ROS Quenching Potential of the Epidermal Cornified Cell Envelope

Wilbert P. Vermeij[1,3], A. Alia[2] and Claude Backendorf[1]

The cornified cell envelope (CE) is a specialized structure assembled beneath the plasma membrane of keratinocytes in the outermost layers of the epidermis. It is essential for the physical and permeability properties of the barrier function of the skin. Our skin is continuously exposed to atmospheric oxygen and threatened by reactive oxygen species (ROS). Here, we identify the CE as a first line of antioxidant defense and show that the small proline-rich (SPRR) family of CE precursor proteins have a major role in ROS detoxification. Cysteine residues within these proteins are responsible for ROS quenching, resulting in inter- and intramolecular S–S bond formation, both in isolated proteins and purified CEs. The related keratinocyte proline-rich protein is also oxidized on several cysteine residues within the CE. Differences in antioxidant potential between various SPRR family members are likely determined by structural differences rather than by the amount of cysteine residues per protein. Loricrin, a major component of the CE with a higher cysteine content than SPRRs, is a weak ROS quencher and oxidized on a single cysteine residue within the CE. It is inferred that SPRR proteins provide the outermost layer of our skin with a highly adaptive and protective antioxidant shield.

Journal of Investigative Dermatology (2011) **131,** 1435–1441; doi:10.1038/jid.2010.433; published online 20 January 2011

INTRODUCTION

Reactive oxygen species (ROS) can have beneficial effects, as they operate as regulatory molecules in multiple intracellular signaling pathways, for instance as the first danger signal during wound healing to attract immune cells, or merely as chemical sterilizers in our host defense mechanism (Finkel and Holbrook, 2000; D'Autreaux and Toledano, 2007; Martin and Feng, 2009). Nevertheless, in general, ROS are considered as toxic compounds. In the mid-1950s, Denham Harman proposed ROS as essential determinants of the ageing process, as excessive ROS can damage lipids, proteins, and nucleic acids, leading to cellular dysfunction and death (Harman, 1956). More recently, altered ROS levels were implicated in diseases such as Alzheimer, Parkinson, atherosclerosis, rheumatoid arthritis, diabetes, psoriasis, cystic fibrosis, hypertension, ischemia, and cancer (Winyard *et al.*, 2005; Benz and Yau, 2008).

Of all tissues, our skin is exposed to the highest ROS levels. It is, besides the lungs and eyes, the only organ in direct contact with atmospheric oxygen, including air pollutants and the natural deleterious ozone gas. The skin has been shown, as early as 1851, to directly uptake oxygen via cutaneous respiration (von Gerlach, 1851). Skin also faces high levels of ROS that are induced during wound healing against invading bacteria (Martin and Feng, 2009). In addition, various types of ROS, such as the superoxide anion ($O_2^{\bullet}-$), the hydroxyl radical ($HO^{\bullet}$), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$) are generated following exposure to UV radiation derived from natural sunlight (Schallreuter and Wood, 1989; Poswig *et al.*, 1999). To cope with excessive ROS and to endow a protective antioxidant barrier, our skin has evolved several detoxification mechanisms (Shindo *et al.*, 1994; Kohen, 1999). These antioxidants can be classified into two major groups, enzymes and low-molecular-weight antioxidants. The low-molecular-weight antioxidant group contains compounds such as ascorbic acid (vitamin C), tocopherol (vitamin E), uric acid, glutathione, and ubiquinol, all capable of directly scavenging ROS (Kohen, 1999). The enzyme group contains superoxide dismutase, catalase, peroxidase, and glutathione reductase, which in turn can actively detoxify ROS (McCord and Fridovich, 1969; Schallreuter and Wood, 1989). Beside the two major antioxidant classes, other proteins function in this detoxification process, either by direct quenching or indirect regulation of signaling pathways that activate the antioxidant defense system (Winyard *et al.*, 2005; D'Autreaux and Toledano, 2007).

Many antioxidants are present at higher levels in the epidermis, as compared with the dermis, correlating with

[1]*Laboratory of Molecular Genetics, Faculty of Science, Leiden Institute of Chemistry, Leiden University, Leiden, The Netherlands and* [2]*Solid State NMR, Faculty of Science, Leiden Institute of Chemistry, Leiden University, Leiden, The Netherlands*

[3]*Current address: DNage BV, Rotterdam, The Netherlands*

Correspondence: Claude Backendorf, Laboratory of Molecular Genetics, Faculty of Science, Leiden Institute of Chemistry, Leiden University, Einsteinweg 55, Leiden 2333CC, The Netherlands.
E-mail: backendo@chem.leidenuniv.nl

Abbreviations: CE, cornified cell envelope; EDC, epidermal differentiation complex; NEM, N-ethylmaleimide; RB, Rose Bengal; ROS, reactive oxygen species; SPRR, small proline-rich protein

Received 9 August 2010; revised 14 December 2010; accepted 14 December 2010; published online 20 January 2011

decreasing ROS levels toward the inner layers of our skin (Shindo *et al.*, 1994). In fact, it was shown that the most external, cornified layer of the skin already provides sufficient antioxidant protection following a challenge with, for instance, ozone (Thiele *et al.*, 1997). Within the cornified layer, a specialized structure surrounding the terminally differentiated keratinocytes, namely the cornified cell envelope (CE), is responsible for the physical and permeability properties of the skin's innate barrier function (Kalinin *et al.*, 2002; Candi *et al.*, 2005). During the epidermal differentiation process, several proteins are expressed from the epidermal differentiation complex (EDC) localized on human chromosome 1q21 (for example, involucrin, loricrin, and the small proline-rich (SPRR) or LCE protein families; Cabral *et al.*, 2001b; Candi *et al.*, 2005). These cornified envelope precursor proteins contain highly similar head and tail domains, rich in lysine and glutamine, which are involved in transglutaminase-mediated crosslinking in the outermost layers of the skin (Backendorf and Hohl, 1992; Kalinin *et al.*, 2002). On crosslinking on the cell periphery, they form, together with lipids, the CE (Rice and Green, 1977; Nemes and Steinert, 1999).

We recently showed that SPRR proteins are able to detoxify ROS during wound healing (Vermeij and Backendorf, 2010). Interestingly, this novel function of SPRR is not only restricted to squamous epithelia. Indeed, on injury, SPRR protein expression massively increases at the edge of the wound in various types of tissues. This increase directly lowers the amount of ROS at the wounded site, and is essential to allow proper cell migration during the wound healing process. As SPRR proteins evolved together with all other EDC genes for their role in the assembly of the CE (Vanhoutteghem *et al.*, 2008), we inferred that the antioxidant potential of the SPRR proteins could also provide the skin with an antioxidant barrier. In this paper, we identify the CE as a first line of antioxidant defense, as it is able to directly quench ROS. We show that the SPRR family of CE precursor proteins, which were originally identified as UV-inducible genes (Kartasova and van de Putte, 1988), have a major role in ROS quenching, both *in vitro* and *in vivo*, mainly due to their cysteine residues.

RESULTS AND DISCUSSION

In the first instance, we examined the potency of purified CEs in ROS quenching. CEs from sunburned peeled skin were isolated, as previously described (Mehrel *et al.*, 1990), and measured with an in-house flash-photolysis setup, which is graphically represented in Figure 1a. With this technique, it is possible to quantitatively measure the time-resolved near-infrared luminescence of singlet oxygen, one of the major oxidizing species in skin (Kochevar, 2004). All reactions were performed in a glass cuvette with magnetic stirrer, containing Rose Bengal (RB), to generate singlet oxygen and D_2O, allowing a longer singlet oxygen lifetime (Keene *et al.*, 1986).

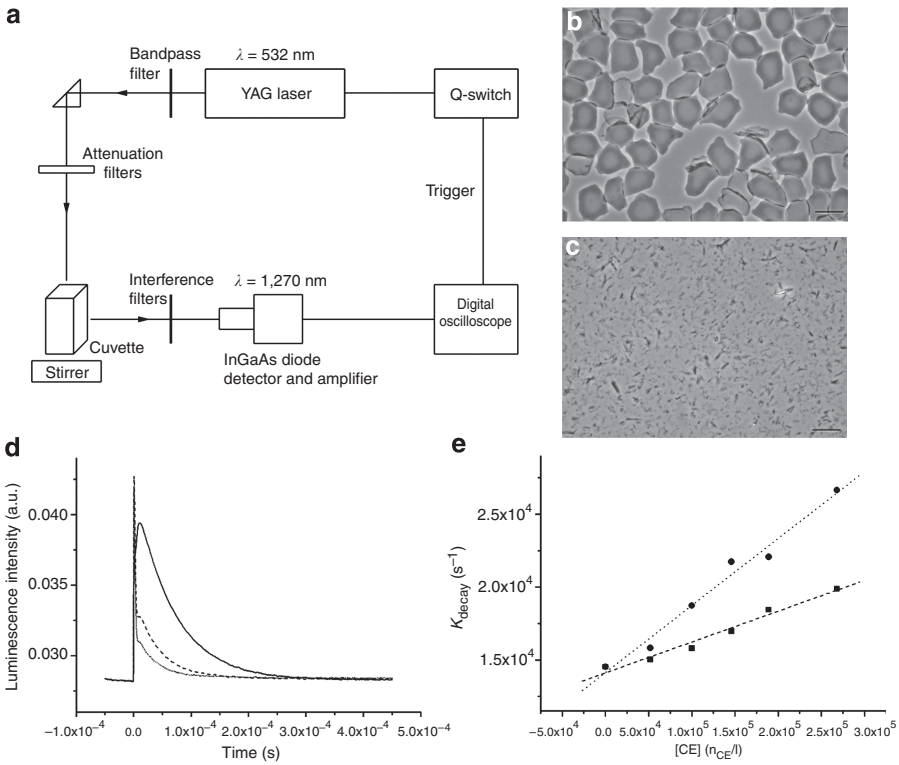

Figure 1. Flash-photolysis detection of the singlet oxygen quenching by cornified cell envelopes (CEs). (**a**) Graphical representation of the used flash-photolysis setup. Singlet oxygen is produced by laser excitation at 532 nm of the samples, containing D_2O and Rose Bengal as sensitizer. The subsequent decay of singlet oxygen is measured at 1,270 nm with a photodiode. (**b**, **c**) Photographs of purified, intact (**b**) and sonicated (**c**) CEs isolated from human skin. Bar = 15 μm. (**d**) Typical time profile of the luminescence of singlet oxygen (solid line), which is significantly reduced after addition of intact (dashed line) or sonicated CEs (dotted line). (**e**) Singlet oxygen decay (k_{decay}) plotted against increasing concentrations of intact (dashed line) or sonicated CEs (dotted line).

The advantage of this system is that the quenching potential of any compound, whether it is a purified protein, a living cell, or, in this case, isolated intact (Figure 1b) or sonicated CEs (Figure 1c), can be quantified. A typical time profile of the singlet oxygen luminescence in D_2O is shown in Figure 1d (solid line). Addition of intact (dashed line) or sonicated CEs (dotted line) both resulted in a substantial decrease in the singlet oxygen lifetime, indicating their direct involvement in ROS quenching. The rate constant of singlet oxygen decay, which can be calculated from the slopes in Figure 1e, is significantly increased in sonicated CEs (dotted line) as compared with intact ones (dashed line). The CE consists of a protein envelope coated by a lipid envelope (Hohl, 1990; Nemes and Steinert, 1999). Sonication is likely to result in a better accessibility of the internal proteinaceous CE components, suggesting that the protein part of the CE might be responsible for the antioxidant properties of the CE. It can, however, not be completely excluded that oxidation products, possibly generated during sonication, also contribute to the higher quenching ability of sonicated CEs. Analysis of CEs from different body sites has previously revealed that loricrin and the SPRR protein family together always comprise about 85–90% of the total CE protein mass, with relative molar ratios ranging from >100:1 in trunk epidermis to 5:1 in footpad epidermis and 3:1 in forestomach epithelium (Steinert *et al.*, 1998; Koch *et al.*, 2000).

To compare the individual antioxidant properties of these major CE precursor components *in vivo*, stable cell lines were established expressing loricrin (HFLor), SPRR1B (HF1B), SPRR2A (HF2A), SPRR3 (HF3), SPRR4 (HF4), or empty vector control (H24) in HeLa cells that do not express these proteins (our unpublished observation). It appeared that each of these ectopically expressed proteins potentiated the ROS quenching ability of the transfected cells (Table 1). The relative percentage of quenching was calculated by dividing the cellular quenching rate constants by the respective protein expression levels determined by western blotting. The highest effect was observed with cells expressing SPRR4 (set at 100%), followed by the other SPRR proteins (~50% efficiency) and loricrin, with a relative effect of 25%. To verify the differences in ROS quenching and relate them directly to the various proteins in question, the flash-photolysis measurements were repeated with purified proteins. Owing to the insolubility of loricrin, it was not possible to purify this protein and include it in the *in vitro* study. As far as SPRRs are concerned, similar rate constants were identified for SPRR1B, SPRR2A, and SPRR3 (Table 2), and again a higher value was observed for SPRR4, consistent with the cellular data. This indicates that the data obtained in living cells (Table 1) are the result of direct ROS quenching by the ectopically expressed proteins.

While performing these *in vitro* experiments, we observed that singlet oxygen, produced via illumination of RB, induced SPRR protein multimerization (Figure 2a, lane 3 arrows). This multimerization was not observed in the presence of RB without irradiation (lane 2), indicating that the production of ROS directly affects the SPRR proteins. Indeed, various other oxidizing compounds induced the formation of similar SPRR multimers (Figure 2b). All SPRR proteins contain, besides proline residues, high amounts of cysteine, a known redox-regulated amino acid involved in ROS quenching and signaling in many proteins (Michaeli and Feitelson, 1994; D'Autreaux and Toledano, 2007). Specific inactivation of the cysteine residues in SPRR1B (Figure 2c), SPRR2A (Figure 2d), SPRR3 (Figure 2e), and SPRR4 (Figure 2f) by *N*-ethylmaleimide (NEM) prevented both inter- and intramolecular S–S bond formation (compare lanes 2 and 4). Addition of ROS to the untreated SPRR proteins (lanes 1) resulted in the formation of dimers and trimers (lanes 2), and tetramers in the case of SPRR4. Interestingly, SPRR1B, SPRR2A, and SPRR3 also appear to be subjected to intramolecular S–S bond formation, as shown by the appearance of protein forms

Table 1. *In vivo* quenching of reactive oxygen species by living cells ectopically expressing loricrin or one of the SPRR proteins

Cell line	Rate constant[1]	SD	Relative expression[2]	Relative % quenching[3]
HFLor	7.540E−02	6.03E−03	1.76	25.3
HF1B	4.169E−01	1.02E−02	4.71	52.3
HF2A	2.274E−01	2.49E−02	2.86	47.0
HF3	1.898E−01	1.14E−02	2.18	51.4
HF4	1.692E−01	1.22E−02	1.00	100.0
H24	6.900E−03	7.72E−04	—[4]	—[4]

Abbreviation: SPRR, small proline-rich.
[1]*In vivo* rate constants ($L \times C^{-1} \times s^{-1}$) obtained by flash photolysis with cultured cells are depicted in singlet oxygen lifetime (L) per cell (C) per second (s).
[2]The relative expression levels were calculated by western blot. The band intensities were quantified after detection with a FLAG antibody and the expression level of FLAG-SPRR4 was set at 1.
[3]The relative % quenching was calculated by correcting the individual cellular rate constants for the expression level of the particular proteins.
[4]The values could not be calculated as no protein was ectopically expressed in this cell line.

Table 2. *In vitro* quenching of reactive oxygen species by purified SPRR proteins

Protein	Rate constant[1]	SD	Relative % quenching	No. of cysteines
Loricrin	ND	ND	ND	19
SPRR1B	6.078E+08	9.75E+07	50.9	8
SPRR2A	6.755E+08	4.62E+07	56.5	11
SPRR3	6.318E+08	1.11E+08	52.9	8
SPRR4	1.195E+09	1.63E+08	100	7
SPRR4NEM	4.650E+07	3.46E+06	3.9	—[2]

Abbreviations: ND, could not be determined; NEM, *N*-ethylmaleimide; SPRR, small proline-rich.
[1]*In vitro* rate constants ($L \times M^{-1} \times s^{-1}$) obtained by flash photolysis with purified proteins are depicted in singlet oxygen lifetime (L) per mole protein (M) per second (s).
[2]All cysteine residues in this sample were inactivated by NEM modification. The extent of modification was confirmed by mass spectrometry.

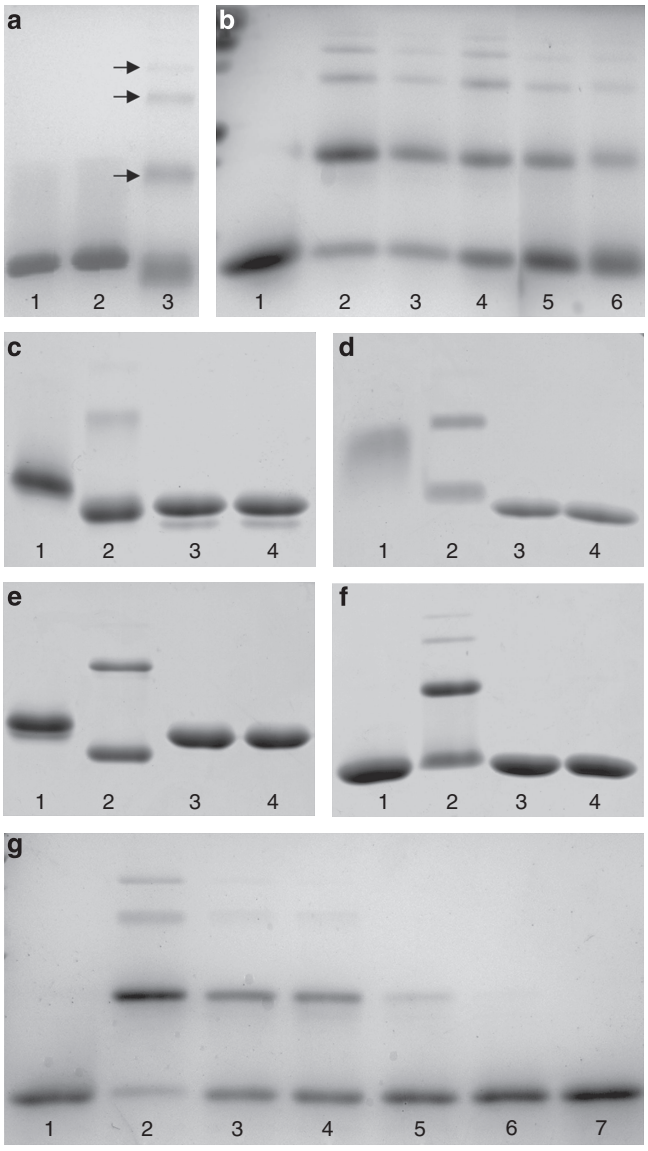

Figure 2. Small proline-rich (SPRR) multimerization induced by cysteine oxidation. (**a**) PAGE analysis of untreated (lane 1), Rose Bengal-treated (lanes 2 and 3), and illuminated SPRR4 (lane 3). Arrows indicate dimer, trimer, and tetramer formation. (**b**) Multimerization of SPRR4 by various types of reactive oxygen species: no treatment (lane 1), hydrogen peroxide (lane 2), peroxyradical (lane 3), bromate radical (lane 4), illuminated toluidine blue (lane 5), and chlorin e6 (lane 6). (**c–f**) H_2O_2-induced multimerization of SPRR1B (**c**), SPRR2A (**d**), SPRR3 (**e**), and SPRR4 (**f**) is inhibited by *N*-ethylmaleimide (NEM): mock-treated (lane 1), H_2O_2 (lane 2), NEM (lane 3); and NEM followed by H_2O_2 (lane 4). (**g**) Reversion of H_2O_2-mediated SPRR4 multimerization (lane 2) with increasing concentrations of β-mercaptoethanol (lanes 3–7). Lane 1: untreated control.

migrating faster than the monomeric form. SPRR multimerization is gradually reverted on addition of increasing amounts of β-mercaptoethanol (Figure 2g). To prove the direct implication of cysteine residues and S–S bond formation in ROS quenching, the singlet oxygen decay rate of NEM-treated SPRR4 was measured. The results revealed that by specifically inactivating all cysteine residues in SPRR4, the quenching activity was almost completely inhibited (Table 2, bottom row).

In summary, we have shown that the CE is directly involved in ROS quenching, and that sonication likely results in a better accessibility of the internal proteinaceous CE components, leading to an increased antioxidant potential in our measuring system. From the major CE protein components, the SPRR family members are capable of directly detoxifying ROS, both *in vitro* and in cultured cells, mainly because of their cysteine residues. The superior ROS quenching by SPRR4 is not because of a higher content of cysteine residues, as all SPRR proteins contain similar amounts of cysteines (Table 2). The same conclusion can be drawn by comparing the quenching potential of loricrin and SPRR4. Loricrin contains almost three times as many cysteines than SPRR4, but is nevertheless the weakest ROS quencher (see Tables 1 and 2).

Structural studies by nuclear magnetic resonance and circular dichroism predicted SPRR1, SPRR2, and SPRR3 proteins to consist of repeating β-turns in their central domain, resulting in an ordered spring-like structure (Steinert *et al.*, 1999) in which the proline content determines the rigidity of the different SPRR proteins (Candi *et al.*, 2005). Secondary structure predictions (BetaTPred2) of SPRR proteins are represented in Figure 3. β-Turns mainly consist of four amino acids stabilized by cross-strand interactions (Marcelino and Gierasch, 2008). As the repeats in the central domain of SPRR consists of eight to nine amino acids, depending on the SPRR isoform, they are likely to form a chain of repetitive turns in which the cysteine residues would be able to form intramolecular S–S bonds to stabilize the structure. The ROS-induced appearance of more compact monomeric protein forms, with a higher mobility in PAGE (Figure 2c–e), indicates that such intramolecular S–S bonds are indeed formed after ROS quenching. Interestingly, SPRR4 predictions disclosed a structure containing less β-turns in the central domain and two α-helices at the N terminus (Figure 3). This conformation, related to the lower proline content, indicates that SPRR4 might be a more flexible protein. As a result, cysteine residues of SPRR4 would be more exposed and as such more eager to directly interact with ROS or engage into intermolecular S–S bonds.

To ascertain whether S–S bond formation is actually responsible for ROS quenching in isolated CEs (shown in Figure 1), the oxidation state of cysteine residues in CE components was analyzed by LTQ-Orbitrap tandem mass spectrometry. Sonicated CEs were first mock- or H_2O_2-treated and subsequently modified with NEM, which only reacts with the free thiol groups. The remaining oxidized cysteines were reduced by addition of dithiothreitol and then modified with iodoacetamide, a second thiol-reactive compound. In this way, cysteine residues engaged in S–S bonds within the CE are selectively modified with iodoacetamide, whereas free thiol groups are modified with NEM. Following tryptic digestion, many peptides containing oxidation-sensitive cysteine residues were found. By only gating iodoacetamide-labeled peptides originating from EDC genes or known cornified envelope precursor genes (Table 3), multiple

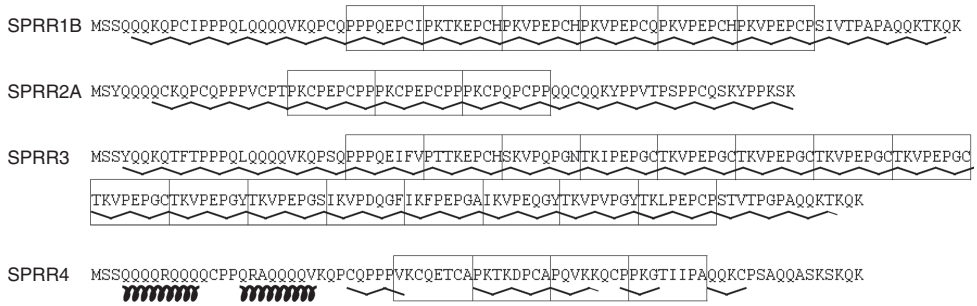

Figure 3. Secondary structure prediction of the highly homologous small proline-rich (SPRR) proteins. Graphical representation of the secondary structure of the various SPRR protein sub-classes. β-Turn sequences predicted in all SPRR proteins are indicated by zigzag structures, and the two α-helices in SPRR4 are also shown. The characteristic SPRR repeats in the central domain of the proteins are boxed.

Table 3. Mass spectrometric identification of CE peptides involved in S–S bonds after oxidation

Sequence	Δp.p.m.	Peptide score	*z*	Protein
CPEPC*PPPK	−0.87	49	2	SPRR2A, SPRR2B, SPRR2D, SPRR2E, SPRR2F, SPRR2G
C*PEPCPPPK	0.91	41	2	SPRR2A, SPRR2B, SPRR2D, SPRR2E, SPRR2F, SPRR2G
C*PPVTPSPPC*QPK	1.48	60	2	SPRR2E, SPRR2F
QPC*QPPPVC*PTPK	0.41	46	3	SPRR2A, SPRR2B, SPRR2D, SPRR2E, SPRR2G
QPTPQPPVDC*VK	−1.25	51	2	Loricrin
FGGQGNQFSYIQSGC*QSGIK	−0.19	94	2	Filaggrin-2
GGQGHGC*VSGGQPSGC*GQPESNPC*SQSYSQR	1.85	127	3	Filaggrin-2
C*PVEIPPIR	1.12	60	2	Keratinocyte proline-rich protein
IEISSPC*C*PR	−1.81	59	2	Keratinocyte proline-rich protein
GRPAVC*QPQGR	0.35	36	3	Keratinocyte proline-rich protein
LDQC*PESPLQR	−1.47	66	2	Keratinocyte proline-rich protein
FSTQC*QYQGSYSSC*GPQFQSR	−0.23	60	3	Keratinocyte proline-rich protein
TSFSPC*VPQC*QTQGSYGSFTEQHR	0.3	80	3	Keratinocyte proline-rich protein
LDTEAPYC*GPSSYNQGQESGAGC*GPGDVFPER	−0.29	117	3	Keratinocyte proline-rich protein

Abbreviations: CE, cornified cell envelope; EDC, epidermal differentiation complex.
Cysteine residues involved in the formation of S–S bonds were identified by iodoacetamide labeling (see Materials and Methods) and are indicated by C*. Mass deviations between measured and theoretical values of the various peptides are given as Δp.p.m. (absolute values <5 are considered as accurate measurements). The respective peptide scores (reflecting the reliability of the assignment of the peptide sequence) and *z*-values (charge of the peptide) are also provided. All Mascot identifications were manually validated and gated against a subset of the SwissProt database containing EDC and known CE precursor proteins (a list of these proteins is provided as Supplementary Information online).

cysteine-containing SPRR peptides appeared to be subjected to H_2O_2-induced oxidation, demonstrating that SPRR proteins fulfil a major role in ROS detoxification within the CE. In addition, cysteine-containing peptides originating from loricrin and filaggrin-2, one of the fused gene family members in the EDC (Presland *et al.*, 1992), were identified to be involved in the formation of S–S bonds. One of the most recently identified EDC genes, keratinocyte proline-rich protein (*KPRP*) (Kong *et al.*, 2003) showed, similar to SPRR, numerous oxidized cysteines residues. To our knowledge, this is the first direct evidence that keratinocyte proline-rich protein is a CE precursor protein and that it is involved in ROS detoxification.

Overall, our data indicate that all SPRR proteins have the ability to contribute to the antioxidant properties of the CE, but to a different extent, depending on the amount and accessibility of their cysteine residues. Our data suggest that the CE, as part of the outermost layer of our skin, constitutes the first line of antioxidant defense and provides protection against the high levels of ROS engendered by atmospheric oxygen and UV irradiation. UV light, for instance, can induce ROS production and trigger lipid peroxidation (Evelson *et al.*, 1997; Nishigori, 2006). Within the stratum corneum, the lipid envelope is in close contact with the proteinaceous CE components (Nemes and Steinert, 1999). In this way, the majority of ROS can be directly secured by the SPRR CE precursor proteins at the periphery of cells. This will permit a more efficient handling of residual cell-infiltrating ROS by the low-molecular-weight antioxidants and enzymatic antioxidants. Besides triggering ROS production, UV irradiation of

the skin has previously been shown to induce SPRR4 expression associated with thickening of the stratum corneum (Cabral *et al.*, 2001a). As the SPRR proteins can be differentially regulated by a complex panel of interdependent transcription factor complexes (Fischer and Backendorf, 2005), this stress-induced activation is likely part of the skin's antioxidant defense against subsequent ROS damage. We have previously inferred that the differential regulation of highly homologous SPRR proteins constitutes the basis for an adaptive epithelial barrier function. The data provided here demonstrate that SPRR proteins also provide the outermost layer of our skin with a highly adaptive and protective antioxidant shield.

MATERIALS AND METHODS

Flash photolysis

Singlet oxygen, $O_2(^1\Delta_g)$, and quenching rate constants (k_q) were determined by monitoring its time-resolved luminescence following Nd:YAG laser (Continuum, Santa Clara, CA) excitation of RB at 532 nm. This generates singlet oxygen via energy transfer from the triplet state of RB to ground state molecular oxygen. The subsequent luminescence at 1,270 nm was detected with a Judson Germanium G-050 photodiode coupled to a Judson preamplifier (Judson Technologies, Montgomeryville, PA). All samples were measured in a glass cuvette with magnetic stirrer in 300 µl D_2O (Merck, Darmstadt, Germany), with 5 µl of 10 mM RB (Sigma-Aldrich, St Louis, MO) added before any quencher was supplied. The luminescence decay of singlet oxygen was averaged over 256 measurements per concentration of quencher and was independently measured for at least six different concentrations per sample. At each concentration, a luminescence trace was obtained and fitted with a single exponential. The quenching rate constants for purified CEs, SPRR proteins, and CE precursor-expressing cells were calculated from the singlet oxygen decay rates (k_{decay}) plotted against the concentrations of quencher. The cellular rate constants were divided by the respective protein expression levels, quantified by western blot with a monoclonal anti-FLAG antibody (clone M5, Sigma-Aldrich), to obtain the relative percentage of quenching.

SPRR protein production and purification

SPRR proteins were produced by using isopropyl-β-D-thio-galactoside induction of *Escherichia coli* BL21 (DE3)*RP (Stratagene, La Jolla, CA) bacteria transformed with a pET vector (Merck) containing a full-length SPRR cDNA sequence. Bacterial pellets were lysed by freeze–thawing in 25 mM sodium citrate (pH 3.6), 1 mM EDTA and 1 mM dithiothreitol in which the SPRR proteins remained soluble. After centrifugation at 37,000 r.p.m. in a Ti60 rotor (Beckman-Coulter, Brea, CA), the supernatant was further purified using a 6 ml Resource S column (GE Healthcare, Diegem, Belgium). The buffer was replaced by 10 mM sodium phosphate (pH 7.0), and the SPRR proteins were stored at −80 °C. The purity of all proteins was confirmed by mass spectrometry.

SPRR multimerization and cysteine modification

All multimerization experiments were performed using identical molar ratios of proteins and oxidizing compounds. Singlet oxygen was generated by illumination of RB, toluidine blue, or chlorine e6, for 2 minutes with a 500-W halogen lamp. Treatments with all other oxidizing compounds were for a period of 10 minutes. The following final concentrations were used: 10 mM H_2O_2; 0.2 mM $FeSO_4$ with 1 mM H_2O_2; 10 mM $KBrO_3$; 10 mM RB; 0.5 mM toluidine blue; and 10 mM chlorine e6. All reactions were performed on ice, and equal amounts of protein were loaded on gel, using loading buffer without β-mercaptoethanol. Cysteine residues of SPRR were specifically inactivated by incubation with 20 mM NEM (Sigma-Aldrich) for 1 hour. The modifications were confirmed by mass spectrometry.

Secondary structure predictions

Secondary structure predictions were performed with the BetaTPred2 web server (Kaur and Raghava, 2003; http://www.imtech.res.in/raghava/betatpred2/).

Cell culture

For the generation of stable cell lines, full-length SPRR or loricrin cDNA sequences were provided with an N-terminal FLAG-tag and introduced into the episomal expression vector pECV25 (Belt *et al.*, 1989). HeLa cells, which do not express any of these proteins, were transfected using DOTAP (Boehringer, Mannheim, Germany) and were grown in DMEM supplemented with 10% newborn bovine serum, penicillin/streptomycin, and 300 µg ml^{-1} hygromycin. Stable cell lines were named HFLor (loricrin), HF1B (SPRR1B), HF2A (SPRR2A), HF3 (SPRR3), HF4 (SPRR4), and H24 (empty vector control). For intracellular protein quantification, the various ectopically expressed proteins were detected on western blots with monoclonal anti-FLAG antibody (clone M5, Sigma).

CE isolation and mass spectrometric characterization

CEs were isolated from sunburned peeled skin, according to the previously described procedure (Mehrel *et al.*, 1990), by boiling the skin pieces in 100 mM Tris-Cl (pH 7.5), 2% SDS, 1 mM EDTA, and 10 mM DTT. Note the reducing character of this buffer (10 mM DTT), which means that CEs will loose all oxidized cysteine residues during the purification procedure and are as such isolated in their native form (as composites of transglutaminase crosslinked proteins and lipids).

For mass spectrometry determination, sonicated CEs were oxidized with 10 mM H_2O_2 followed by NEM treatment. In this way, all cysteine residues that are in a position to form S–S bonds will become oxidized, whereas all non-oxidized cysteines will be modified with NEM. The samples were subsequently reduced with dithiothreitol and treated with iodoacetamide to modify the originally oxidized cysteines. The subsequent procedures and treatments for tryptic digestion, stage-tip purification, LTQ-Orbitrap tandem mass spectrometry, and Mascot analysis are described elsewhere (Florea *et al.*, 2010). Within Mascot, all identified peptides were characterized via the SwissProt database. All protein identifications were manually validated and gated for EDC and known CE precursor proteins. A list of these reference proteins is provided as Supplementary Information online.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We would like to thank J. Arts and W. Sol for protein production and purifications. We would also like to thank Dr B. Florea (LIC, Leiden) for help with mass spectrometry and Dr P. Gast (LION, Leiden) for advice

and assistance with flash photolysis. Professor J. Brouwer and Professor M. Noteborn (LIC, Leiden) are acknowledged for stimulating discussions. This research was financed by the Leiden Institute of Chemistry.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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