SPRR4, a novel cornified envelope precursor: UV-dependent epidermal expression and selective incorporation into fragile envelopes

Adriana Cabral[1], Arzu Sayin[1], Sandrine de Winter[2], David F. Fischer[1], Stan Pavel[2] and Claude Backendorf[1,*]

[1]Department of Molecular Genetics, Leiden Institute of Chemistry, Leiden University, PO Box 9502, 2300 RA Leiden, The Netherlands
[2]Department of Dermatology, Leiden University Medical Centre, The Netherlands
*Author for correspondence (e-mail: backendo@chem.leidenuniv.nl)

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SUMMARY

The cornified cell envelope (CE), a structure formed in the outermost layers of stratified squamous epithelia, provides a physical barrier against environmental insults. It is composed of several structural proteins, which are irreversibly crosslinked by calcium-activated transglutaminases. The small proline rich proteins (SPRRs) are one set of CE precursors. *SPRR4*, a novel member of this gene family, displayed very low or undetectable expression levels in normal human skin or other stratified squamous epithelia, but was clearly induced by UV light both in vivo and in vitro. High epidermal expression of SPRR4 was monitored only after chronic UV exposure and was concomitant with a thickening of the stratum corneum, which is believed to provide protection against subsequent damage. The calcium-dependent translocation of an SPRR4-GFP fusion protein to the cell periphery in living keratinocytes and its integration into both rigid and fragile cornified envelopes proved that SPRR4 is a novel CE precursor. Interestingly, after UV irradiation, SPRR4 was selectively incorporated into fragile CEs. Our results show for the first time that UV-induced cornification is accompanied by qualitative changes in CE precursor assembly. SPRR4 is part of an adaptive tissue response to environmental stress, which is likely to compensate for UV induced impairment of the epidermal barrier function.

Key words: Small proline rich protein, UV light, Adaptive tissue response, Compensatory mechanisms, Rigid/fragile cornified envelope

INTRODUCTION

The epidermis provides an efficient barrier against a large variety of environmental hazards. Keratinocytes, the major epidermal cell type, are committed to a process of terminal differentiation, which results in the multilayer structure of the skin. The cornified cell envelope (CE), a structure formed beneath the plasma membrane of terminally differentiated cells, is the major element responsible for the protective function of the skin (Hohl, 1990; Matoltsy and Matoltsy, 1966; Rice and Green, 1977). The CE assembly occurs as an orchestrated sequence of events, in which structural proteins are irreversibly crosslinked by calcium-dependent transglutaminases, resulting in a highly insoluble 15 nm-thick layer (Candi et al., 1995; Martinet et al., 1988). Biochemical studies have identified several CE precursor proteins, including cystatin α, desmoplakin, elafin, envoplakin, filaggrin, involucrin, keratins, loricrin and the small proline rich proteins (SPRRs) (Steinert and Marekov, 1999).

The human *SPRR* genes consist of a multigene family, previously mapped to a 170 kb region on human chromosome 1q21, within the epidermal differentiation complex (EDC) (Cabral et al., 2001; Gibbs et al., 1993; South et al., 1999). The genomic structure of all *SPRRs* is characterized by a short first exon and an exon 2 comprising the complete open reading frame. The head and tail domains of the SPRR proteins are highly homologous among all family members and other CE precursors (e.g. involucrin and loricrin) (Backendorf and Hohl, 1992). The distinction among the different SPRRs relies on the number and consensus sequence of the internal repetitive units (Gibbs et al., 1993). Direct sequencing of peptides recovered from CE proteolysis revealed that glutamine and lysine residues in the head and tail domains of the SPRRs are crosslinked during CE assembly (Steinert and Marekov, 1995). The CE formed at different body sites varies in protein content. While loricrin is the major component (80%) in CEs derived from normal epidermal tissues, SPRR1 constitutes 60-70% of oral epithelia (Lee et al., 2000). It has been proposed that the variable amounts of SPRRs in different epithelia affect the structure of the CE and consequently alter the biomechanical properties of the tissue, in accordance with the specific barrier function requirements (Candi et al., 1999; Steinert et al., 1998a; Steinert et al., 1998b; Tarcsa et al., 1998). In this paper, we describe the isolation and characterization of a new member of the *SPRR* gene family, whose expression is restricted to cells and tissues exposed to UV radiation, suggesting that this specific gene, *SPRR4*, is part of an adaptive response to environmental stress.

MATERIALS AND METHODS

Identification, cloning and mapping the *SPRR4* gene

A human expressed sequence tag (EST) homologous to the *SPRR1*

coding region (Gibbs et al., 1993) was identified using the BLASTn program (NCBI) and cloned from epidermal RNA in pBluescriptSK(–) (Stratagene), using PCR primers GGGAATTCAC-CTGTTCCTAGAGCAATGT (sense) and GGGCTCGAGAGCATC-GGGGTGGGATA (antisense). A gridded flow-sorted chromosome 1 cosmid library was used to identify a *SPRR4*-positive clone (ICRFc112k0695) (Cabral et al., 2001), which was completely digested with *Eco*RI and hybridized with *SPRR4* (cDNA and genomic probes) for mapping purposes.

Cell culture

Primary cultures of human epidermal keratinocytes were established from foreskin and cultured in the presence of lethally γ-irradiated 3T3 cells. Confluent passage three cultures grown in complete medium were incubated for 2 days in strip medium (Fischer et al., 1996), which resulted in the detachment of the differentiated cells. The remaining monolayers were either sham-irradiated or irradiated with 500 J/m^2 UV-A, 300 J/m^2 of UV-B or 30 J/m^2 of UV-C, and incubated for 24-48 hours in complete medium without growth factors.

Generation of an SPRR4-GFP construct, transient transfection and CE isolation

The *SPRR4* cDNA was amplified by PCR using the primers GGGTCCGGAGGTGGCATGTCTTCCCAGC (sense) and GTAAT-CTGCAGTCCATCCTTACTTCTG (antisense) and cloned into pEGFP-C1 (Clontech), a eukaryotic expression vector with an enhanced green fluorescent protein (GFP). In the resulting construct a hinge region with three consecutive glycine residues was introduced between GFP and SPRR4. Transient transfection experiments were performed either with the SPRR4-GFP construct or with the pEGFP-C1 vector. Monolayers of normal human keratinocytes (see above) were transfected using DOTAP (Boehringer Mannheim). Cells were either maintained in strip medium or induced to stratify for 40 hours in DMEM supplemented with 5% serum (1.8 mM Ca^{2+}). Cells were examined using a laser scanning confocal microscope (Leica SP DM IRBE). For CE isolation, transfected cells were maintained in high calcium medium for 24 hours and incubated with 25 μM of the calcium ionophore A23187 (Sigma) for an additional 48 hours. UV-C irradiation was performed 24 hours after transfection, just prior to ionophore treatment. The cultures were washed with PBS, treated with lysis buffer (2% SDS, 0.1 M Tris-HCl pH 7.5, 1 mM EDTA, 10 mM DTT) and incubated at room temperature for 10 minutes. CEs were analysed under a fluorescence microscope (Zeiss Axiovert 135). CE morphology (rigid, fragile) was assessed either by Nomarski or phase contrast microscopy (Michel et al., 1988) or by Nile Red staining (Hirao et al., 2001).

Northern blot analyses

Total RNA (20 μg/lane) was separated on a 1% denaturing agarose gel and transferred to Genescreen (Biotech Systems, Dupont) and hybridized with the above mentioned *SPRR4* cDNA probe, *SPRR1*, *-2*, or *-3*-specific probes (Gibbs et al., 1993), a 645 bp *Pst*I fragment derived from the modern repeat region of human *involucrin* (a gift from P. Djian, Paris), or with a *28S* rRNA probe (loading control). Human normal tissue blots (Northern Territory I, II, III) were purchased from Invitrogen.

RT-PCR

Total epidermal RNA was either isolated from tissue obtained after breast reduction or purchased from Invitrogen. Samples were treated with 20 Units of RNase-free DNaseI (Roche) for 30 minutes at room temperature and phenol:chloroform extracted. Total RNA (400 ng) was reverse-transcribed with Super-RT (SphaeroQ) using random hexamer primers (Amersham Pharmacia Biotech). PCR was performed for 35 cycles with *SPRR4*-specific primers (forward: GGTCCAGCTTGTCGCCT; reverse: GGCTCAAGAGCTGGAGG), *involucrin* (forward: TCCTCCAGTCAATACCCATC; reverse: CTTCATTCCCAGTTGCTCATC) and *GAPDH* (forward: CGGAG-TCAACGGATTTGGTCGTAT; reverse: AGCCTTCTCCATGGTGG-TGAAGAC), which generated fragments of 371 bp, 373 bp and 307 bp, respectively. Products were visualized on a 1.5% agarose gel.

Skin specimens and in vivo UV irradiation

Two different UV treatments were performed, each with two Caucasian volunteers with either fairly light (phototype II) or darker skin (phototype III). A sunbed equipped with Cleo Natural® UV lamps (Philips), simulating the spectrum of sunlight, was used as a light source. In a short-term experiment, subjects were exposed to a single dose of 1.2 minimal erythema dose (MED), and biopsies were taken 24 and 48 hours after the irradiation. In a long-term experiment, individuals received irradiations three times a week (Monday, Wednesday and Friday) for 3 weeks. The first dose was 0.5 MED, the second 1 MED, followed by dose increments of 20%. These exposures caused in both individuals a fourfold increase in MED values. Punch biopsies were taken before and after the irradiation on the buttocks or lower back. In all cases the biopsies were taken under local anaesthesia using solutions containing 2% xylocaine and 12.5 μg/ml adrenaline. Specimens were fixed in 4% formaldehyde for 6 hours, dehydrated and embedded in paraffin.

In situ hybridization

Tissue sections were deparaffinized, washed with PBS and incubated with proteinase K (20 μg/ml) for 15 minutes at 37ºC followed by 0.2% glycine treatment. Overnight incubation at 62ºC with digoxigenin-labeled riboprobes was followed by detection with the DIG-Detection-Kit (Boehringer Mannheim) according to the manufacturer's recommendations. Sense and antisense RNA probes were generated using either *SPRR4* (470 bp) or *SPRR2* (680 bp) cDNAs cloned in pBluescriptSK(–). No cross-hybridization of the *SPRR4* probe with any other *SPRR* gene on the previously described cosmid contig (Cabral et al., 2001; South et al., 1999) was detected.

RESULTS AND DISCUSSION

Identification and characterization of the human SPRR4 gene and protein

The human EST database at NCBI was searched for sequences homologous to the *SPRR1* coding sequence. An EST (accession no. W77994), originating from a human fetal heart cDNA library, encoded a putative member of the *SPRR* gene family. This EST was cloned from epidermal RNA and a genomic clone was obtained by screening a 1q21 cosmid contig (South et al., 1999) with the cDNA probe (underlined in Fig. 1B). The chromosomal localization at the proximal end of the *SPRR* cluster, between *SPRR1A* and *involucrin* (Fig. 1A), strongly suggested that this gene is a novel member of the *SPRR* gene family (Cabral et al., 2001). This assumption was further supported by the genomic organization and the deduced amino acid sequence of the *SPRR4* gene as shown in Fig. 1B. Comparison of the cDNA and the genomic clone revealed the presence of two exons separated by an intron of 1173 bp. Although the first exon of *SPRR4* is very short (43 bp) and contains most of the 5′ untranslated region, exon 2 comprises the entire open reading frame. A similar genomic organization is found in all other members of the *SPRR* gene family (Gibbs et al., 1993), as well as in other genes encoding cornified envelope precursors, such as *involucrin* and *loricrin*. The unique open reading frame encodes a 79 amino acid protein, rich in glutamine (29%), proline (16%) and lysine (13%) residues. Among all described SPRR proteins, SPRR4 has the

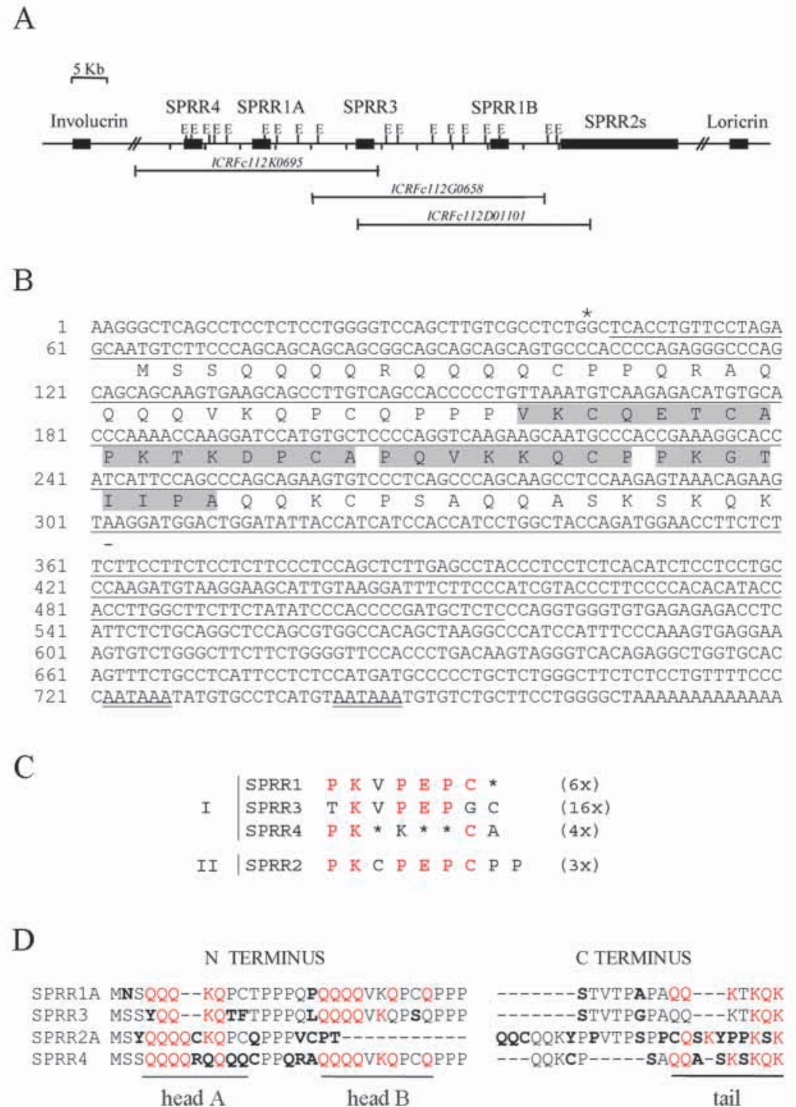

Fig. 1. Molecular characterization of the *SPRR4* gene and protein. (A) Physical map of the *SPRR4* gene in the *SPRR* locus. Three overlapping cosmid clones (South et al., 1999) are represented. Black boxes indicate either the genomic sequences of individual genes or the *SPRR2* cluster as a whole. *Eco*RI restriction sites (E) are indicated. (B) Nucleotide and deduced amino acid sequence of the *SPRR4* gene. The sequence used as a probe is underlined, and the position of the single intron is indicated by an asterisk. Grey boxes indicate the four internal octapeptide repeats. Putative polyadenylation sites are double underlined. GenBank accession number AF335109. (C) Comparison of internal repeat consensi from the two groups (I/II) and four classes of human SPRR proteins (Cabral et al., 2001). The number of repetitive units for each class is represented between brackets. Amino acids conserved in three out of four classes are in red. (D) Amino acid sequence of N- and C-terminal SPRR domains. Differences between various classes are in bold. Amino acids involved in the transglutaminase mediated crosslinking reaction in SPRR1, -2 or -3, and conserved in SPRR4, are in red.

highest glutamine content (SPRR1A: 20.2%; SPRR2A: 16.7%; SPRR3: 10.1%). In contrast to involucrin, which contains a high number of glutamines in the central repetitive region (Eckert and Green, 1986), the majority (65.2%) of these residues in SPRR4 was found in the N-terminal domain (especially the head A region) (Fig. 1D). The SPRR4 protein has a central region of short tandem repeats (Fig. 1B), which are specific for each SPRR class (Gibbs et al., 1993). In the case of SPRR4, the octamer PK*K**CA is present four times. This repetitive unit is more degenerate than the corresponding domain in other SPRRs (Fig. 1C). In addition, the PEP sequence present in all other SPRRs is not found in SPRR4, indicating that this gene has diverged at an earlier stage from a common SPRR ancestor (Backendorf and Hohl, 1992). It seems plausible that, during evolution, SPRR4 was not subjected to gene conversion, which has resulted in the homogenization of the other (especially SPRR2) family members (Cabral et al., 2001). Contrasting with the divergence in the internal repetitive domain, the N- and C-termini of SPRR4 and other SPRRs are highly homologous (Fig. 1D). Biochemical studies have identified head A, head B and tail domains which harbour amino acids (Q,K) involved in transglutaminase-mediated crosslinking in SPRR1, -2 and -3 (Candi et al., 2000; Candi et al., 1999; Tarcsa et al., 1998). Notably, these functional amino acids (represented in red in Fig. 1D) are highly conserved in SPRR4, which directly suggests an involvement of this protein in cornified envelope assembly. Additionally, the presence of head A and head B domains suggests that SPRR4 might be crosslinked by both TGase 3 and TGase 1, respectively (Candi et al., 1999).

To investigate a possible function of SPRR4 in CE assembly, monolayers of basal keratinocytes were transfected with an SPRR4-GFP construct. Cultures were either maintained in medium without calcium or induced to differentiate in medium containing 1.8 mM Ca^{2+}. This experiment allowed the monitoring of SPRR4 in living cells. While the fused protein was dispersed in the cytoplasm in medium without calcium (Fig. 2A, panel 1), a clear translocation to the cell periphery was observed in 1.8 mM Ca^{2+} (panel 2). Such a pattern was not displayed by the control GFP protein (panels 3,4). Interestingly, all cells expressing the SPRR4-GFP fusion protein at the cell periphery were found suprabasally, indicating their commitment to terminal differentiation (panel 2, middle). Such a subcellular location can be expected for a cornified envelope precursor protein, since CE assembly is catalyzed at the cell membrane by calcium-dependent transglutaminases (Thacher and Rice, 1985). In order to prove that the fusion protein was actually incorporated into CEs, we purified these structures by incubating transfected and ionophore-treated cells in 2% SDS and 10 mM DTT. Fluorescent CEs could be identified only in extracts from cells expressing the SPRR4-GFP construct (Fig. 2B), but not in lysates from GFP control cells (not shown). Note that SPRR4 was incorporated into both fragile and rigid CEs (Michel et al., 1988) (Fig. 2B; Table 1). All evidence presented indicates that *SPRR4* is a new member of the *SPRR* gene family, which functions as a cornified envelope precursor.

Expression of SPRR4 transcripts in vivo and in vitro

Commercial blots (Invitrogen) containing total RNA from normal human tissues were hybridized with a *SPRR4* cDNA probe. No expression was found in 24 different RNA samples, derived from esophagus, uterus, bladder, stomach, lung, prostate, testis, ovary, heart and brain, among others (data not shown). Similarly, no *SPRR4* expression could be monitored by a more sensitive RT-PCR analysis of RNA isolated from both stratified squamous epithelia (esophagus, cervix), which expressed high levels of all other SPRR members. Two non-squamous tissues (uterus, ovary), which were positive for several SPRRs (SPRR1A, SPRR2B, 2D, 2E, 2F, SPRR3), were also clearly negative for SPRR4 (Cabral et al., 2001). In Fig. 3A, RT-PCR experiments revealed variable *SPRR4* expression levels in seven epidermal RNAs and four primary human keratinocyte cultures, ranging from very low to undetectable levels (compare with the expression of *involucrin*). A similar picture emerged from in situ hybridization experiments, performed on seven additional Caucasian skin samples (Fig. 3B). Whereas *SPRR4* transcripts were below the detection level

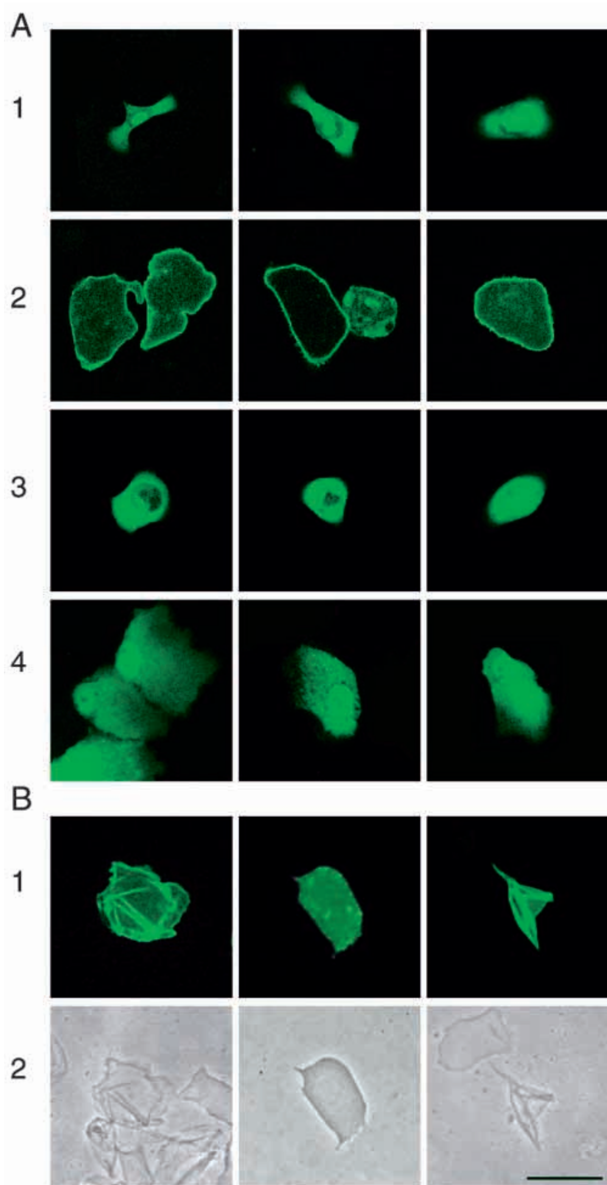

Fig. 2. SPRR4 is a CE precursor protein. (A) Subcellular location of SPRR4-GFP and GFP in living cells. Monolayers of normal human keratinocytes were transfected with either SPRR4-GFP (1,2) or GFP (3,4), as a control. Cultures were either maintained in medium without calcium (1,3) or induced to differentiate for 40 hours in 1.8 mM Ca^{2+} (2,4). Cells were analyzed with a laser scanning confocal microscope. (B) Isolation of cornified envelopes from keratinocytes transfected with SPRR4-GFP. Transfected cells were incubated for 48 hours with A23187 ionophore, treated with 2% SDS and 10 mM DTT and examined using a fluorescence microscope (1, fluorescence; 2, phase-contrast). The middle image in panels 1 and 2 shows a rigid envelope and the flanking images show fragile envelopes. Bar, 15 μm.

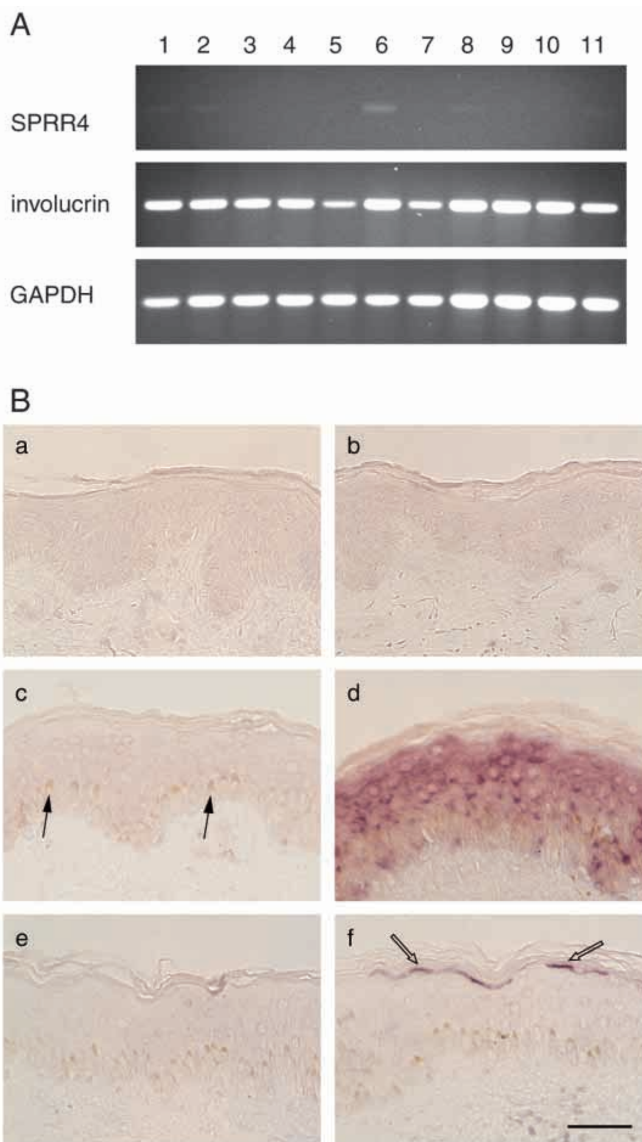

Fig. 3. In vivo screening for *SPRR4* expression. (A) RT-PCR analysis of *SPRR4* expression. Epidermal RNA (lanes 1-7) and RNA isolated from primary cultured keratinocytes (lanes 8-11) were analyzed with *SPRR4*, *involucrin* and *GAPDH*-specific primers. (B) In situ hybridization performed on different skin samples. Sense (a,c,e) and antisense (b,d,f) probes from either *SPRR4* (a-d) and *SPRR2* (e,f) were analyzed. Melanin caps (c, filled arrows) and SPRR2-positive staining (f, open arrows) are indicated. Bar, 30 μm.

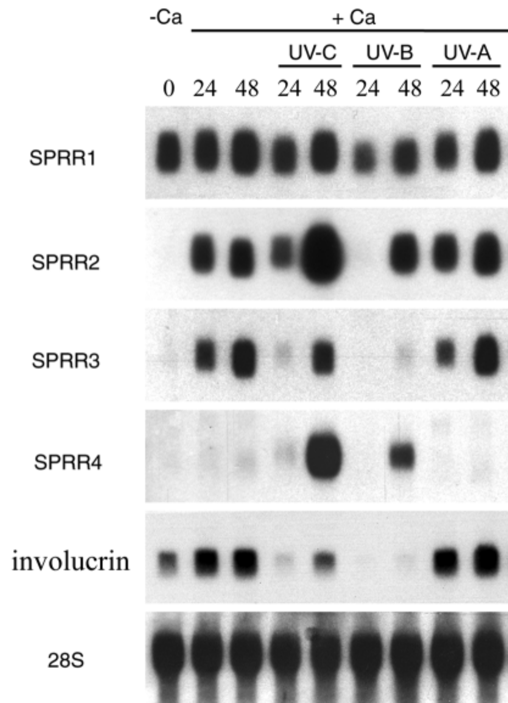

Fig. 4. Influence of calcium-induced differentiation and UV irradiation on *SPRR* expression in vitro. Normal human keratinocytes (NHK) were cultured in complete medium to confluency and subsequently incubated with calcium-free medium to remove differentiated cells. Cells were either irradiated with 500 J/m^2 UV-A, 300 J/m^2 UV-B or 30 J/m^2 UV-C, or mock irradiated, and maintained for 24 or 48 hours in medium containing 1.8 mM Ca^{2+}. Total RNA was isolated from the different cultures and hybridized with either *SPRR* class specific, *involucrin*, or *28S* ribosomal RNA probes.

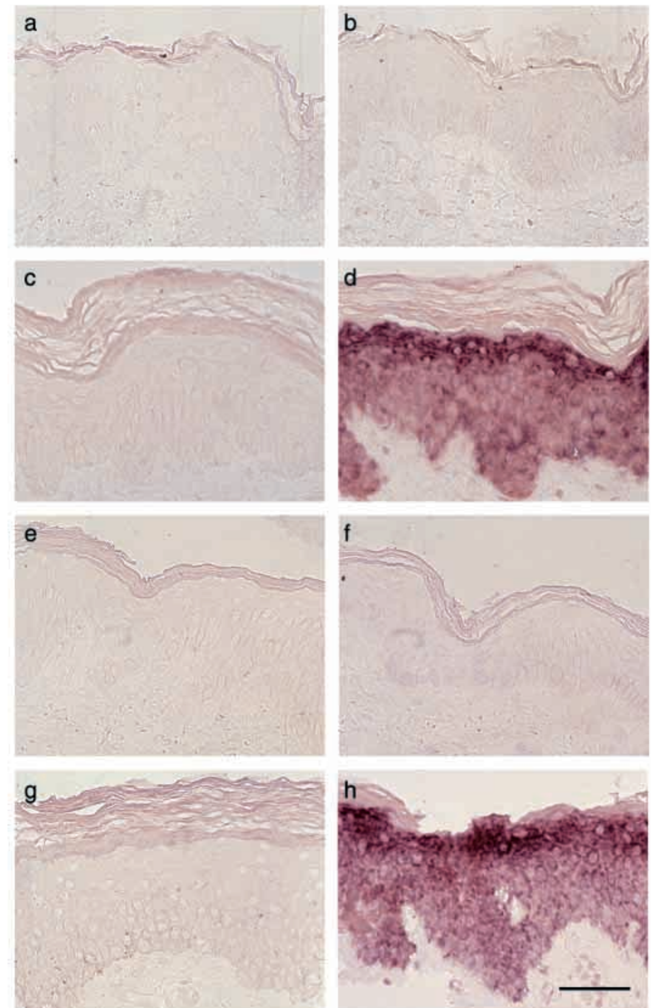

Fig. 5. In vivo *SPRR4* expression after chronic UV exposure. In situ hybridization was performed with *SPRR4* sense (a,c,e,g) and antisense (b,d,f,h) probes. Results for chronic exposure from phototype II (a-d) and phototype III (e-h) volunteers, before (a,b,e,f) and after (c,d,g,h) the UV treatment are represented. Bar, 30 μm.

in 6 samples (Fig. 3Ba,b), one biopsy revealed high *SPRR4* expression, which was preferentially located in the spinous and granular layers (Fig. 3Bc,d). The same skin sample was hybridized with a *SPRR2* riboprobe (Fig. 3Be,f), which revealed an overtly different expression pattern, with a weak, patchy staining restricted to the granular layer (open arrow), as previously documented (Hohl et al., 1995). This particular skin biopsy was characterized by a high content of melanin caps (filled arrows), suggestive of prior solar exposure. Indeed, after UV exposure, melanocyte dendricity is increased, which results in high melanin contents in keratinocytes (Hara et al., 1995). This pigment is often located over the nuclei and forms supranuclear melanin caps, which protect from incident UV rays (Kobayashi et al., 1998). To investigate a possible involvement of UV light in *SPRR4* expression, normal human keratinocytes were cultured under stratifying conditions and irradiated with either UV-A, UV-B or UV-C light and analysed on northern blots (Fig. 4). While *SPRR1*, *-2*, *-3* and *involucrin* showed increasing expression levels following calcium-induced differentiation, *SPRR4* levels were hardly detectable under these experimental conditions (in accordance with the RT-PCR analysis of Fig. 3A). However, a drastic increase in *SPRR4* mRNA levels was seen 48 hours after exposure to either UV-B or UV-C. *SPRR2* was also induced after UV-C irradiation, in agreement with our previously published results (Gibbs et al., 1990), but it clearly differed from *SPRR4* by high expression in unirradiated differentiating cultures. None of the other genes (*SPRR1*, *-3* and *involucrin*) were induced after UV irradiation. Intriguingly, *SPRR3* and *involucrin* expression were strongly downregulated, especially after UV-B treatment, indicating that various CE precursors can be differentially modulated by UV exposure. UV-A did not affect the expression of any of the analysed genes. This experiment revealed a characteristic property of *SPRR4* expression: the absence of calcium responsiveness and a complete dependence on the pretreatment of cells with UV-B or UV-C light. Consequently, we questioned whether the same regulation is also operative in vivo.

Volunteers with either skin phototype II or III were exposed to different UV treatments. In the first experiment, individuals were submitted to chronic UV exposure (nine irradiations over a three-week period). Punch biopsies were taken before and after the treatment and analyzed by in situ hybridization (Fig. 5a-h). Note that UV exposure resulted in the thickening of the stratum corneum (Fig. 5c,d,g, h). In contrast to the unirradiated controls (Fig. 5b,f), *SPRR4* transcripts were strongly induced

Table 1. Influence of UV-C irradiation on the distribution of fragile (F) versus rigid (R) CEs in cultured ionophore-treated keratinocytes

UV dose (J/m^2)	Total population (%) F	Total population (%) R	SPRR4$^+$ (%) F	SPRR4$^+$ (%) R	P*	n‡
0	49	51	60	40	<0.2	309
5	71	29	80	20	<0.2	317
10	75	25	90	10	<0.01	191
15	68	32	82	18	<0.025	288

*Probability value (Chi-squared test) for the difference in fragile/rigid distribution between the total and the SPRR4$^+$ population. The increase of fragile envelopes in the total population was significant ($P<0.01$). P values <0.05 were considered to be significant.

‡Total amount of CEs counted per experiment.

after treatment in both light (Fig. 5d) and darker (Fig. 5h) skin phototypes. For both samples, moderate levels in the basal and lower spinous layer contrasted with strong expression in the upper spinous and granular layers. This preferentially suprabasal expression pattern is in line with our finding that SPRR4 functions as a CE precursor. We were not able to detect SPRR2 expression in these specimens, probably due to the previously reported low and patchy epidermal expression pattern of this family member (Hohl et al., 1995) (see also Fig. 3B). In the second experiment, phototype II and III volunteers were submitted to an acute UV treatment, which consisted of a single 1.2 MED exposure. Biopsies were taken before, as well as 24 and 48 hours after the exposure. No *SPRR4* expression was observed in any of the two skin types (results not shown), indicating that a single UV exposure was not sufficient for *SPRR4* induction in vivo. Apparently, *SPRR4* induction reflects a long-term adaptation of the cornified cell envelope to external insults. Different skin phototypes, which were submitted to the same stress conditions (identical MED), responded similarly (compare Fig. 5d and 5h), suggesting that *SPRR4* induction is directly related to the damaging effect of UV exposure, and not to the degree of pigmentation, which is characteristic for each skin phototype and a major determinant of UV sensitivity (Lock-Andersen et al., 1997). Such a view is also in line with our in vitro experiments (Fig. 4), where *SPRR4* induction correlates with the damaging ability of the UV wavelength used (UV-C > UV-B > UV-A). It is worth noting that while the other *SPRR* genes are restricted to the uppermost layers of different squamous epithelia, indicating strict differentiation-dependent expression (Fig. 3f) (Hohl et al., 1995), *SPRR4* revealed a gradual increase in expression from the basal to the granular layer. This characteristic pattern suggests that UV-induced stress is the major stimulus for *SPRR4* expression, which might be modulated by the differentiation state of the cell. Conversely, the observed gradient in *SPRR4* expression might simply reflect higher stress in more superficial living layers. The absence of calcium responsiveness in vitro (an inducing signal for keratinocyte terminal differentiation) (Fig. 4) underlines the distinct regulation pattern of this new member of the SPRR gene family.

SPRR4 and keratinocyte cornification after UV irradiation

An interesting observation is that *SPRR4* induction after chronic UV exposure correlated with a clear thickening of the stratum corneum (Fig. 5). It is generally believed that increased keratinocyte cornification after UV exposure has a protective role, as it functions, together with enhanced pigmentation, as a shield against subsequent damage (Dissanayake and Mason, 1998; Fitzpatrick, 1986). In order to investigate a possible link between UV-induced modulation of keratinocyte cornification and SPRR4 incorporation into CEs, we have transfected cultured human keratinocytes with the SPRR4-GFP construct, and irradiated the cells with 0, 5, 10 or 15 J/m^2 of UV-C, just prior to ionophore treatment (see Materials and Methods). CEs were isolated 2 days later, and analysed for SPRR4 fluorescence. The morphological properties of CEs were assessed both by Nomarski and phase contrast microscopy and by staining with Nile Red, a lipophilic dye. Rigid envelopes are polygonal, smooth (Nomarski), slightly opaque (phase-contrast) and react strongly with Nile Red. Conversely, fragile envelopes are irregular in shape, appear transparent under phase-contrast microscopy and react weakly with Nile Red (Hirao et al., 2001) (see also Fig. 2B). Results are compiled in Table 1 and lead to several conclusions. First, the fraction of fragile CEs increased significantly with UV-C irradiation ($P<0.01$ for each UV dose). Fragile CEs are generally believed to be indicative of barrier function impairment (Hirao et al., 2001), which has also been observed in vivo after UV-B exposure, but in this case the permeability characteristics and lipid composition of CEs were studied (Haratake et al., 1997; Holleran et al., 1997). Second, the distribution of SPRR4 among fragile and rigid envelopes changes significantly after higher UV doses ($P<0.01$ and $P<0.025$ for 10 and 15 J/m^2, respectively) (Table 1). Apparently, SPRR4 is preferentially incorporated into fragile envelopes after UV damage. Third, it is not likely that crosslinking of SPRR4 is the cause of this destabilisation because in sham-irradiated cells the same protein is also found at high frequency (up to 40%) in rigid envelopes (Table 1). Selective incorporation of SPRR4 into fragile envelopes might have a compensatory function and counteract the impairment of the barrier, which is likely to be concomitant with UV-induced epidermal hyperplasia and the thickening of the stratum corneum (Haratake et al., 1997). At present it is not clear whether the increase in fragile envelopes, which we have observed, is directly linked to the alterations in barrier permeability described by others (Holleran et al., 1997). The finding that immature fragile envelopes are poorly reactive to the lipophylic dye Nile Red suggests that these envelopes are likely to differ in their covalently bound lipids. The UV-induced downregulation of involucrin (Fig. 4), which constitutes a major support for ceramide attachment via TGase-mediated esterification of glutamine residues (Nemes et al., 1999), could be a possible cause for this observation. Whether SPRR4 incorporation will help to restitute subsequent lipid crosslinking remains to be determined. At least the higher glutamine content of SPRR4 (see above), the documented increase in stratum corneum ceramides after UV irradiation (Wefers et al., 1991) and the observed reversibility of the UV-induced barrier disruption (Haratake et al., 1997) are in line with such a speculation. The recently described compensatory mechanisms in a loricrin-deficient mouse model (Koch et al., 2000), are likely to constitute an extreme example of the adaptive tissue responses described here (Cabral et al., 2001).

In conclusion, our research shows that a novel CE precursor protein, SPRR4, not expressed in skin under normal

conditions, is highly induced after UV irradiation both in vitro and in vivo, and is selectively incorporated into immature, fragile and poorly hydrophobic CEs. These qualitative changes in CE precursor incorporation are likely to improve the maintenance of skin integrity after UV exposure and/or prevent early desquamation. Apparently, SPRR4 is part of an adaptive tissue response that compensates for the transient disruption of the epidermal barrier function, which is inherent to UV-induced hyperproliferation and the generation of a protective thickened stratum corneum.

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