to show the sortilin protein with the expected molecular weight of 95 kDa (Figure 1b). Preabsorption assays were carried out to verify the specificity of the sortilin antibody. Using blocking peptide preabsorbed with sortilin antibody, we did not observe a signal for either the keratinocyte extract or the human brain extract (data not shown).

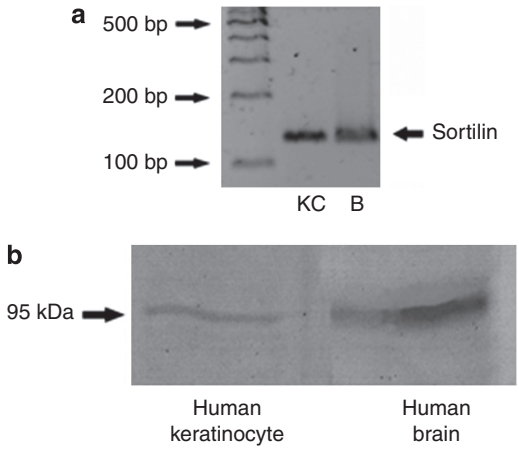

Figure 1. Expressions of sortilin mRNA and protein in cultured human keratinocytes. Reverse transcription (RT)-PCR was carried out on mRNA derived from human keratinocytes and human brain extract to show the presence of sortilin mRNA. The 129-bp PCR product of sortilin was detected with the use of either keratinocyte complementary DNA (cDNA) or human brain cDNA (**a**; KC = human keratinocytes, B = human brain). The presence of sortilin protein was detected by using western blotting in protein extracts from cultured human keratinocytes and human brain. (**b**) Shows the presence of sortilin protein with the expected molecular weight (95 kDa) in the lysates from both human keratinocytes and human brain. Absorption control experiments were performed with the sortilin blocking peptide to test the specificity of the primary antibody (data not shown).

Immunocytochemical investigation of the expression of sortilin in cultured human keratinocytes

In the next step, immunocytochemical investigations were performed on cultured human keratinocytes. Immunocytochemical assays on cultured human keratinocytes with the use of polyclonal anti-sortilin antibody revealed considerable immune staining both in the cytoplasm and on the surface of the cells (Figure 2b and c). The distribution of immune staining proved to be cytoplasmic, but in certain areas the surfaces of the cells were also stained. Specific immune staining was not found on the isotype control (Figure 2a).

In situ expression of sortilin in human skin

On skin samples from five persons undergoing plastic surgery, we carried out immunohistochemical investigations to show the presence of sortilin in the different layers of the human skin, and to examine how the level of intensity varies. With polyclonal anti-sortilin antibody, the highest sortilin expression was found in the basal layer of the epidermis, while in the spinous layer there was only faint staining, and

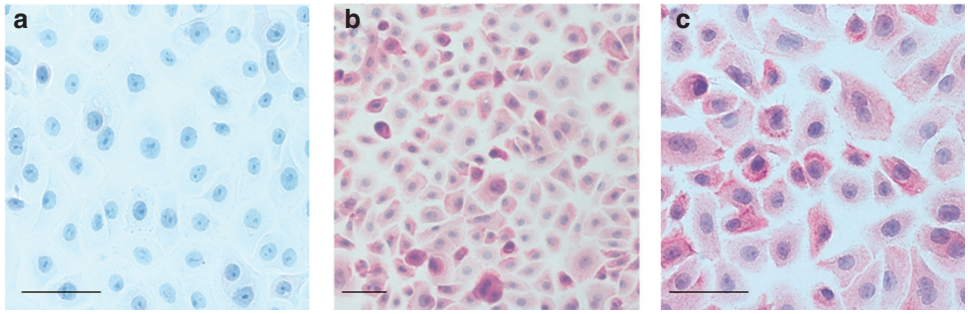

Figure 2. Immunocytochemical analysis of sortilin in cultured human keratinocytes. Cultured normal human keratinocytes (derived from adult human breast skin) were subjected to immunocytochemical studies to analyze the expression of sortilin. No specific staining was found for the negative control (**a**). (**b**, **c**) Illustrate the immune staining of the sortilin in the cytoplasm and on the surface of the keratinocytes at various magnifications. Nuclei were counterstained with hematoxylin. Scale bar = 50 μm (**a**–**c**).

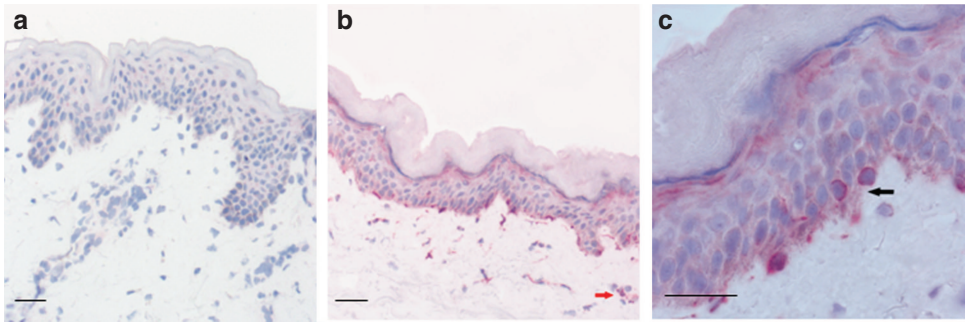

Figure 3. Localization of sortilin in the different layers of normal human skin by immunohistochemistry. The figure shows the expression of sortilin in normal human female breast skin obtained from a patient undergoing plastic surgery. A negative control was performed with normal rabbit IgG instead of primary antibody (**a**). (**b**, **c**) Show the more intensive coloration of the basal keratinocytes (black arrow), but immunostaining was additionally detected in the other layers of the epidermis. Positive blood vessel cells and lymphoid elements (red arrow) can also be observed in the dermis. Nuclei were counterstained with hematoxylin. Section thickness: 4 μm. Scale bar = 50 μm (**a**–**c**).

the granular layer also showed moderate sortilin positivity (Figure 3b and c). In the isotype control, there was no specific staining at all (Figure 3a). Some sortilin-positive cells were also found in the dermis.

Influence of neuropeptides (SP, CGRP, VIP, or GAL) on the expressions of sortilin mRNA and sortilin protein in cultured human keratinocytes

After the induction of subconfluent cultured keratinocytes with SP (10^{-8} M), significant decreases of approximately 40–50% were experienced in the amounts of sortilin mRNA as compared with the time-matched controls at 3 and 6 hours after the treatment (Figure 4a). Investigation of sortilin protein revealed an increase at 24 hours in the neuropeptide-treated cells, but the difference was not significant. Later, the amount of sortilin protein gradually decreased, and after 72 hours in the SP-treated cultures the decrease (60%) was significant relative to the controls (Figure 4b).

After the induction of keratinocyte cultures with CGRP (10^{-8} M), there was no significant difference in the expression of sortilin mRNA (Figure 4c). Interestingly, the sortilin protein levels in the CGRP-treated cultures were dramatically decreased (60 and 70%) at 48 and 72 hours after the treatment (Figure 4d).

The induction of cultured keratinocytes with VIP (10^{-8} M) resulted in a significant decrease (70%) in the expression of sortilin mRNA at 6 hours after the treatment. Later, at 12 and 24 hours, the sortilin mRNA expression in the VIP-treated samples had reached the same levels as in the controls (Figure 4e). The amount of sortilin protein in the VIP-treated cultures showed a slight, but significant decrease (30%) only 72 hours after the treatment (Figure 4f).

The effects of GAL (10^{-8} M) on the expression of sortilin mRNA of cultured keratinocytes were noteworthy at 6 and 12 hours after the treatment, when 80% decreases in sortilin mRNA expression were shown (Figure 4g). A significant decrease (50%) in sortilin protein level was detected at 72 hours after the neuropeptide treatment (Figure 4h).

Neuropeptides modulate the proNGF-induced apoptosis in cultured human keratinocytes

Normal human keratinocytes were cultured in keratinocyte serum-free medium in 96-well plates. After 24 hours, the cells were induced with proNGF (100 ng ml^{-1}) alone, or together with the combinations of proNGF and SP, or CGRP, or VIP, or GAL. After 48 hours, the extent of apoptosis was detected by using an *in situ* apoptosis detection kit. The cell cultures treated with only proNGF showed a considerable increase in apoptotic cell index (1.4) as compared with the untreated cultures (1.0). In each of the keratinocyte cultures treated with proNGF supplemented with one or other of the

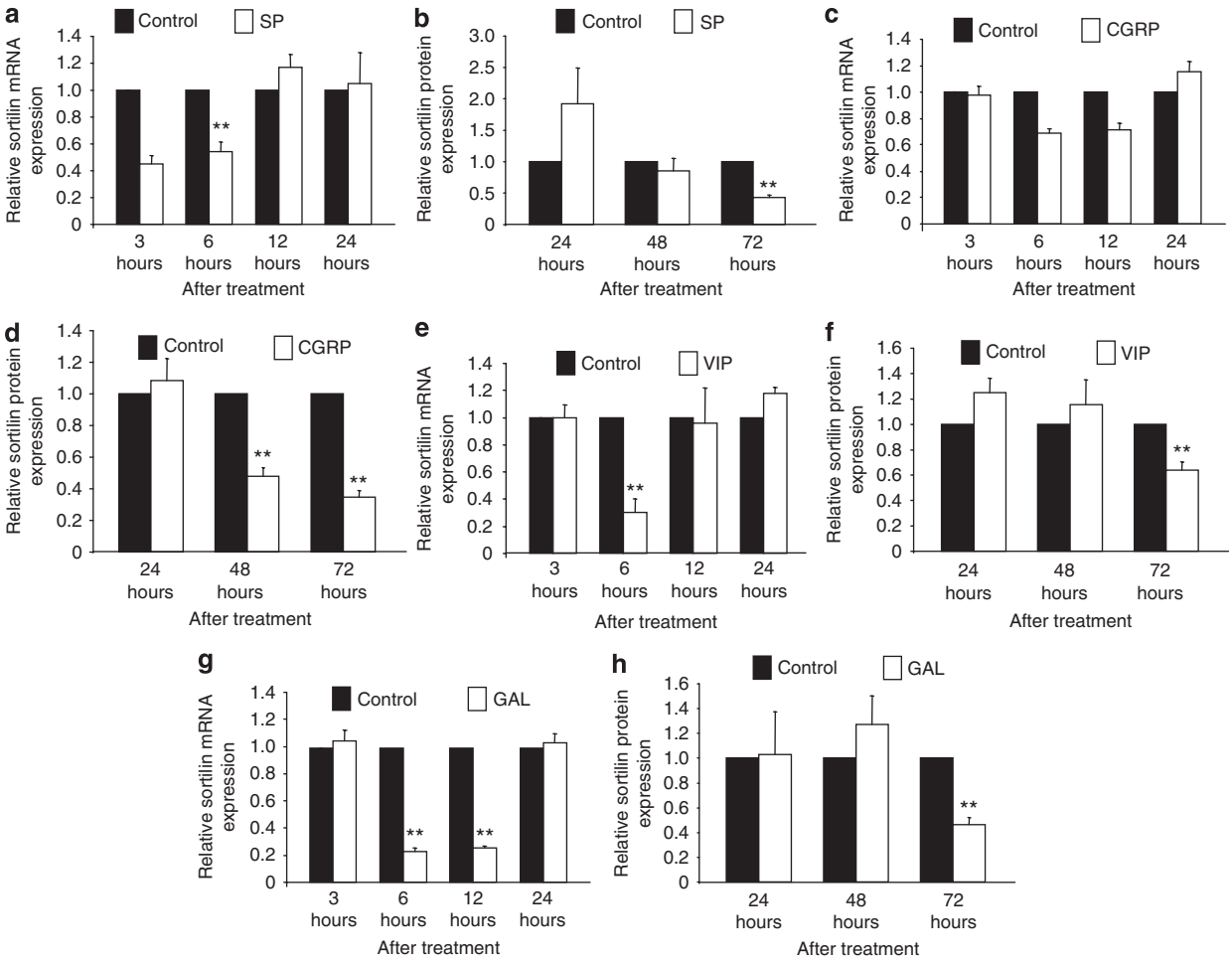

Figure 4. The effects of cutaneous neuropeptides on the expressions of sortilin mRNA and sortilin protein in cultured human keratinocytes. Cultured human keratinocytes were treated with SP, CGRP, VIP, or GAL at 10^{-8} M for 30 minutes. (**a**) Depicts the changes in sortilin mRNA expression after treatment with SP, relative to the untreated time-matched controls. The changes in the relative amount of sortilin protein in the lysate from human keratinocytes after SP induction are observed in (**b**). (**c**, **d**) Present the sortilin mRNA and protein changes caused by CGRP, (**e**, **f**) caused by VIP, and (**g**, **h**) caused by GAL. The reported data were calculated as the means ± SD of the results of three independent experiments. $^{**}P<0.01$; ■, control; □, neuropeptide-treated samples.

neuropeptides, a significant decrease was shown in the apoptotic index relative to the cells induced with proNGF alone (0.7–0.9 vs 1.4) (Figure 5a). To reconfirm the results, an Annexin V apoptosis assay was also carried out by the above-detailed method; this indicated a similar tendency of the proNGF-induced apoptosis (Figure 5b).

DISCUSSION

Sortilin is expressed in cultured human keratinocytes and in normal skin

To date, the expression of sortilin in human keratinocytes or in human skin has not been clearly shown. Our study revealed the presence of sortilin at both the mRNA and protein levels in cultured human keratinocytes. Immunocytochemical investigations showed that the expression of sortilin in the keratinocytes is mostly cytoplasmic, and some cells show sortilin-specific cell surface staining. In normal human skin, the highest sortilin expression was found in the basal layer of the epidermis, but moderate or slight sortilin positivity was also observed in the upper dermis. A majority of the cells showed cytoplasmic staining, but cell-surface sortilin expression was additionally detected on the keratinocytes of the epidermal basal layer.

Sortilin is expressed in diverse cell types, the highest amount of sortilin being found in the vesicles within the cells (Lin *et al.*, 1997). However, several studies have revealed the presence of sortilin on the surface of various cells (Nielsen *et al.*, 1999; Navarro *et al.*, 2001); its expression on the surface of human keratinocytes may be of considerable biological significance. It is well documented that, in neuronal cells, the NGF pathway (neurotrophin pathway) has a central role in the maintenance of the balance between neuronal cell proliferation, differentiation, and apoptosis (Nykjaer *et al.*, 2004). NGF and its precursor proNGF are very important in this process. ProNGF elicits opposite biological effects to those of mature NGF; while mature NGF promotes cell survival and cell proliferation, proNGF can cause cell death (Ibanez, 2002). These opposite effects are mediated by the interactions with different transmembrane signaling systems. The TrkA receptor preferentially

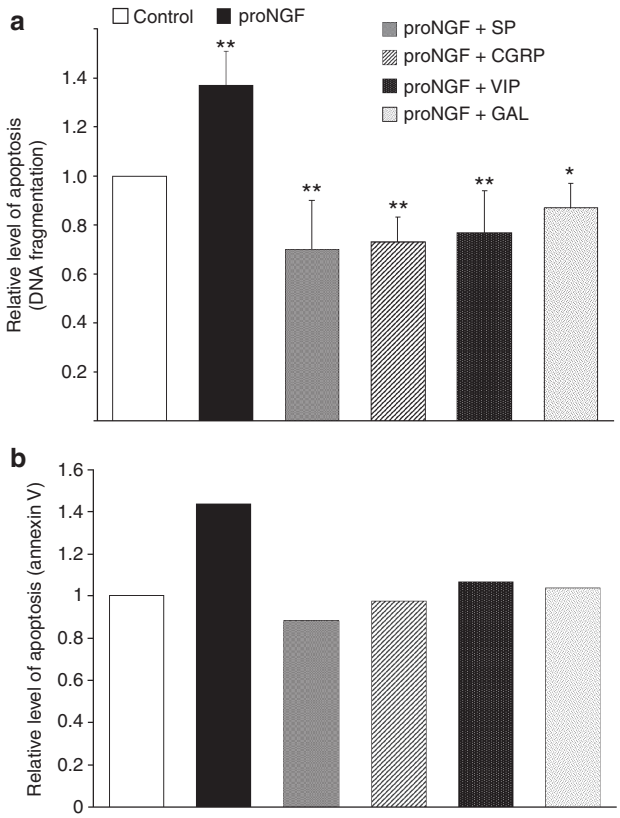

Figure 5. The neuropeptides SP, CGRP, VIP, and GAL modulate the proNGF-induced apoptosis in cultured human keratinocytes. Apoptosis in human keratinocytes was detected by using an *in situ* apoptosis detection kit on the basis of the measurement of DNA fragmentation (**a**) and by using the Annexin V apoptosis assay (**b**). Human keratinocytes were induced with proNGF (100 ng ml^{-1}) alone or together with the combinations of proNGF and SP, CGRP, VIP, or GAL. The reported data were calculated as the means ± SD of the results on at least triplicate wells of three independent experiments (*$P<0.05$; **$P<0.01$) (**a**). The results (**b**) are expressed as the means of two independent experiments (□, control; ■, proNGF, or proNGF + neuropeptide-treated cultures).

binds mature NGF, while a receptor complex sortilin–p75^{NTR} interacts with proNGF to activate the proNGF-mediated cell death signaling pathway (Lee *et al.*, 2001; Nykjaer *et al.*, 2004, 2005). This NGF pathway has been shown to operate in neuronal cells (Nykjaer *et al.*, 2004; Volosin *et al.*, 2006) and has been hypothesized in some other tissues (Peters *et al.*, 2006), but it is as yet unknown in keratinocytes.

It is obvious that NGF is a very important and potent growth factor in the skin. Normal human keratinocytes synthesize and release high amounts of biologically active NGF. In addition, human keratinocytes express both the high-affinity (TrkA) and the low-affinity (p75^{NTR}) receptors (Pincelli *et al.*, 1994). NGF is the key component of an autocrine loop, acting as a mitogen and as a survival factor for human keratinocytes (Di Marco *et al.*, 1993; Pincelli and Yaar, 1997). Moreover, NGF is a crucial neurotrophin molecule for skin innervation (Albers *et al.*, 1994). Besides mature, biologically active NGF, proNGF is also produced and secreted by cultured human keratinocytes (Dallos *et al.*, 2006a). We have now shown that keratinocytes can express sortilin, which seems to be essential for the proNGF-induced apoptotic process. All the participants in the NGF pathway are therefore present in the epidermal milieu and can operate. We have also established that proNGF is able to induce apoptosis in cultured human keratinocytes, which is indirect evidence that the NGF/proNGF balance exists and may be important in skin homeostasis. This idea is supported by the fact that the production of mature NGF is increased in inflammatory skin conditions such as psoriasis accompanied by keratinocyte hyperproliferation (Pincelli and Marconi, 2000; Raychaudhuri and Raychaudhuri, 2004). Even less is known regarding the NGF receptor expression. In some studies on psoriasis, TrkA and p75^{NTR} were found to be upregulated, while in other studies the opposite was the case (Bull *et al.*, 1998). It was recently reported that the p75^{NTR} level is significantly lower in psoriatic than in normal keratinocytes, and p75^{NTR} protein seems to be absent in psoriatic lesional skin. These results indicate that p75^{NTR} acts as a pro-apoptotic receptor in human keratinocytes and the decrease or absence of p75^{NTR} receptors in psoriasis could account for the resistance of psoriatic keratinocytes to apoptosis (Truzzi *et al.*, 2006; Marconi *et al.*, 2008).

Cutaneous neuropeptides modulate sortilin expression and proNGF-induced apoptosis in cultured human keratinocytes

In the second part of our study, we examined the effect of the neuropeptides SP, CGRP, VIP, and GAL on the expression of sortilin by cultured human keratinocytes and on the proNGF-induced apoptosis of cultured keratinocytes. Under both normal and pathological conditions, various neuropeptides are released from free nerve endings. The secreted neuropeptides can interact with keratinocytes to modulate their function. One of the well-known characteristics of SP, CGRP, and VIP is their ability to promote the proliferation of keratinocytes (Haegerstrand *et al.*, 1989; Takahashi *et al.*, 1993; Benrath *et al.*, 1995), i.e., they promote the survival of cells rather than cause their apoptosis. Currently, little knowledge is available on the effects of GAL on human keratinocytes. In neural cells, GAL may exert a proliferative effect (Wang *et al.*, 1998). SP, CGRP, VIP, and GAL are known to increase the production and secretion of mature NGF to different extents in cultured human keratinocytes (Dallos *et al.*, 2006a); and the keratinocytes produce and secrete proNGF. As neuropeptides can modulate the NGF production of keratinocytes, it seemed reasonable to analyze the effects of these neuropeptides on the sortilin expression of keratinocytes. Sortilin with p75^{NTR} represents the opposite arm of the NGF/proNGF neurotrophin pathway.

Throughout the study, we used a constant concentration of stimulating neuropeptide, 10^{-8} M. We took into consideration the concentrations reported for each neuropeptide to occur physiologically in the serum (Koch *et al.*, 1991; Schifter, 1991; Bondy *et al.*, 2003). We included relatively high concentrations of stimulating peptides because serum concentrations of growth factors, hormones, and neuropeptides not necessarily reflect the concentrations in the direct pericellular microenvironment, which do can exceed serum

levels. The findings of our previously published experiments and the other studies led us to choose 10^{-8} M as the constant concentration of the stimulating peptide (Kiss *et al.*, 1999; Burbach *et al.*, 2001; Brenneman *et al.*, 2003; Dallos *et al.*, 2006a).

Evidence emerged that each of the analyzed neuropeptides was able to reduce both sortilin mRNA and protein expression in cultured human keratinocytes. However, there were some discrepancies between the timing of the sortilin mRNA decrease and the protein decrease brought about by the neuropeptides. Although the sortilin mRNA expression was decreased significantly relative to the untreated controls 6–12 hours after neuropeptide treatment, the decrease in sortilin protein content could be shown only after 48–72 hours. Our mRNA measurements were normalized for 18S ribosomal RNA as housekeeping gene; there were no significant differences in 18S ribosomal RNA expression between neuropeptide-treated samples and their time-matched controls (data not shown). There must obviously be some explanation for the apparently contradictory sortilin mRNA and protein data. The half-life of sortilin is not yet known, but it is possible that sortilin protein has a relatively long lifetime, which might explain why we experienced a sortilin protein decrease only 48–72 hours after the neuropeptide treatment.

We earlier showed that human keratinocytes can secrete proNGF (Dallos *et al.*, 2006a). Accordingly, to study the functionality of sortilin as a coreceptor, we induced cultured keratinocytes with proNGF and measured the apoptotic index in the cultures. There are merely a few studies in the literature regarding proNGF-induced apoptosis. The concentration of proNGF in different apoptosis assays varied in the range 2–300 ng ml^{-1}, depending on the experimental system (Nykjaer *et al.*, 2004; Althaus and Klöppner, 2006). In our preliminary experiments, we used 20, 100, and 500 ng ml^{-1} proNGF for keratinocyte apoptosis induction and, on the basis of our results, we finally chose 100 ng ml^{-1} proNGF. In agreement with our hypothesis that the presence of sortilin on keratinocytes may facilitate a pro-apoptotic process, there was a considerable increase in the apoptotic index of the proNGF-induced cells as compared with that of the untreated cells. This accords well with our findings, as each of the analyzed neuropeptides was able to reduce the amounts of both sortilin mRNA and sortilin protein in cultured human keratinocytes. In our previous study, we also showed that cutaneous neuropeptides enhance the mature NGF production of keratinocytes, which may lead to the survival and differentiation of the surrounding cells in the human skin (Dallos *et al.*, 2006a). Our results that keratinocytes express sortilin and that neuropeptides can decrease the sortilin expression in keratinocytes, supported our hypotheses, that (1) the complete proNGF/NGF pathway can operate in keratinocytes and (2) neuropeptides released from cutaneous nerves shift the balance to cell proliferation not only by increasing mature NGF secretion from the keratinocytes, but also by decreasing their sortilin expression. Nonetheless, further studies are required to investigate the exact mechanism of the process leading to apoptosis after the induction of human keratinocytes with proNGF. It seems definitely worthwhile to study the biological role of the NGF/proNGF pathway in the skin homeostasis and in different dermatological disorders.

MATERIALS AND METHODS

Cell cultures

Adult epidermal keratinocytes were isolated from breast skin specimens from healthy female donors (age group 20–40 years) undergoing plastic surgery. The investigation was approved by the ethical committee for biomedical trials at the University of Szeged. Written informed consent was obtained from all donors, and the study was conducted according to the Principles of the Declaration of Helsinki. To separate the dermis from the epidermis, overnight incubation in dispase solution (Grade II, Roche Molecular Biochemicals, Indianapolis, IN) was carried out at 4 °C. The epidermis was then incubated in 0.25% trypsin-EDTA (Sigma, St Louis, MO) for 20 minutes at 37 °C. The cells were seeded in keratinocyte serum-free medium (Gibco BRL, Eggstein, Germany). The medium was changed every 2 or 3 days. The cultures were maintained at 37 °C, under a humidified atmosphere, containing 5% CO_2.

Cell treatment

On the attainment of approximately 80% confluence after the third passage, the cultured human keratinocytes were treated with SP, CGRP, VIP, or GAL at 10^{-8} M for 30 minutes. For control purposes, cells were handled as mentioned above, but without neuropeptide induction. Total RNA was isolated 3, 6 12, and 24 hours after neuropeptide treatment. Cell lysates were collected 24, 48, and 72 hours after neuropeptide treatment.

Reverse transcription-PCR and semiquantitative real-time PCR

Total RNA was isolated from cultures by using the TRIzol reagent method (Invitrogen, Carlsbad, CA), as described by the manufacturer. One microgram of total RNA was reverse-transcribed with the iScript complementary DNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. After reverse transcription, amplifications were carried out with an iCycler machine (iCycler IQ Real-Time PCR, Bio-Rad Laboratories). To detect the presence of sortilin receptor mRNA and of the endogenous control 18S ribosomal RNA, Human Sortilin TaqMan Gene Expression Assays (reference numbers Hs00907094_m1 and Hs03003631_g1) (Applera Hungary, Budapest, Hungary) were used. After amplification, 15 µl of PCR products was run on 1.5% agarose gel, stained with ethidium bromide, photographed, and evaluated with Kodak ID Digital Science software (Scientific Imaging Systems, New Haven, CT). Each of the PCR reactions was carried out in duplicate. The amount of complementary DNA was calculated on the basis of the threshold cycle (C_T) value, and was standardized through the amount of housekeeping gene (18S ribosomal RNA) by means of the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

Western blotting

Cultured normal human epidermal keratinocytes and human brain tissue from a cadaver donor were lysed in 1 ml of lysis buffer containing 62.5 mM TRIS (pH 6.8), 5 mM EDTA, 1.5% SDS, 5% β-mercaptoethanol, and 0.2% proteinase inhibitor (Sigma). After separation by electrophoresis on 10% SDS, polyacrylamide gel

proteins were transfered to blotting membrane (Bio-Rad Laboratories). Rabbit polyclonal antibody against human sortilin (Abcam, Cambridge, UK) was used at a dilution of 1:50 in 2% skim milk in Tris-buffered saline (TBS), as primary antibody. Next day, the membrane was washed in TBS containing 0.5% Tween 20 (TTBS) and incubated for 75 minutes with anti-rabbit IgG-conjugated alkaline phosphatase (Sigma) at a dilution of 1:2,000. After extensive washing, the alkaline phosphatase activity was detected with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma) as substrate. The specificity of the antibody used against sortilin was verified by preabsorbing the antibody with a 5-fold excess concentration of blocking peptide (Abcam) for 4 hours before addition to the blot. To check on the equal loading of the total protein in all lanes, blots were developed with a 1:100 dilution of rabbit polyclonal anti-actin antibody (Sigma). Otherwise, the conditions were as described above. The results of the western blotting for sortilin and actin expressions of each sample were filmed with a Kodak DC290 Zoom Digital Camera (Scientific Imaging System) and were quantitated densitometrically with Kodak 1D Digital Science software (Scientific Imaging System).

Immunocytochemistry and immunohistochemistry

Cells were either grown on culture slides or harvested by trypsinization for cytospin preparation. For cytospins, after centrifugation the cell pellet was resuspended in phosphate-buffered saline and the cell concentration was set at 1×10^6 cells ml^{-1}. In all, 100 µl of cell suspension was placed into plastic tubes, cytocentrifuged onto glass slides (Cytopro, Wescor, Logan, UT), and then dried overnight at 25 °C. Cultured slides were fixed in 2% paraformaldehyde for 20 minutes, and cytospin air-dried slides and frozen sections from normal human skin were fixed in ice-cold acetone for 10 minutes. Nonspecific binding was blocked with goat serum (Vectastain ABC Kit, Vector, Burlingame, CA) diluted in TBS-T (Tris-buffered saline containing 0.1% Triton-X), following the manufacturer's instructions. Slides were incubated with rabbit primary antibodies specific for human sortilin (Abcam) at a dilution of 1:400 in a staining solution containing 0.5% BSA (Sigma) and goat serum in TBS-T. Control slides were incubated with rabbit IgG (NeoMarkers, Fremont, CA) as an isotype control. Endogenous peroxidase activity was removed by treatment with 0.3% H_2O_2/methanol for 20 minutes. Slides were then incubated with a biotinylated secondary antibody for 1 hour at room temperature, followed by incubation with horseradish peroxidase-conjugated streptavidin for 1 hour at room temperature and finally peroxidase activity was detected by using 3,3-amino-9-ethylcarbazole (Sigma) as a substrate. Slides were counterstained with hematoxylin. The cells and sections were visualized with a Zeiss Axio Imager microscope (Carl Zeiss MicroImaging, Thornwood, NY) and photographed with a PixeLINK digital camera (PixeLINK, Ottawa, ON, Canada).

Evaluation of keratinocyte apoptosis by DNA fragmentation and Annexin V assay

Apoptosis in human keratinocytes was detected by using an *in situ* apoptosis detection kit (TiterTacs *In situ* Apoptosis Detection Kit, R&D Systems, Minneapolis, MN). Briefly, normal human keratinocytes were cultured in keratinocyte serum-free medium in 96-well plates (6,000 cells per well). After 24 hours, the cells were induced with proNGF (100 ng ml^{-1}) as an apoptosis-inducing factor, together with a combination of proNGF and GAL, VIP, SP, or CGRP. Each of the neuropeptides was used at a concentration of 10^{-8} M. Cells left without treatment served as control. After 48 hours, the extent of the apoptosis was determined according to the manufacturer's instructions. All reactions were repeated at least six times. Annexin V assay was performed by using FITC Annexin V and propidium iodide (both from BD Biosciences, San José) according to the manufacturer's instructions. Cells were treated with proNGF and neuropeptides in a 24-well plate (5×10^4 cells per well) by the above-detailed method; after a 6-hours incubation the fluorescence of apoptotic cells was detected with a FACSCalibur and analyzed with CellQuest Software (BD Biosciences).

Statistical analysis

The data were collected from at least three independent experiments. Quantitative data are expressed as means ± SD. Statistical significance was determined by using one-way analysis of variance followed by Dunnett's and Tukey's *post hoc* tests (SPSS, Chicago, IL). Differences were considered to be statistically significant when $P<0.05$.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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