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Small proline-rich protein 1 is the major component of the cell envelope of normal human oral keratinocytes

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Abstract Oral keratinocytes of buccal and gingival tissues undergo a terminal differentiation program to form a protective epithelial barrier as non-keratinized or parakeratinized stratified cells. We have examined the protein composition of cell envelopes (CEs) from normal human buccal and gingival tissues as well as keratinocytes from normal human gingival cells grown in culture. Biochemical and sequencing analyses reveal that the CEs contain 60–70% small proline-rich protein 1a/b (SPR1a/b), together with smaller amounts of involucrin, annexin I and several other known CE proteins. The data imply a specialized role for SPR1 proteins in the unique barrier function requirements of oral epithelia.

Key words: Oral keratinocyte; Barrier function; Transglutaminase; Cell envelope; Small proline-rich protein

1. Introduction

A primary function of stratified squamous epithelia is to provide a physical and chemical barrier against the environment. This is afforded by a specialized structure termed the cell envelope (CE), which is an insoluble protein layer formed beneath the plasma membrane [1–3]. The CE is formed during terminal differentiation by a well-defined program of events involving the sequential deposition of envelope precursor proteins. These proteins are crosslinked into an insoluble amalgam by disulfide bonds and by N^{ε}-(γ-glutamyl)lysine and/or bis-(γ-glutamyl)-spermidine isopeptide bonds formed by the action of transglutaminases (TGases). TGases are a family of Ca^{2+}-dependent enzymes that catalyze the crosslinking of proteins through the formation of isopeptide bonds between protein-bound glutamines and amines such as polyamines or the ε-NH_2 of protein-bound lysine residues [1–3,5,6]. Keratinocytes are known to contain at least three major isoforms, TGases 1, 2 and 3. TGase 1 exists largely as a membrane-bound form and is induced during epidermal differentiation in skin and other squamous epithelia where it plays a key role in the formation of CEs. TGase 3 is primarily cytosolic and is expressed in certain orthokeratinizing epithelia where it too is important for CE assembly. TGase 2, also termed tissue TGase, is a cytosolic enzyme found in most cells and tissues. While it has been implicated in several cellular processes including apoptotic body formation, cell adhesion, metastasis, extracellular matrix stabilization and G-protein-mediated signal transduction, its role in the terminal differentiation of program of keratinocytes remains unclear.

Many proteins, including annexin I, cystatin α, desmoplakin, elafin, envoplakin, filaggrin, involucrin, type II keratin intermediate filament chains, loricrin, periplakin and the small proline-rich proteins (SPRs) are now known by protein sequencing analyses to be components of CEs and are crosslinked by TGases 1 and/or 3 [3,7,8]. Recent data suggest that CEs from various squamous epithelia may be built from a common scaffold consisting of envoplakin, involucrin, desmoplakin and SPRs, but the proteins deposited for reinforcement appear to vary widely depending on the epithelium [9]. For example, loricrin is the major component of the so-called rigid CEs derived from normal epidermal tissues, accounting for 80% of the total CE protein, but it is much less expressed in fragile envelopes prepared from psoriatic epidermis or cultured skin keratinocytes, or many other internal epithelia. Similarly, SPRs vary widely in amount in epithelia, from $<1\%$ in interfollicular epidermis, 5% in foreskin epidermis, about 10% in lip, palm and sole/footpad epidermis, to $>20\%$ in rodent forestomach epithelium [9,10]. Thus the different specialized barrier functional requirements of various epithelia may be reflected by the protein composition of their CEs, and especially the SPRs.

SPRs consist of three families of about 10 related proteins: SPR1 (two members SPR1a and SPR1b), SPR2 (7–8 expressed members) and one SPR3 protein [3,4]. All SPRs contain head and tail domains enriched in glutamines and lysines which are involved in crosslinking. Their central domains consist of varying numbers of proline-rich peptide repeats. Individual SPRs are differentially expressed in stratified squamous epithelia and most epithelial cell types express only a subset of SPRs. For example, in the human upper digestive tract, SPR1 proteins are expressed in sublingual, tongue, gingival and buccal epithelia, SPR2 in lingual papillae and SPR3 in esophageal epithelium [11–15]. Together, such differential expression of SPRs suggests that the SPR multigene family evolved to serve specialized roles in barrier function [9,10]. SPR proteins become crosslinked to themselves as well as many protein partners and thereby seem to function as crossbridging proteins in CEs [9,10].

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Abbreviations: CE, cell envelope; NHGK, normal human gingival keratinocytes; TGase, transglutaminase

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Extensive biochemical characterization of epithelial differentiation has been carried out using human and mouse epidermal tissue. Epidermal keratinocytes in culture can be induced to undergo terminal differentiation by high Ca^{2+} concentration and to express marker proteins as well as CE precursors such as keratins 1 and 10, involucrin, filaggrin, SPRs and TGase 1 [16]. In contrast, there is only limited information on the differentiation programs of oral epithelia and in particular the molecular composition of their CEs. Expression of TGase 1, involucrin and SPRs have been reported in human oral and tracheal epithelia [11–15,17], and a terminal differentiation program and CE formation similar to those in epidermis are presumed to occur. Oral epithelia are constantly exposed to salivary proteins, growth factors, proteases, other bioactive molecules in saliva, as well as various microorganisms and substances released by these organisms that may have toxic effects on the tissue. Thus, it bears an important and specialized barrier function against physical, mechanical, chemical and biological insults. In this report, we present evidence that the major protein component of human buccal and gingival keratinocyte CEs are SPR1a/b proteins.

2. Materials and methods

2.1. Cell culture

Normal human gingival keratinocyte (NHGK) cells were isolated from small pieces of gingival tissue excised from a healthy volunteer during retromolar extraction at the Clinical Research Core of the National Institute of Dental and Craniofacial Research. The excised tissue was washed in Hanks balanced salt solution and treated with dispase (2.4 U/ml, Boehringer Mannheim) at 37°C for 30 min. The epithelial sheet was separated from the underlying dermal tissue and digested with a 0.05% trypsin solution containing 0.02% EDTA (trypsin–versene mixture, Biowhittaker, Walkersville, MD, USA) at 37°C for 30 min. The detached epithelial cells were cultured in keratinocyte growth medium (KGM) (Clonetics, San Diego, CA, USA) containing 0.15 mM Ca^{2+} in a 60 mm tissue culture dish. After 1–2 weeks, when cultures were near confluence, the cells were subcultured into 10–20 dishes (60 mm). This secondary culture was used for experiments.

2.2. Preparation of CEs

When NHGK cells reached confluence, terminal differentiation was induced by switching the medium to KGM containing 1.2 mM Ca^{2+}. After 5 days, cells were harvested by centrifugation. Cell scrapings using a dental brush were collected from the buccal mucosa of normal human subjects, and cells were pelleted by centrifugation. Buccal or cultured cell pellets and gingival epithelial fragments were resuspended and boiled in a buffer of 0.1 M Tris–HCl (pH 9.0) containing 2% SDS and 1% DTT for 15 min, and centrifuged at $5000 \times g$ for 10 min to remove soluble proteins in the supernatant [18]. The pellets were resuspended in the same solution and the boiling and centrifugation cycle was repeated 5–6 times with intermittent sonication of the pellets for the complete extraction of soluble proteins.

2.3. Protein chemistry procedures

Amino acid compositions were determined following acid hydrolysis for 22 h in 5.7 N HCl in vacuo. Compositions were modeled for protein amounts as before [19,20] using the amino acids Asx, Ser, Glx, Pro, Gly, Ala, Val, Cys, Leu and Lys. Suspensions of CEs (1 mg/ml) were digested with 1:100 (w/w) trypsin (Sigma, sequencing grade) in 0.1 M NH_4HCO_3, pH 8.3, for 17 h at 37°C. The insoluble pellet was subjected to a second round of digestion with 1:100 (w/w) proteinase K (Sigma, sequencing grade) in the same buffer for 17 h at 37°C. Soluble peptides were separated by reverse phase high performance liquid chromatography (HPLC) on a 4.6×150 mm C_{18} column (Phenomenex) with a gradient of acetonitrile as described [9,20]. Selected peptides were covalently attached to Sequalon-AA membranes (Millipore) and sequenced on a Porton LF 3000 gas phase protein sequencer (Beckman Instruments) [21,22].

2.4. TGase analyses

NHGK cells (8×10^5) were resuspended and sonicated in 1 ml of 0.1 M Tris–acetate buffer, pH 7.5, containing 1 mM EDTA, and the protease inhibitors (5 μg/ml leupeptin, 5 μg/ml aprotinin, 50 μg/ml calpain inhibitor I, 100 μg/ml bestatin and 1 mM PMSF). TGase enzymes were assayed from the supernatant (cytosol) or membrane fraction following release with 0.1% Triton X-100 [23–26]. TGase activity assays were performed by measurement of the incorporation of [1,4-^{14}C]putrescine into succinylated casein [21]. Immuno-slot blotting of the cytosolic and membrane-bound fractions of NHGK cells were done using a standard procedure [24,25] followed by reaction with specific TGase 1 and 2 antibodies, and detected using enhanced chemiluminescence (Pierce, Rockford, IL, USA).

2.5. Reverse transcription-polymerase chain reaction (RT-PCR) analyses

Total RNA was isolated from NHGK cells using the TRIZOL reagent (Gibco BRL, MD, USA). The reverse transcription reaction was carried out at 42°C using AMV reverse transcriptase (Clontech, CA, USA). One μl of this reaction mixture was used as template in all PCR reactions. The oligonucleotide primer pairs used were: TGase 1 (361 bp) sense: GAT TGT CTT CAA GAA CCC CCT TCC C; antisense: TCA TCT GAC TCC AGT CCC ATT GCT C; TGase 2 (310 bp) sense: CTC GTG GAG CCA GTT ATC AAC AGC TAC; antisense: TCT CGA AGT TCA CCA CCA GCT TGT G; β-actin (838 bp) sense: ATC TGG CAC ACC TTC TAC AAT GAG CTG CG; antisense: CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC; SPR1a/b (385 bp) sense: CAC ACC AGG ACC AGC CAC TGT TGC AGC; antisense: TGC AAG GCA AAT GGG ACT CAT ACA CAG; annexin I (474 bp) sense: ATG GCA ATG GTA TCA GAA TTC CTC AAG; antisense: TTA CTC TCT GTA GAC CCT GTT AAT GTC, and involucrin (339 bp) sense: AAG GGA GAA GTA TTG CTT CCT GTA GAG; antisense: TGG GGA AGT AAG GAT TCC TAT AGT AAC. Primers were designed for specific regions and constructed to span an intron in order to distinguish cDNA from DNA transcribed from a genomic DNA template. The conditions for PCR were as described elsewhere [24–26]. PCR products were separated on 6% TBE gel (Novex, CA, USA) and radioactive bands detected using a phosphoimager (Molecular Dynamics Co., MN, USA). The PCR products corresponded to the expected sizes of cDNA (see Fig. 1D, inset).

3. Results

3.1. Ca^{2+}-dependent formation of CEs in NHGK cells

In Fig. 1, we document a series of experiments to demonstrate that NHGK cells, like epidermal keratinocytes, can be induced to assemble a CE structure in a Ca^{2+}-dependent manner in vitro in confluent cultures containing 1.2 mM Ca^{2+} concentration. First, as defined by use of a light-scattering assay, the NHGK cells formed CEs within 3–5 days (Fig. 1A). This increased CE formation correlated precisely with marked increases in TGase activity in the membrane fraction of the NHGK cells, whereas cytosolic activity changed little (Fig. 1B). This activity increase was due to the membrane-bound TGase 1 isoform (Fig. 1C) rather than the cytosolic TGase 2 or 3 (not expressed) isoforms. Furthermore, measurement of the amounts of mRNAs by RT-PCR of three important CE precursor proteins and the TGase 1 and 2 enzymes revealed coincident modest increases for SPR1 and involucrin, major increases for annexin I and TGase 1, but a marked decrease for TGase 2 mRNAs. Together, these data indicate that NHGK cells can be induced to undergo a terminal differentiation program that involves production of a CE barrier structure.

3.2. CEs from gingival and buccal tissues and cultured NHGK cells are biochemically very similar

First, we performed amino acid analyses on CEs isolated

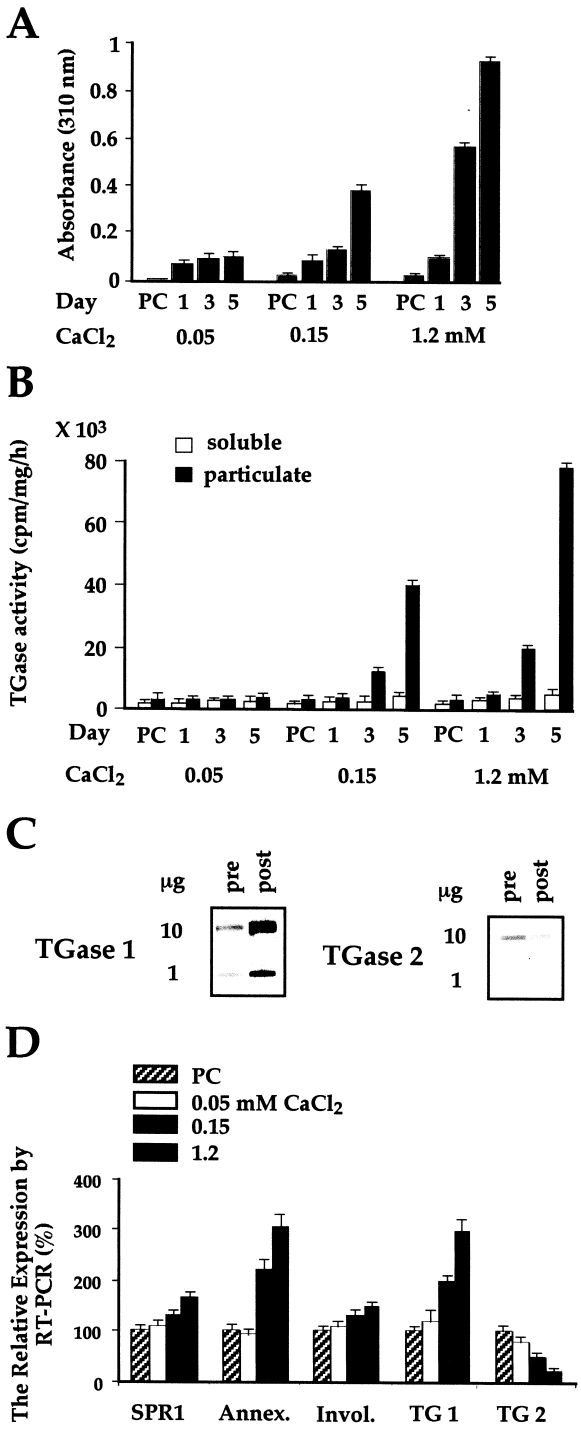

Fig. 1. CE formation in NHGK cells. A: Pre-confluent (PC) NHGK cell or post-confluent cells cultured in media containing 0.05, 0.15 and 1.2 mM Ca^{2+} for 1, 3 and 5 days were used for the detection of CE formation by light-scattering. CEs from 1×10^7 cells were resuspended in 1 ml of buffer and turbidity measured at 310 nm. B: TGase activity in the cytosolic and membrane fractions were measured in pre- or post-confluent NHGK cells incubated as above. C: The membrane and cytosolic fractions were used for immuno-slot blotting with specific antibodies for TGase 1 and TGase 2 for pre- or post-confluent (5 days after confluence in 0.15 mM Ca^{2+}) cultures of NHGK cells. D: Total RNA was isolated from NHGK cells pre-confluent (PC) or after 5 days of confluence in media containing 0.05, 0.15 or 1.2 mM Ca^{2+} and used for RT-PCR analyses of expression of SPR1, annexin I, involucrin, TGase 1 and TGase 2 mRNAs.

from intact gingival and buccal mucosal tissues, as well as NHGK cells cultured to confluency for 5 days. Using established modeling algorithms [19,20], we then estimated the amounts of several major protein species likely to be present (Table 1). The CE preparations were generally very similar and notably enriched in Pro residues, from which we can predict that they contain similarly high levels (60–65%) of SPRs together with smaller amounts of involucrin, desmoplakin/envoplakin, annexin I and loricrin. Their crosslink contents were 1.3–1.5 residues/100. Together, these data suggest that the CEs formed in terminally differentiating cultured NHGK cells are biochemically similar to those of intact buccal or gingival tissues. While the amount of crosslink is comparable to levels seen in epidermal [22] or forestomach [9] CEs, the estimated extraordinarily high content of SPRs in these oral epithelial CEs is unique.

3.3. Protein sequencing analyses of peptides recovered from CEs of NHGK cells

Protein sequencing analyses were performed in order to characterize the nature and extent of proteins crosslinked in the NHGK CEs. We recovered 210 μg of CEs from cells cultured for 5 days in high Ca^{2+} medium. These were first digested with trypsin, and the tryptic-soluble products (110 μg) were resolved by HPLC (Fig. 2A). Trypsin-insoluble material (100 μg) was digested further with proteinase K, after which 70 μg remained insoluble and the solubilized 30 μg were used for fractionation by HPLC (Fig. 2B). Aliquots of each protease fraction were used for crosslink quantitation, and amino acid analysis followed by modeling of protein amounts (Table 1).

The soluble tryptic peptides (Fig. 2A) were dominated by two overlapping peaks eluted at 31–33 min of sequence VPEPCQPK and VPEPCHPK, respectively, and which correspond exactly to the two repeat motifs characteristic of the central domain of human SPR1a/b proteins. Assuming that none of these sequences was involved in crosslinking, as found before [10], and based on the numbers of these repeats in human SPR1a/b proteins [27], the yields of the two peptides indicate that the original 210 μg of NHGK CEs contained at least 60% SPR1a/b proteins. In addition, a broad poorly-resolved peak eluted near the end of the chromatogram. This is reminiscent of in vitro experiments with SPR proteins [28,29], and is suggestive of the presence of densely crosslinked material. While, it could not be further analyzed by protein sequencing, its amino acid composition was similar to that expected for SPR1 head and tail domains, and aliquots across the peak were enriched in Pro- and Gln-rich sequences (data not shown). Also, it contained about 2.8 mol/mol of crosslink (Table 1), which corresponds to about 5.2 mol of crosslink per head+tail domain of SPR1 proteins. This is the highest density of head/tail domain crosslinking of SPRs reported so far, although this broad peak is likely to contain crosslinked sequences of other CE proteins as well.

However, the soluble proteinase K peptides recovered from the trypsin pellet yielded a number of well-resolved peptide peaks, in addition to another broad unresolved peak late in the chromatogram (Fig. 2B). Table 2 lists the sequences obtained, as well as an estimate of their molar recoveries. In no case did we find two or more sequences adjoined by a crosslink, which means the listed peptides originated from uncrosslinked parts of CE proteins that nevertheless had been linked

Table 1
Amino acid compositions (residues/100 residues) and modeled amounts of proteins of CEs

Amino acid[a]	Gingival tissue	Buccal tissue	NHGK cells				
			cells	trypsin		proteinase K	
				S[b]	P	S	P
Asp+Asn	4.1	4.0	5.4	6.5	4.3	4.3	4.1
Thr	3.6	3.4	3.8	3.7	3.2	3.6	2.8
Ser	3.2	3.3	3.5	5.4	4.1	5.2	5.3
Glu+Gln	17.2	17.0	17.5	16.9	17.0	17.2	16.4
Pro	20.8	19.3	19.9	15.0	20.2	19.4	21.1
Gly	6.7	6.6	5.6	6.4	6.4	4.7	8.2
Ala	5.1	5.0	5.2	3.9	5.6	4.9	5.7
Cys	5.8	7.4	4.6	7.9	8.2	7.9	8.2
Val	5.4	5.2	5.1	6.7	4.0	4.9	3.2
Met	0.9	0.8	1.5	1.7	0.6	0.7	0.4
Ile	3.3	3.1	2.3	2.3	2.3	2.5	1.4
Leu	7.7	7.5	6.5	5.7	6.7	5.5	6.8
Tyr	1.0	1.0	2.2	1.0	2.3	1.2	2.4
Phe	0.7	0.8	2.0	1.1	2.1	2.7	1.5
His	3.7	3.6	3.3	4.5	2.5	3.3	1.2
Lys	9.0	8.7	8.4	7.9	8.6	7.5	8.6
Arg	1.9	2.1	3.2	1.7	3.6	4.4	2.8
Crosslink	1.5	1.4	1.3	1.0	1.5	0.0	4.8
Protein	Modeled amounts[a] (%)						
SPRs	70	70	65	50	≈70[e]	≈60	≈70[e]
Loricrin	5	10	<5		5		10
Annexin I	5	5	10	10	<5	≈10	<5
Involucrin	10	10	10	10	<5	≈10	<5
Plakins[c]	5	5	5	10	<5	≈10	<5
GenBank[d]	<5	<5	10	20	>20[e]	≈10	>20[e]

[a]Numbers may not add up to 100 due to rounding.
[b]S, supernatant; P, undigested pellet.
[c]Plakins means average composition of envoplakin and desmoplakin.
[d]This is included to rationalize the modeled amounts: in this study, a high content probably suggests a poor fit due to loss by proteolysis of some sequences of proteins.
[e]These numbers are unreliable, probably due to loss of some sequences of proteins.

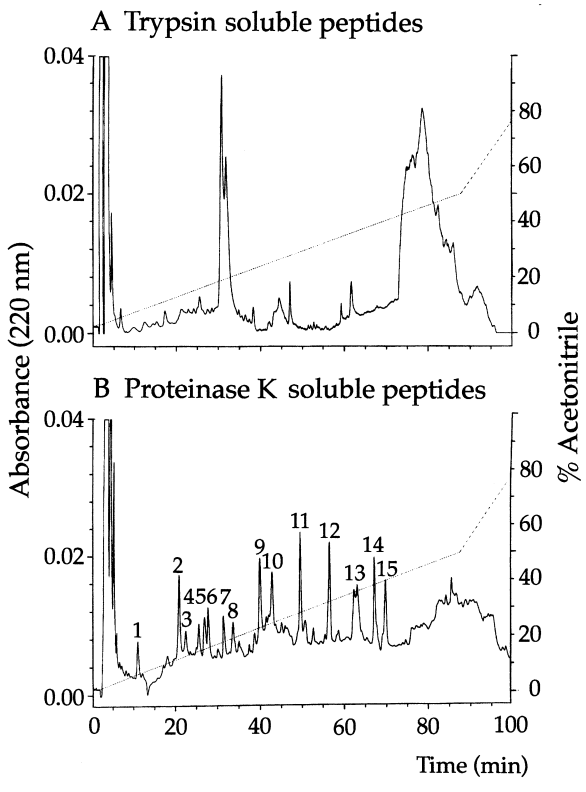

Fig. 2. HPLC profiles of trypsin (A) and proteinase K (B) soluble peptides.

together through residues elsewhere in their sequences. The most abundant protein species was involucrin (13% of total), followed by annexin I (7%), loricrin, envoplakin and SPR1 (4% each), desmoplakin and cystatin α (≤2% each).

Table 2
Amino acid sequences of peptides from Fig. 2B

Peak	Sequence[a]	Protein	Estimated % amount[b]
1	86-QETGK	annexin I	1
2	504-EQQEK	involucrin	4
3	22-VQTVK	annexin I	1
4	303-HQTQQK	loricrin	1
5	581-PPKHK	involucrin	1
6	301-VTGPGG	desmoplakin	2
7	25-QLEEK	cystatin α	2
8	131-GTDEDTL	annexin I	1
9	1971-QDESSY	envoplakin	4
10	53-SGGGGY[c]	loricrin	3
11	93-EQHEEY	involucrin	4
12	560-PTKGEVL	involucrin	4
13	?-VPEP[E/Q][E/Q][E/Q][d]	SPR1	4
14	69-PEPCPSI	SPR1	4
15	317-GISLC	annexin I	4

[a]Sequence of only the first 5–7 Edman degradation cycles is shown; numbers refer to location in protein as reported in databases.
[b]Based on total initial amount of NHGK CE protein.
[c]Also present in keratin 1, which is not expressed in NHGK cells (data not shown).
[d]Sequence uncertainty due to mixed sequences or partial deamidation of glutamines.

Finally, the proteinase K pellet (33% of total CE protein mass) retained extraordinarily high contents of crosslink (4.8 residues/100 residues), as well as Pro and Glx residues. However, mathematical modeling yielded ambiguous results, presumably due to the fact that after two cycles of proteolysis, many proteins had been severely pruned and could thus no longer be used for meaningful predictions. The data nevertheless suggest extensively crosslinked SPR protein head/tail remnants, as well as other minor protein constituents. In addition, the higher contents of Gly and Cys were suggestive of the presence of loricrin.

4. Discussion

The present experiments were initially designed to assess whether oral keratinocytes undergo a program of terminal differentiation and form a CE barrier structure as do other types of stratified squamous epithelia. The expression characteristics in NHGK of three ubiquitous CE precursor proteins as well as the TGase 1 enzyme are consistent with the formation of large amounts of CEs following 5 days of culture in high Ca^{2+} medium, are somewhat similar to other epithelia such as epidermal keratinocytes, and are consistent with numerous earlier studies [11–15]. Modeling analyses of the compositions of CEs recovered from gingival and buccal tissues and cultured NHGK cells revealed near identities in biochemical properties. Furthermore, the amounts of various CE precursor proteins found following amino acid sequencing were similar to those estimated to be present in the intact tissues and NHGK cells by modeling (cf. Tables 1 and 2). The contents of desmoplakin, envoplakin and involucrin were similar to those of other cultured keratinocytes [8,22], suggesting that CE assembly in NHGK cells is initiated by a pathway which is common to many other types of stratified squamous epithelia [8]. However, NHGK CEs differed from those of epidermal keratinocytes by much lower contents of loricrin and the TGase 3 enzyme, somewhat higher contents of annexin I and the TGase 1 enzyme, and notably by the extraordinarily high contents of SPR1a/b proteins. Indeed, our modeling and sequencing analyses reveal that NHGK CEs contain by far the highest content of SPR proteins reported in any epithelial cell type. This is unlikely to be due to an artifact of culturing, since buccal and gingival tissue CEs were estimated to contain similarly high levels (Table 1).

The question therefore is, why? Based on extensive sequencing analyses of the CEs of several different epithelia, we have documented that SPR proteins in general function as cross-bridgers between other proteins within the CE structure [9,10]. Furthermore, we have noted that the sum of SPR+loricrin contents of CEs of many epithelia is constant at about 80%, but that the amount of SPRs varies in direct proportion to the presumed requirements of the epithelium to withstand mechanical abrasion and wear-and-tear [9,10]. In this way, we have proposed that SPRs function to modify the biomechanical properties of the CEs and thus of the entire epithelium. As examples, CEs of trunk epidermis contain few SPR proteins, those of lip and footpad epidermis contain about 10% SPRs, and CEs of rodent forestomach contain about 20% SPRs. In buccal and gingival tissues and derived NHGK cells, the sum of SPRs+loricrin is also about 65–75% (Table 1). Therefore, we conclude that buccal and gingival CEs contain high levels of SPRs specifically for biomechanical purposes; indeed we are unaware of other epithelial tissues that are likely to be subjected to more mechanical stresses and insults from the environment.

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