Isolation and Characterization of Human Repetin, a Member of the Fused Gene Family of the Epidermal Differentiation Complex

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The human repetin gene is a member of the ''fused'' gene family and localized in the epidermal differentiation complex on chromosome 1q21. The ''fused'' gene family comprises profilaggrin, trichohyalin, repetin, hornerin, the profilaggrin-related protein and a protein encoded by c1orf10. Functionally, these proteins are associated with keratin intermediate filaments and partially crosslinked to the cell envelope (CE). Here, we report the isolation and characterization of the human repetin gene and of its protein product. The repetin protein of 784 amino acids contains EF (a structure resembling the E helix-calcium-binding loop-F helix domain of parvalbumin) hands of the S100 type and internal tandem repeats typical for CE precursor proteins, a combination which is characteristic for ''fused'' proteins. Repetin expression is scattered in the normal epidermis but strong in the acrosyringium, the inner hair root sheat and in the filiform papilli of the tongue. Ultrastructurally, repetin is a component of cytoplasmic non-membrane ''keratohyalin'' F-granules in the stratum granulosum of normal epidermis, similar to profilaggrin. Finally, we show that EF hands are functional and reversibly bind Ca^{2+}. Our results indicate that repetin is indeed a member of the fused gene family similar to the prototypical members profilaggrin and trichohyalin.

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The skin protects our body against diverse environmental hazards. The frontline defense against microorganisms, physical stress, ultraviolet radiation, chemical irritation, or water loss is constituted by the epidermal barrier (Eckert *et al*, 1997; Presland and Dale, 2000; Fuchs and Raghavan, 2002). This barrier is established during epidermal terminal differentiation, a complex biochemical process changing keratinocytes from the proliferating phase in the basal layer to the mitotically inactive cells producing the cornified cell envelope (CE), the outermost protecting structure of our body. Thereby, keratinocytes migrate through the four epidermal layers, constantly modulating their proteomic profiles to the specific needs of the respective cell layer. During final maturation of the keratinocyte, the formation and subsequent dissolution of keratohyalin granules, the simultaneous alignment of keratin intermediate filament bundles and the formation of the CE are major structural changes. Their components constitue the bulk of proteins synthesized during late epidermal differentiation. The functional consequences of disturbed epidermal differentiation leading to diverse inherited disorders of keratinization are well demonstrated by the various genetic defects of epidermally expressed keratins, connexins, calcium pumps, enzymes, or protease inhibitors (Arin *et al*, 2002). The crucial role of the late stages of differentiation for epidermal homeostasis is demonstrated by lamellar ichthyosis resulting from keratinocyte transglutaminase deficiency (Huber *et al*, 1995).

In recent years, a number of genes specifying structural proteins expressed late during epidermal differentiation, e.g. proteins forming keratohyalin granules and CE precursor proteins, have been identified and found to be clustered on chromosome 1q21 (Backendorf and Hohl, 1992; Volz *et al*, 1993; Marenholz *et al*, 1996, 2001). Therefore, this region has been named the epidermal differentiation complex (EDC) (Mischke *et al*, 1996). The proteins encoded by the EDC genes can be, based on the primary sequence, combined into three groups.

The members of the first group are precursor proteins of the CE. The CE is a structure of 15 nm width consisting primarily of loricrin, involucrin, and small-proline rich proteins (SPRR), and to a lesser extent of proteinase inhibitors, keratins, desmosomal components, and keratohyalin proteins (Steinert and Marekov, 1995; Robinson *et al*, 1997; Steinert, 2000) that are crosslinked by the action of keratinocyte transglutaminase at the cell periphery (for a recent review, see Grenard *et al*, 2001). Simultaneously, ceramides are extruded from lamellar bodies into the intercellular space and become covalently attached to an involucrin scaffold on the outer surface of the CE (Swartzendruber *et al*, 1988; Marekov and Steinert, 1998). This leads to the formation of a bicomposite protein–lipid structure that progressively replaces the plasma membrane. Loricrin, involucrin, SPRR, the more recently identified xp5 or late

Abbreviations: CE, cell envelope; SPRR, small-proline rich proteins

envelope proteins (LEP) (Zhao and Elder, 1997; Marshall *et al*, 2001), and the distantly related NICE-1 (Marenholz *et al*, 2001) are encoded by genes with similar structures. The proteins encoded have homologies in the terminal protein domains and contain a variable number of internal tandem repeats specific for each protein, and are major precursors for the building of the CE. The human EDC contains one gene for loricrin and involucrin, 11 SPRR genes (two SPRR1, seven SPRR2, one SPRR3, and one SPRR4), 16 xp5/LEP genes (Marshall *et al*, 2001), and 1 NICE-1 gene (Marenholz *et al*, 2001). It is therefore thought that this gene family emerged from a common ancestor (Backendorf and Hohl, 1992).

The second group comprises 14 membres of the S100 family whose genes flank the EDC (Heizmann, 2002). S100 proteins are calcium-binding proteins because of the presence of two EF hands. They are regulatory proteins primarily involved in different steps of the calcium signal transduction pathway regulating cell shape, cell cycle progression, and differentiation (Eckert *et al*, 2004). They may play a role in the pathogenesis of epidermal diseases such as psoriasis, skin cancer, and skin inflammation (Eckert *et al*, 2004). Some S100 proteins (e.g. S100A10 and S100A11) have been isolated from purified CE and thus are crosslinked by transglutaminases (Robinson *et al*, 1997).

The third group combines EF hands and internal tandem repeats, the reason why these proteins are called "fused" members of CE precursor proteins. This group comprises profilaggrin, trichohyalin, repetin (Krieg *et al*, 1997), hornerin (Makino *et al*, 2001), and the protein encoded by c1orf10 (Xu *et al*, 2000). Profilaggrin, trichohyalin, and possibly other protein products of the fused gene group are components of cytoplasmic non-membrane "keratohyalin" granules in the stratum granulosum of normal epidermis, hair follicles, and mucosal keratinizing epithelia (Dale *et al*, 1994). Functionally, they are associated to keratin intermediate filaments and partially crosslinked to the CE. Profilaggrin is processed to functional filaggrin units in the terminal phase of epidermal differentiation (Resing *et al*, 1995). Further degradation of the filaggrin monomers to amino acids is thought to have an important role in the water retention capabilities of the skin. The "fused" gene, repetin, was recently cloned from mouse epidermis (Krieg *et al*, 1997). In this paper, we report the isolation and characterization of the human repetin gene and its protein product.

Results

Isolation of the gene and chromosomal assignment The mouse repetin gene was localized to chromosome 3F, a region that is syntenic to human chromosome 1q21 (Krieg *et al*, 1997). To clone the human homologue, we screened 40,000 clones from a chromosome 1-specific cosmid library (Nizetic *et al*, 1994) with a mouse repetin probe (Genbank X99251 nt 7198–7441). One positive cosmid, 31H23, was further investigated by Southern blot analysis using the same mouse repetin probe localizing the human repetin gene to a 7 kbp fragment flanked by *Hind*III and *Eco*RI sites (Fig 1*A*). Sequence analysis of this fragment yielded 6679

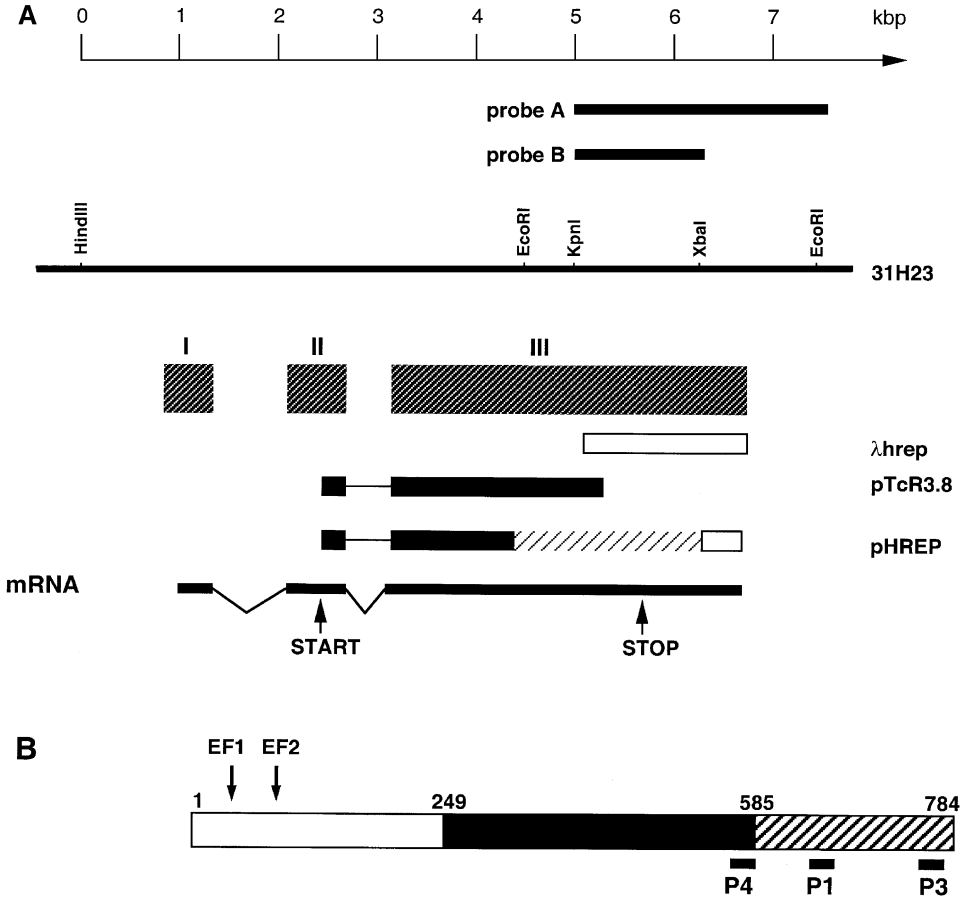

Figure 1
Genomic organization of the human repetin gene. (*A*) The genomic structure of cosmid 31H23 containing the repetin gene with exons I–III and the different cDNA clones are shown. Probes A and B designate DNA fragments used for cDNA cloning and hybridization experiments. (*B*) Depicts the repetin domain structure with the N-terminal part (amino acids 1–248) containing EF hands, the central repetitive domain (amino acids 249–584), and the C-terminal part (amino acids 585–784). P1, P3, and P4 indicate the positions of three peptides that were used for production of affinity-purified polyclonal antibodies.

nucleotides (Fig 2). This sequence has been deposited in Genbank under accession number AY219924. Comparison of this sequence to the genomic sequence of mouse repetin identified the three exons of the human repetin gene as those regions with the highest homologies to the mouse repetin gene. Comparing the banding pattern of Southern blot analysis between 31H23 and human genomic DNA suggested that no DNA rearrangement had occurred during the amplification of the cosmid clone (data not shown).

FISH hybridization with cosmid 31H23 revealed that the human repetin gene was localized on chromosome 1q21 (data not shown). Southern blot analysis of several yeast

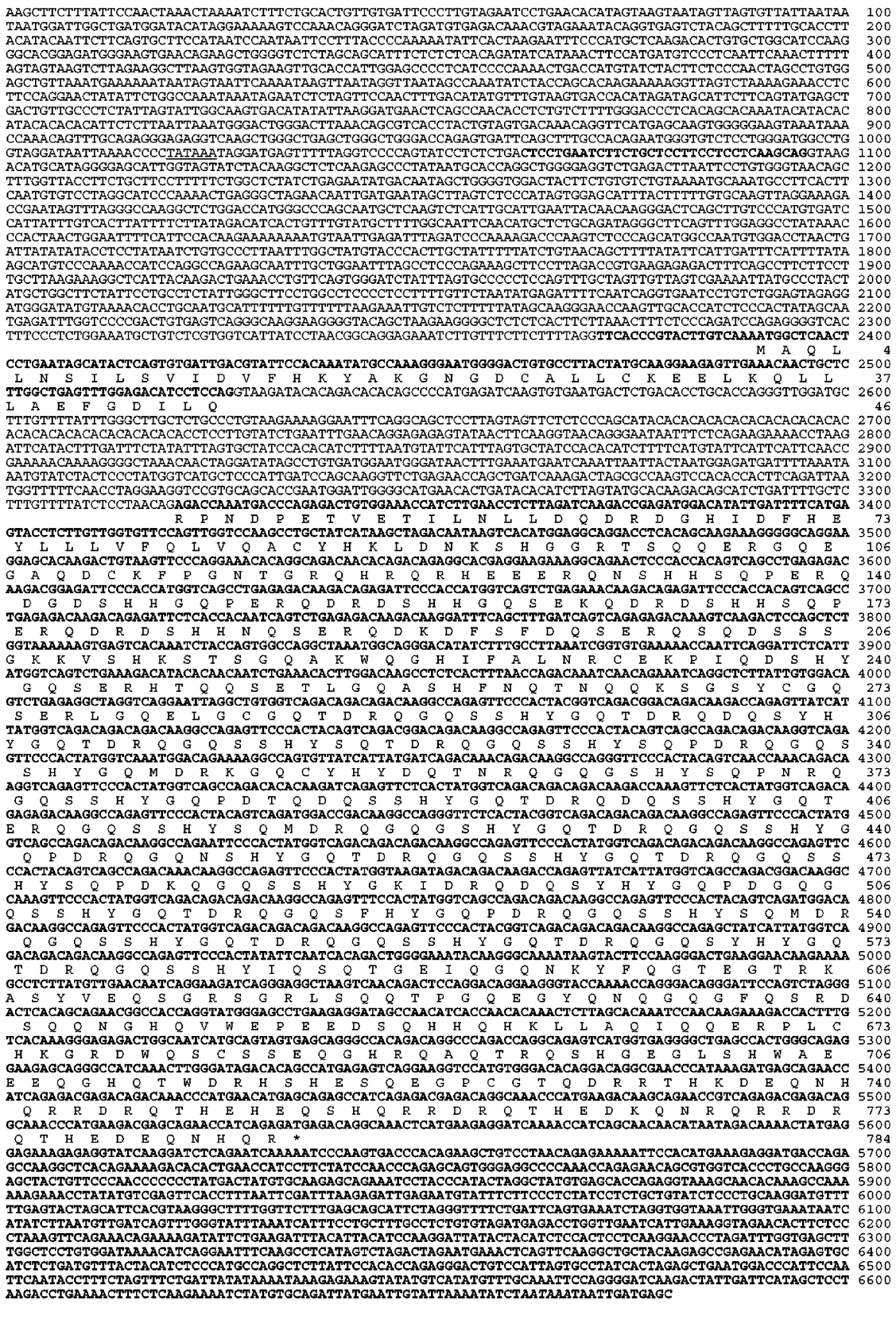

Figure 2
Nucleotide and deduced amino acid sequence of human repetin. These data have been compiled from cosmid clone 31H23 and cDNA clones λhrep and pTcR3.8 (Fig 1*A*). The single-letter code amino acid sequence is numbered with the initiation methionine as amino acid 1. Exons I–III are marked in bold, the putative TATA box is underlined, and the polyadenylation signal sequence is in italics. This sequence has been deposited in Genbank under accession number AY219924.

artificial chromosomes (YAC) clones and human genomic DNA with probe A (Fig 1*A*) allowed to assign the human repetin gene to the region between the genes for trichohyalin and profilaggrin (data not shown).

Characterization of the repetin cDNA To further analyze the gene structure, 6.6×10^5 independent clones of a λZAPII cDNA library constructed with RNA isolated from cultured keratinocytes (South *et al*, 1999) were screened with probe B from cosmid clone 31H23 (Fig 1*A*). Clone λhrep, the longest clone isolated, contained an insert of about 1400 bp. Sequence analysis revealed that λhrep sequence spanned the region from nucleotides 5233–6679 of the human repetin gene (Fig 2). The 5′ end of the cDNA was isolated using RACE strategy with RNA isolated from suspension-induced cultures of human keratinocytes and the forward primer HR-44, located upstream of the putative start codon in exon 2 sequence, and reverse primer HR-37, derived from the 5′ end of the λhrep insert. The resulting 2.2 kbp fragment was subcloned into the pGEM-T Easy vector yielding plasmid pTcR3.8 (Fig 1*A*). The sequence of the plasmid insert was in complete agreement with the regions of 31H23 displaying the highest homologies with the exons of the mouse repetin gene, thus confirming length and position of exons 2 and 3 of the human repetin gene. Plasmid pHREP was constructed by joining a 1.4 kbp fragment of clone pTcR3.8 from the 5′ end to the *Eco*RI site was joined with the *Eco*RI/*Xba*I fragment of 31H23 and the fragment from the *Xba*I site to the 3′ end of the insert in λhrep (Fig 1*A*).

To investigate whether we had indeed cloned a cDNA encoding the full-length repetin polypeptide, the open reading frame of the pHREP insert was subcloned by PCR into the bacterial expression vector pET28a(+) yielding plasmid p28HR. Cell lysates of IPTG induced p28HR transformed bacteria and suspension-cultured keratinocytes were then analyzed using western blot analysis with anti-repetin antibody AF646. The results showed that there was no major size difference between the repetin proteins expressed in keratinocytes and bacteria (Fig 3*D*). This argues strongly that clones p28HR and pHREP contain inserts specifying a full-length human repetin protein and that the cognate start codon is located in exon II. In summary, the human repetin gene is composed of exon I (34 bp), exon II (159 bp), and exon III (3356 bp), separated by introns I (1273 bp) and II (792 bp) (Fig 2).

Sequence and properties of human repetin protein Human repetin consists of 784 amino acids (including the start methionine). The apparent molecular weight of 100 kDa on SDS-PAGE (Fig 3) was in excellent agreement with the 91 kDa calculated from the amino acid sequence (Fig 2). The repetin sequence contains high numbers of glutamine (19.4%), serine (11.7%), and glycine residues (10.5%). The protein can be divided into three domains (Fig 1*B*): first, an N-terminal domain (amino acids 1–248) with significant homology to the calcium-binding region found in S100 proteins, profilaggrin and trichohyalin, and mouse repetin (Fig 4), second, the central part (amino acids 249–584) contains 28 repeats of 12 amino acids with the consensus sequence QXDRQGQSSHYG, which is very rich in glutamine (23.8%), serine (15.5%), glycine (14.0%), and acidic and basic amino acids but almost devoid of non-polar amino acids,and third, the C-terminal domain (amino acids 585–784) which is again rich in glutamine (20.5%) and arginine (11.0%). The central and C-terminal part showed no homology to other proteins in the database with the exception of mouse repetin and glutamine-rich proteins.

Human repetin binds Ca^{2+} BLAST analysis of human repetin revealed that the N-terminal part has a high homology to the N-terminal EF hand regions of mouse repetin and human profilaggrin, trichohyalin, and S100 proteins (Fig 4). To explore further whether human repetin was capable of binding calcium, the N-terminal region containing both putative EF hands (amino acid residues 1–145; p28HREF; Figs 1*B* and 2) was expressed in bacteria using the pET28b(+) vector. Crude extracts from IPTG-induced and non-induced cultures of p28HREF transformed bacteria were electrophoresed and blotted to a membrane that was subsequently tested for the presence of calcium-binding proteins by an $^{45}Ca^{2+}$ overlay assay (Siegenthaler *et al*, 1997). Extracts from psoriatic scales known to harbor S100A7 and S100A8 proteins were used as positive controls (Fig 5*A*, *lane 1*). Autoradiography showed a 20 kDa band only in induced cultures (Fig 5*A*, *lane 3*) corresponding to the expected molecular weight of the N-terminal part of human

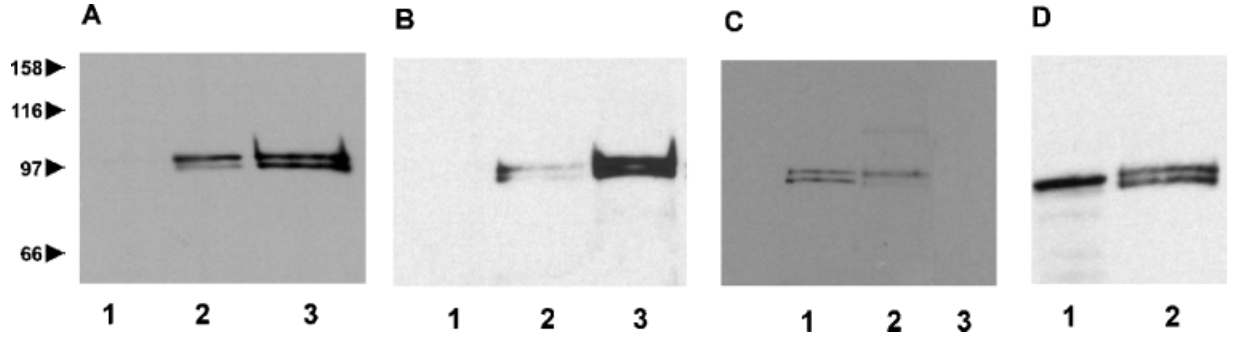

Figure 3
Protein expression of human repetin. Immunoblot analysis of repetin expression in cytosolic (*lane 1*), membrane (*lane 2*) and cytoskeletal (*lane 3*) extracts from suspension-induced human keratinocytes (*A*) and human foreskin biopsy (*B*) detected with antibody AF646. (*C*) Depicts cytoskeletal fractions of suspension-induced human keratinocytes immunoblotted with antibodies anti-peptide P1 (*lane 1*), anti-peptide P3 (*lane 2*), and reaction omitting the first antibody (*lane 3*). (*D*) Demonstrates immunodetection using AF646 with whole cells extracts of p28HR transformed BL21 CodonPlus(DE3)-RIL bacteria 180 min after IPTG induction (*lane 1*) and the cytoskeletal fraction of suspension-induced human keratinocytes (*lane 2*). The positions of molecular weight markers in kDa are indicated on the left.

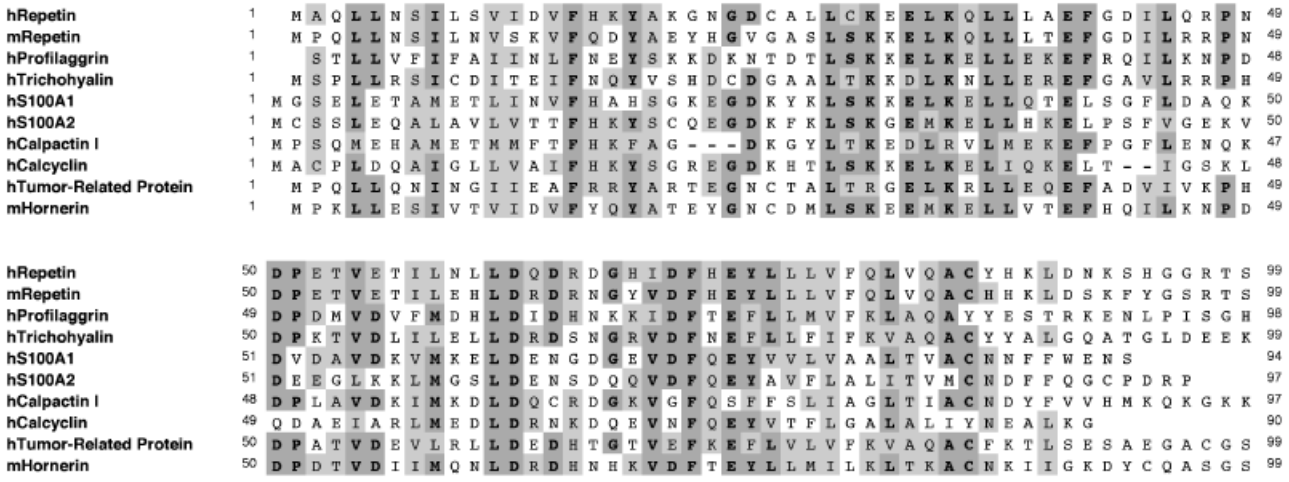

Figure 4
Amino acid comparison of EF hand proteins. Comparison of the N-terminal amino acid sequences of human repetin with mouse repetin (Genbank T30251), human profilaggrin (A48118), human trichohyalin (A45973), human S100A1 (NP_006262), human S100A2 (NP_005969), human calpactin I light chain (JC1139), human calcyclin (P06703), human tumor-related protein (AAD55747), and mouse hornerin (AAK15791). The letters h and m stand for human and mouse, respectively.

repetin (Fig 5*A*, *lane 2*). A second band of lower molecular weight was present in extracts from both induced and non-induced bacteria, suggesting that this protein is of bacterial origin. The 20 kDa band disappeared after extensive washing of the membrane with cold calcium, indicating that the N-terminal region of human repetin contains reversible calcium-binding site(s) (Fig 5*B*).

Expression analysis In order to study the expression of human repetin, total RNA was isolated from keratinocytes cultured in high-calcium medium on lethally irradiated 3T3 feeder layer for 7 d after confluency and from keratinocytes in suspension cultures with 1.75% methylcellulose. Northern blot analysis using hybridization probe B (Fig 1*A*) revealed that repetin was only expressed at very low levels in adherent keratinocytes whereas loricrin mRNA was strongly expressed (Fig 6*A*). But repetin mRNA of approximatively 4 kb was detected in suspension cultures after 24–48 h (Fig 6*B*). This signal disappeared at later time points, probably because of generalized RNA degradation. The observed size corresponded well to the expected one derived from the cloned cDNA sequence (Fig 2). A repetin message of identical size was also found in total RNA isolated from human epidermis and foreskin (Fig 6*C*) using the probe B (Fig 1*A*). Hybridizing tissue blots (Human Multiple Tissue Northern Blot I and II; CLONTECH, Basal, Switzerland) with probe B showed no repetin expression in all tissues tested except in thymus where a low intensity signal at approximatively 4 kb was detected (data not shown).

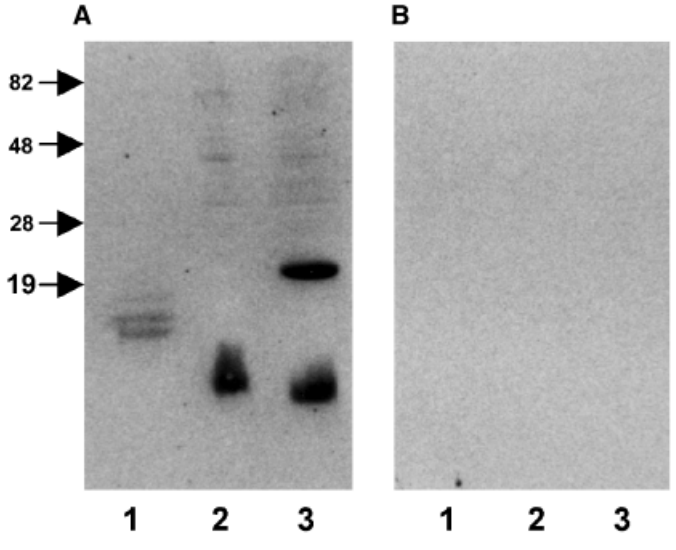

Figure 5
$^{45}Ca^{2+}$ overlay assay. (*A*) Autoradiograph after $^{45}Ca^{2+}$ overlay assay with protein extracts from psoriatic scales (*lane 1*), pET28HREF transformed BL21 (DE3) pLysS bacteria before IPTG induction (*lane 2*), and 180 min after IPTG induction (*lane 3*). (*B*) Autoradiograph after removing radioactive Ca^{2+} by incubation with cold Ca^{2+}. The positions of molecular weight markers in kDa are indicated on the left.

To further analyze repetin expression, a polyclonal antibody against peptide P4 from the C-terminal end of the repeat region (amino acids 570–584; Figs 1*B* and 2) was raised in rabbits and affinity purified. The corresponding antibody, called AF646, detected double bands with apparent molecular weights of 100 kDa both in the membrane and cytoskeletal cell fractions (Fig 3*A* and *B*) from human keratinocytes from suspension cultures and human foreskin. Bands with identical sizes became apparent (Fig 3*C*) with two other affinity-purified antibodies directed against peptides P1 or P3 (Fig 1*B*). A single band of 100 kDa (Fig 3*D*) was detected in lysates of IPTG-treated bacteria expressing full-length repetin from plasmid p28HR, indicating that repetin most likely undergoes post-translational modifications in mammalian cells, the nature of which is not yet known. Another explanation is the variation of the number of repeats between the different repetin alleles similar to what is observed with filaggrin repeats (Gan *et al*, 1990). Because we could not see any low molecular weight bands in western blots with these antibodies in extracts from human foreskin, it is unlikely that human repetin undergoes proteolytical processing during epidermal differentiation.

Expression in epithelial tissues was examined by immunofluorescence analysis using the affinity-purified antibody AF646 (Fig 7). Repetin was expressed in a scattered pattern in the upper granular layer of human interfollicular

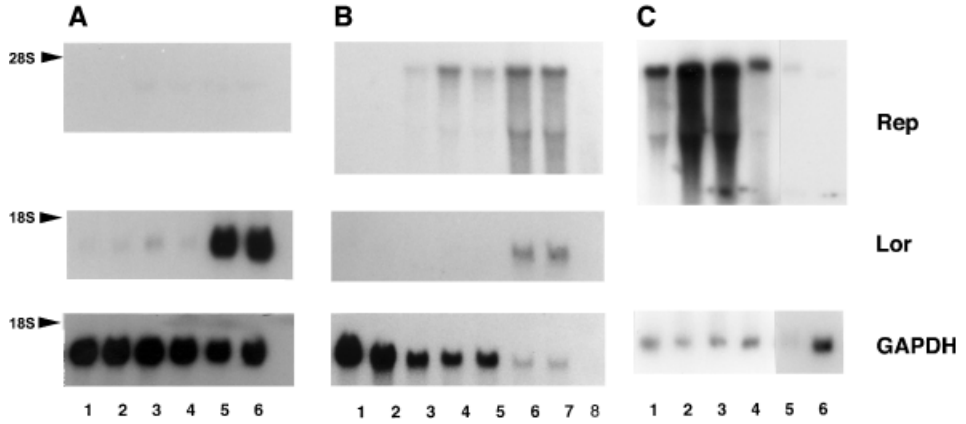

Figure 6
Repetin expression analysis in cultured human keratinocytes and epidermal biopsies. (*A*) RNA isolated from normal human keratinocytes cultured in high calcium medium on irradiated 3T3 feeder layer at confluency (*lanes 1 and 2*), 5 d (*lanes 3 and 4*) and 7 d (*lanes 5 and 6*) postconfluency. (*B*) Normal human keratinocytes were suspended in 1.75% methylcellulose and RNA isolated before suspension (*lanes 1 and 2*), and 17 h (*lane 3*), 24 h (*lanes 4 and 5*), or 48 h (*lanes 6 and 7*) after suspension. (*C*) Total RNA from keratinocytes after suspension culture of 48 h (*lanes 1–4*) and from biopsies from human skin (*lane 5*) or human foreskin (*lane 6*) was hybridized with probes for human repetin (Rep; probe B; Fig 1*A*), loricrin (Lor; Hohl *et al*, 1991), and glyceraldehyde-phosphate dehydrogenase (GAPDH). The positions of 28S and 18S rRNA are indicated on the left.

epidermis (Fig 7*A*) and strongly in the acrosyringium. A more prominent staining was found in the upper cell layers of human foreskin epidermis (Fig 7*B*). A characteristic granular pattern of strong repetin expression was detected in filiform papillae of the human tongue both in the anterior and posterior part of the papillae (Fig 7*C* and *D*). In lingual interpapillae tissue staining was present rarely and then only in upper layer cells. Repetin expression was also found in the inner root sheat of the hair follicle (Fig 7*E* and *F*).

Immunoelectron microscopy with antibody AF646 in human skin showed that basal and spinal cell layers of interfollicular epidermis were negative for repetin expression. The strongest repetin immunoreactivity was found in the granular layers, closely associated with keratohyalin granules (Fig 8*A*). In the transition zone between stratum granulosum and stratum corneum repetin, expression was diffusely distributed in the cytoplasma but not associated with any particular cellular structure or the cell membrane (Fig 8*B* and *C*). Repetin reactivity disappeared in the upper cornified layers (Fig 8*D*).

Discussion

In this report, we show the cloning and molecular characterization of human repetin, which has common structural features with the fused group of cornified CE precursor proteins such as profilaggrin, trichohyalin, and the recently described hornerin. This group of proteins is characterized by an N-terminal domain containing two EF hands which are followed by a central portion of repetitive units that vary in length and composition between the different group members.

The full-length sequence of the human repetin gene was deduced by cDNA and genomic DNA cloning. The gene consists of three exons, an overall genomic organization found also in other members of the fused gene family. The sizes of exons 1 and 2 have been conserved between the mouse and human gene whereas the third murine exon is about 1200 bp longer. Human repetin protein is considerably smaller in size than its murine homologue, mainly because of reductions in the number of repeats (28 in human *vs* 49 in mouse) and the size of the C-terminus. A species comparison of the repeat structure showed that in the human protein the sequences B and B′ (Fig 5b in Krieg *et al*, 1997) have been conserved, whereas the other murine repeat sequences have been lost. The repeats of the human protein showed positional conservation of glutamine residues at positions 1, 5, and 7, glycine at positions 6 and 12, serine residues at positions 8 and 9, and histidine residues at position 10. This is very similar to the murine consensus repeat sequence (B and B′ in Fig 5b in Krieg *et al*, 1997) with the exception of glutamine (additional residue at position 11) and glycine (position 3 instead of 6). This suggests that the amino acid positions and repeat length rather than the total number of repeats have been conserved during evolution. Secondary structure algorithms predict a β-sheet structure for the central domain of human and mouse repetin, suggesting that conserved arrangement of specific residues within the 12 amino acid repeat is important for stability and structural functions. Furthermore, repetin contains only one type of repeat unlike involucrin repeats, which clearly belong to two different types. Interestingly, involucrin, SPRR's, trichohyalin, and LEP featuring short repeats are not proteolytically processed during epidermal differentiation in contrast to the long repeats in profilaggrin, which are degraded to smaller subunits. This is consistent with the results in human foreskin extracts showing no processing of repetin (Fig 3*B*).

Protein motif searches and sequence comparison with known fused proteins (Fig 4) provided evidence for the presence of two EF hand motifs at the N-terminus of human repetin. The homologies are the highest around those residues implicated in Ca^{2+} binding in calmodulin (lower part of Fig 4; Falke *et al*, 1994). To test the functionality of these EF hands, an overlay assay with radioactive Ca^{2+} was performed, which clearly demonstrated that this part of human repetin binds Ca^{2+} in a reversible manner (Fig 5). Although we did not test *in vitro* the calcium binding of the full-length protein, we strongly think, based on the highly similar sequence of EF hands from repetin, other "fused" proteins, and S100 proteins (Fig 4), that also full-length repetin is a calcium binding protein. The stretch between the end of the EF hands and the beginning of the repetitive region in profilaggrin has been shown to contain functional nuclear localization signals important for the nuclear transfer of the N-terminal domain of profilaggrin after cleavage into filaggrin units (Pearton *et al*, 2002; Zhang *et al*, 2002). Searching the

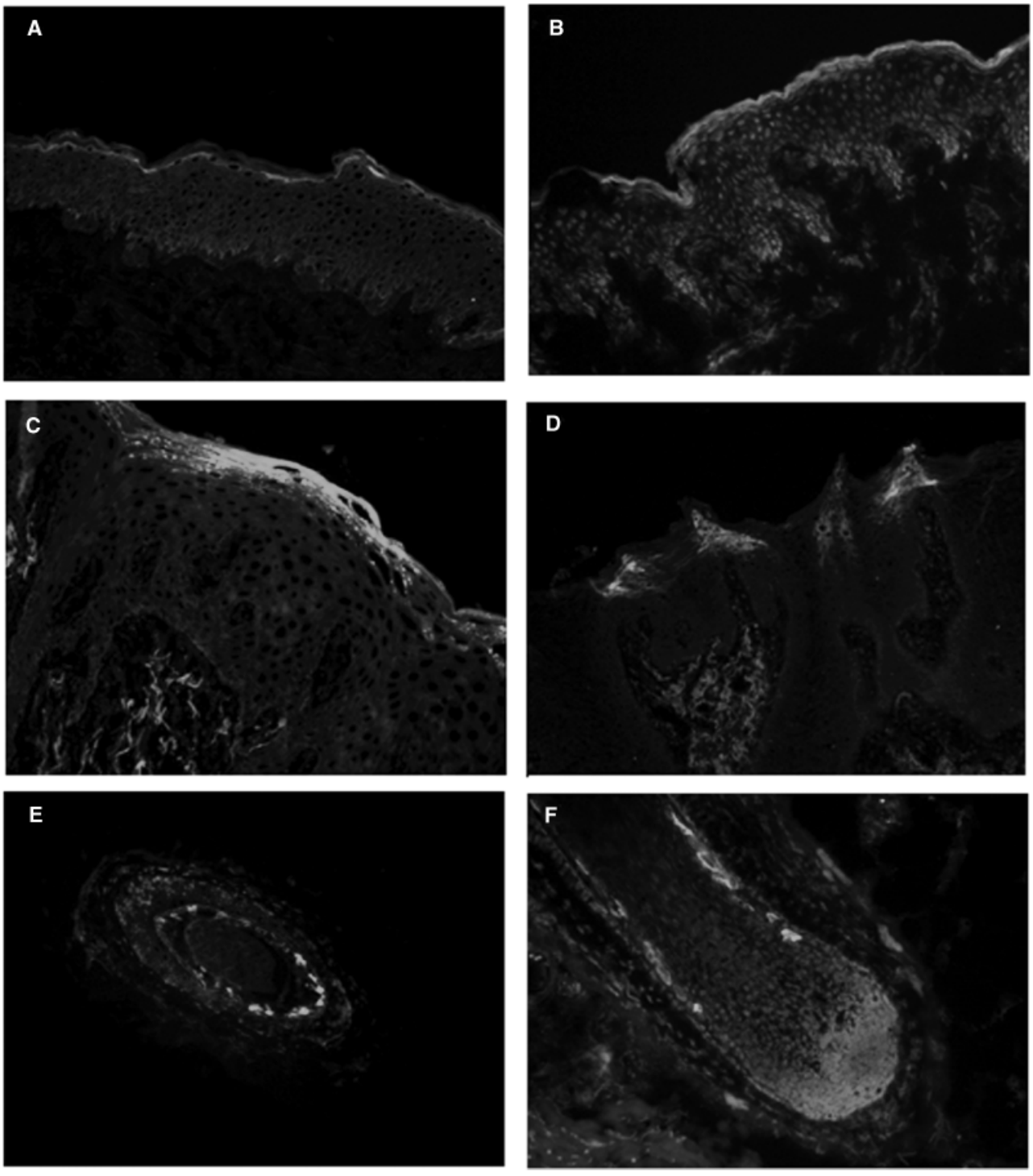

Figure 7
Repetin is a late epidermal differentiation marker highly expressed in tongue. Immunofluorescence analysis of repetin was carried out using antibody AF 646 and FITC-labeled anti-rabbit IgG antibody on frozen section of human skin (*A*), human foreskin (*B*), human tongue (*C* and *D*), and human hair follicles (*E* and *F*).

corresponding region in human repetin with the PSORTII program did not reveal any such signal indicating that human repetin most likely does not enter the nucleus.

In adherent human keratinocytes cultured in high-calcium medium human repetin is not expressed even at 7 d postconfluence (Fig 6*A*) whereas loricrin mRNA, a gene expressed late in terminal differentiation, is present after 5 d (Hohl *et al*, 1991). Keratinocytes in suspension express repetin mRNA with a maximum between 24 and 48 h after the start of the suspension culture (Fig 6*B*). Thus, human repetin is synthesized late in differentiation, probably in keratinocytes undergoing the final steps of the transformation from granular cells into stratum corneum cells. *In vivo*, repetin mRNA is expressed in the trunk and foreskin epidermis only at very low levels (Fig 6*C*), consistent with data from immunolocalization showing patchy, punctuate stain-

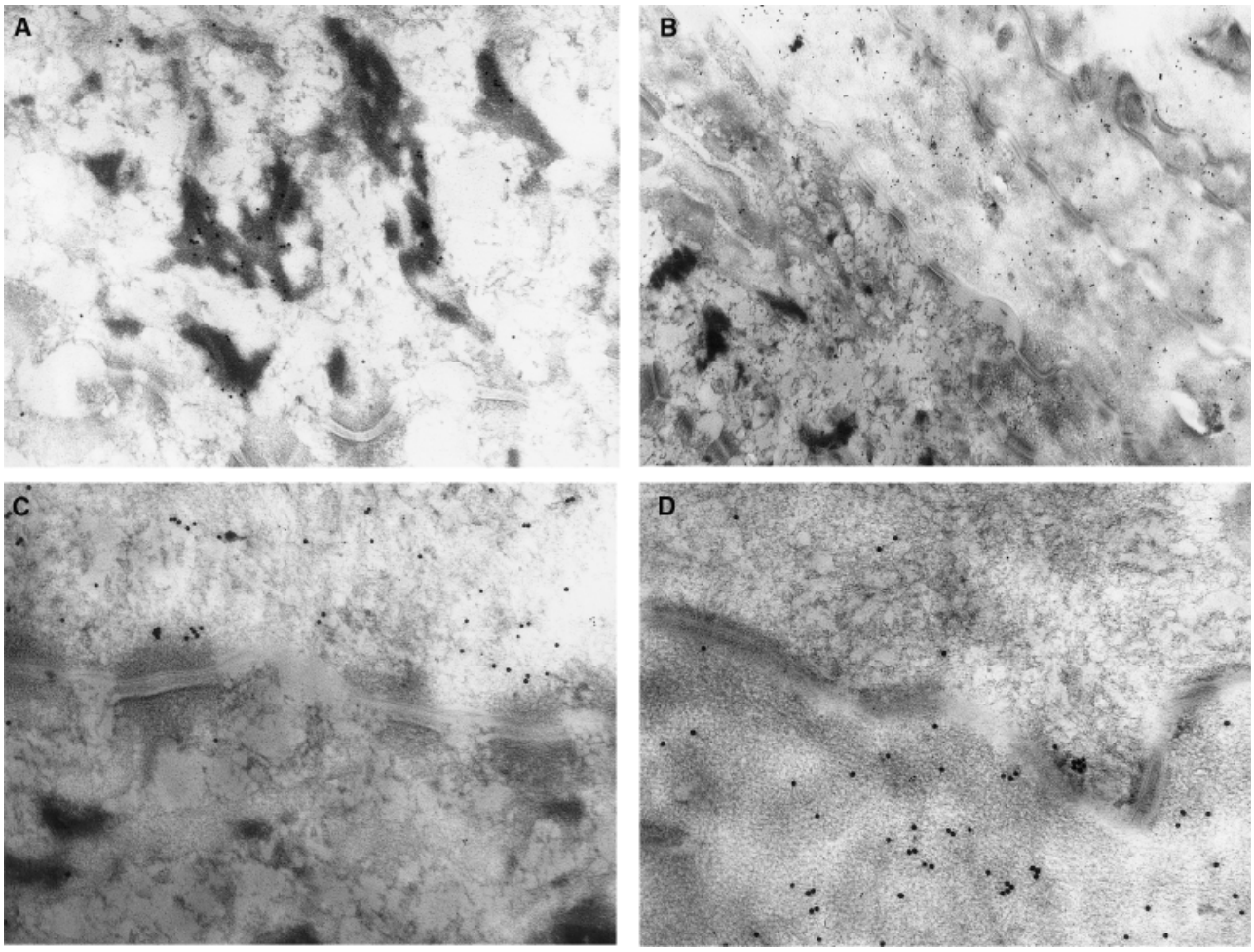

Figure 8
Repetin is associated with keratohyalin granules and is expressed in the transition zone in human skin. Immunelectron microscopic analysis was carried out with anti-repetin antibody AF646 and gold-labeled (15 nm) secondary antibody. (*A*) Association of repetin with keratohyalin granules in the upper granular layers. (*B*) Repetin expression in the zone between upper granular layer and the stratum corneum. (*C*) Higher magnification of the same region as in (*B*). (*D*) Demonstration that repetin immunoreactivity is drastically reduced in the upper stratum corneum. *Scale bar*: 440 nm (*A*), 1100 nm (*B*), 360 nm (*C*), 250 nm (*D*).

ing in the human trunk, and foreskin epidermis (Fig 7*A* and *B*). Higher expression levels of repetin were found in the keratinized area of filiform papilli of the tongue (Fig 7*C* and *D*) overlapping with the reported staining for loricrin (Hohl *et al*, 1993). Surprisingly, we found expression of repetin in the inner root sheath of human hair follicles where also trichohyalin, another member of the "fused" gene family, is expressed (Rothnagel and Rogers, 1986). It remains to be seen whether the two proteins colocalize in the trichohyalin containing granules of the inner root sheath. Protein chemical data demonstrated that repetin is crosslinked to trichohyalin in the inner root sheath of mouse hair follicle (Steinert *et al*, 2003), and to loricrin, SPR1, and SPR2 in the human foreskin and oral mucosa (P. Steinert, unpublished). This raises the possibility that repetin is crosslinked by transglutaminases, which may help to provide mechanical stability. Previous work has shown that the same cell compartment expresses involucrin but not loricrin (Hohl *et al*, 1993; de Viragh *et al*, 1994), suggesting a specific function for repetin in hair follicle biology.

In conclusion, repetin expression is highly variable among different tissues with good expression in tongue tissue. Moreover, expression occurs very late during terminal differentiation, indicating that repetin might be incorporated into the cornified CE. But experiments to show that repetin is a substrate for keratinocyte transglutaminase failed because of difficulties in expressing the full-length protein in mammalian cells. Nevertheless, results of knockout experiments correlate very well with the role of repetin as cornified CE precursor. Mice with an ablated loricrin gene, which are phenotypically normal, show strong upregulation of repetin expression, suggesting a role in substituting for the loss of loricrin in the formation of the cornified CE (Koch *et al*, 2000). Mice deficient in the transcription factor Krüppel-like factor 4 were reported to upregulate the expression of repetin, SPRR2A, and plasminogen activating inhibitor 2 which, however, does not rescue the animals from perinatal death and defects in skin barrier function (Segre *et al*, 1999). Finally, transgenic mice expressing claudin 6 in the suprabasal epidermal layers die perinatally because of excessive water loss and manifest aberrant expression of late epidermal differentiation markers including repetin (Turksen and Troy, 2002). In conclusion, repetin is a fused gene that is not highly expressed in homeostatic ep-

idermis but that can be upregulated several fold upon dysregulation of terminal differentiation because of inactivation of structural or regulatory proteins.

Materials and Methods

Isolation of repetin genomic clones Membrane filters with a high-density gridded flow-sorted chromosome 1-specific cosmid library (Nizetic *et al*, 1994) were hybridized with a ^{32}P-labeled mouse repetin probe (Genbank X99251, nt 7198–7441) (Krieg *et al*, 1997) in Church buffer at 65°C (Nizetic and Lehrach, 1995). The membranes were washed at a final stringency of 30 mM NaCl, 3 mM sodium citrate, 0.1% SDS, pH 7 at 56°C for 45 min. Positive cosmid clones were purified by Qiagen Midi columns (Qiagen, Hombrechtikon, Switzerland) and further analyzed by Southern blot hybridization using the same mouse repetin probe.

Chromosomal localization Fluorescence *in situ* hybridization on metaphase chromosomes was carried out as described (Marenholz *et al*, 1996). YAC and human genomic DNA digested with various rare cutting restriction enzymes were separated by rotating field gel electrophoresis and transfered to Genescreen Plus (Perkin Elmer, Schwerzenbach, Switzerland) membranes (Volz *et al*, 1993; Marenholz *et al*, 1996). Hybridization with radiolabeled probes was performed as described (Volz *et al*, 1993).

Cloning of repetin cDNA For cloning the repetin cDNA, a keratinocyte lambdaZAPII cDNA library (South *et al*, 1999) was hybridized with a ^{32}P-labeled probe B (Fig 1*A*) isolated from cosmid clone 31H23. Plasmid DNA was excised from the phages using the ExAssist Helper Phage system (Stratagene, Switzerland) and purified using Qiagen Midi columns (Qiagen, Amsterdam, The Netherlands). The 5′ part of the cDNA was isolated by 5′-RACE with the Marathon cDNA amplification kit (CLONTECH) using RNA isolated from human keratinocytes cultured for 24 h in suspension. Then, PCR amplification was performed with the primers HR-37 (5′-CTTTATGGGTTCGCCTGTCCTGTGT-3′) and HR-44 (5′-AATGGCTCAACTCCTGAATAGCAT-3′) and the Expand High Fidelity System (Roche, Rotkreuz, Switzerland). The amplification product was cloned into the pGEM-T Easy vector (Promega, Wallisellen, Switzerland) and subjected to sequence analysis.

Cell culture Punch biopsies obtained from normal volunteers were used to establish primary keratinocytes on lethally irradiated murine 3T3 fibroblasts in keratinocyte complete medium (Rheinwald and Green, 1975; Rheinwald and Green, 1977; Green *et al*, 1979). Suspension cultures were carried out in Dulbecco's modified Eagle's medium, 10% fetal calf serum, 1.75% methylcellulose, penicillin 100 U per mL, and streptomycin 0.1 mg per mL. Ten million cells were seeded in six-well plates previously coated with 0.6% polyHEMA to inhibit cell attachment.

Isolation of RNA and northern blot analysis Total RNA was isolated using the guanidine–thiocyanate method (Chomczynski and Sacchi, 1987). Northern blot analysis using 13 μg of total RNA per lane was carried out as described (Huber *et al*, 1997).

Bacterial expression A full-length expression clone of human repetin was constructed by PCR amplification with primers HR-50 (5′-CATATGGCTCAACTCCTGAATAGC-3′) and HR-52 (5′-CTCGAGCTAGTATGGGTAGGATTTCTGC-3′) and plasmid pHREP (Fig 1*A*) and subcloning into *Nde*I and *Xho*I sites of pET28a(+) (Novagen, Lucerne, Switzerland) yielding plasmid p28HR. For the expression of the EF hands domain (amino acids 1–145), primers HR46 (5′-CCATGGCTCAACTCCTGAATAGC-3′) and HR47 (5′-CTCGAGGTGGGAATCTCTGTCTTG-3′) were used for amplification on plasmid pTcR3.8 (Fig 1*A*). The fragment was cloned into *Nco*I and *Xho*I sites of pET28b(+) vector (Novagen) yielding plasmid p28HREF. Constructs were verified by sequencing. Recombinant proteins were expressed in BL21-CodonPlus (DE3)-RIL bacteria (Stratagene) by induction with 1 mM IPTG and incubation for 3 h at 37°C. Cell extracts were prepared by denaturation in Laemmli buffer.

Expression analysis by immunodetection Polyclonal rabbit antibodies against human repetin were developed against peptides P1, P3, and P4 (Fig 1*B*) (QCB, Hopkinton; Eurogentec, Liege, Belgium). Immunofluorescence analysis was carried out as described (Hohl *et al*, 1998). Proteins in differential cell extracts were analyzed by SDS-PAGE using 40 μg proteins, transfer to Hybond-C membrane, and detection of the primary antibody with peroxidase-linked secondary antibody and the ECL reagent (Amersham, Otelfingen, Switzerland).

Bioinformatics Sequence analyses and comparisons (ClustalW alignment) were carried out with the MacVector 6.5.3 software package (Oxford Molecular, Oxford, UK). Protein motifs were analyzed using the PSORTII program (http://psort.ims.u-tokyo.ac.jp)

Immune electron microscopy Immune electron microscopic examination of a biopsy from clinically unaffected skin was performed using a Zeiss 10B electron microscope (Carlzeiss, Oberkocher, Germany) (Ishida-Yamamoto *et al*, 1993; Frenk *et al*, 1996; Breitkreutz *et al*, 2004). Biopsies were obtained after written informed consent and the study, approved by the medical ethical committee of the University of Lausanne Medical Faculty, was conducted according to Declaration of Helsinki Principles.

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