SLURP1 Is a Late Marker of Epidermal Differentiation and Is Absent in Mal de Meleda

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SLURP1 is a secreted member of the LY6/PLAUR protein family. Mutations in the *SLURP1* gene are the cause of Mal de Meleda (MDM), a rare autosomal recessive genetic disease, characterized by inflammatory palmoplantar keratoderma. In this study, we have analyzed the expression of SLURP1 in normal and MDM skin. SLURP1 was found to be a marker of late differentiation, predominantly expressed in the granular layer of skin, notably the acrosyringium. Moreover, SLURP1 was also identified in several biological fluids such as sweat, saliva, tears, and urine from normal volunteers. In palmoplantar sections from MDM patients, as well as in their sweat, mutant SLURP1, including the new variant R71H-SLURP1, was either absent or barely detectable. Transfected human embryonic kidney 293T cells expressed the MDM mutant SLURP1 containing the single amino-acid substitution G86R but did not tolerate the MDM mutation W15R located in the signal peptide. Thus, most MDM mutations in *SLURP1* affect either the expression, integrity, or stability of the protein, suggesting that a simple immunologic test could be used as a rapid screening procedure.

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INTRODUCTION

Mal de Meleda (MDM, OMIM 248300) is a rare recessive palmoplantar keratosis (Neumann, 1898). This disorder is characterized by transgressive palmoplantar keratoderma, keratotic lesions over the joints, perioral erythema, brachydactyly, nail abnormalities, hyperhydrosis, and inflammation (Durmus *et al.*, 1999). At the histological level, hyperkeratosis, hypergranulosis, and acanthosis are observed (Frenk *et al.*, 1996). Mutations in the gene *SLURP1* (secreted LY6/PLAUR-related protein 1) located on chromosome 8q24.3 were found as the cause of MDM (Fischer *et al.*, 2001).

SLURP1 is a secreted member of the LY6/PLAUR family of proteins, characterized by a distinct disulfide bonding pattern between 10 cysteine residues and the presence of a consensus motif CCX_4CN (Casey *et al.*, 1994; Ploug and Ellis, 1994). These features lead to a three-finger folding in a snake toxin-like form. On the basis of the presence or absence of a glycosyl-phosphatidylinositol-anchoring signal sequence, secreted or membrane-linked (via a glycosyl-phosphatidylinositol-anchor) protein subfamilies can be distinguished (Adermann *et al.*, 1999). The LY6/PLAUR family regroups proteins containing a common three-finger protein binding domain but without functional link (Rollins *et al.*, 1991; Ioka *et al.*, 2003, Ploug, 2003).

SLURP1 potentiates the action of acetylcholine on the α7 nicotinic receptor (Chimienti *et al.*, 2003), which plays an important role in the differentiation of stratified squamous epithelium (Arredondo *et al.*, 2002; Kurzen *et al.*, 2004). This receptor is also blocked by neurotoxins such as bungarotoxins and is modulated by LYNX1-C, a glycosyl-phosphatidylinositol-anchored LY6/PLAUR protein (Miwa *et al.*, 1999; Ibanez-Tallon *et al.*, 2002). It was reported that recombinant SLURP1 has a proapoptotic effect on keratinocytes and upregulates the expression of transglutaminase 1, cytokeratin 10, p21, and caspase-3 (Arredondo *et al.*, 2005). SLURP1 was first isolated from human blood and urine (Adermann *et al.*, 1999). Its mRNA was later identified in skin and fetal bladder (Fischer *et al.*, 2001) and the protein was detected in uterus and trachea (Mastrangeli *et al.*, 2003). In mouse *SLURP1*, transcripts were also found in the cornea (Norman *et al.*, 2004), lung, stomach, trachea, and esophagus (Mastrangeli *et al.*, 2003).

As SLURP1 plays an important role in palmoplantar skin homeostasis, as revealed by the MDM clinical pattern, we have further characterized its expression and localization in human skin, identified a new mutation causing MDM, analyzed the expression of SLURP1 in MDM patients at the protein level, and investigated the properties of two-point mutated SLURP1 associated with MDM. Our results indicate that most MDM cases could be identified by a simple immunologic test.

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Abbreviations: Ab, antibody; ISH, in situ *hybridization; MDM, Mal de Meleda; RT-PCR, reverse transcription-PCR; SLURP1-MH, SLURP1 myc and H6-tagged at the carboxy-terminus; WB, Western blot and Western blotting*

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RESULTS

SLURP1 expression in cultured keratinocytes is dependent on calcium-induced differentiation

SLURP1 gene transcription and translation was analyzed in cultured keratinocytes by reverse transcription-PCR (RT-PCR) and Western blotting (WB) with thoroughly characterized polyclonal anti-SLURP1 antibodies (see Materials and Methods and Figure S1). Before total confluence and calcium shift, corresponding to time points D-2 and D0, respectively, *SLURP1* transcripts and the protein could not be detected in cultured keratinocytes (Figure 1). Three days after the calcium shift both *SLURP1* mRNA and the protein could be detected in plantar keratinocytes. The same phenomenon was observed in foreskin keratinocytes but occurred later at D7. SLURP1 expression was slightly delayed when compared with the expression of keratin 10, a marker of differentiation, while the ratio of collagen 17, a hemidesmosomal protein expressed in the basal layer of epidermis, to total keratinocyte protein was decreasing during the differentiation process (Figure 1). Thus, SLURP1 is only expressed in differentiated keratinocytes.

SLURP1 is expressed in the granular layer of human skin

To further characterize the expression of *SLURP1* during differentiation of skin epithelium, we performed *in situ* hybridization (ISH) analysis. Both in foreskin and plantar skin, *SLURP1* transcripts were predominantly detected in the granular layer (Figure 2). SLURP1 mRNA was also seen in eccrine gland conducts (indicated by an arrow in Figure 2). Cell culture and ISH results were coherent with each other. As immunofluorescence results obtained with anti-SLURP1 antibody (Ab) on skin sections were not consistent (see legend of Figure S1), we used laser-captured microdissection on plantar skin sections to separate upper and lower epidermis (Figure S2a–c). WB analysis revealed that SLURP1 was indeed only present in the upper layers of the epidermis, as cornulin (Contzler *et al.*, 2005) (Figure S2d) or loricrin (data not shown). SLURP1 is therefore expressed late in differentiation of the epidermis.

SLURP1 is present in several biological fluids

As ISH experiments revealed that *SLURP1* was expressed in eccrine sweat gland conducts and SLURP1 is secreted, we tested whether the protein was present in sweat. In all four analyzed samples from healthy volunteers, SLURP1 was clearly detected by WB (Figure 3). SLURP1 was also present in saliva and tears. However, in tears the level of SLURP1 was uneven, and the concentration of SLURP1 in saliva was lower than in sweat (Figure 3). SLURP1 was originally isolated from urine and blood (Adermann *et al.*, 1999). In urine, SLURP1 was sufficiently concentrated to be detected by WB (Figure 3). In contrast, we were unable to identify SLURP1 in serum by WB (data not shown), suggesting that its concentration, estimated to be in the range of 13 ng/ml with an ELISA test (Mastrangeli *et al.*, 2003), was less than in the other biological fluids we tested.

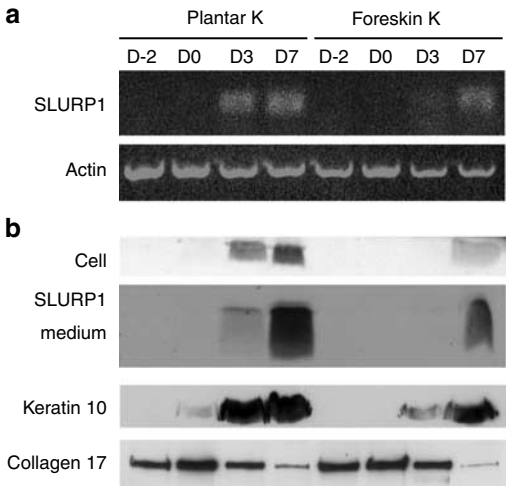

Figure 1. *SLURP1* is expressed in primary keratinocytes only after induction of differentiation. (**a**) Total RNA was isolated from cells at time D-2, D0 (corresponding to near confluence and calcium shift in the culture medium), D3 and D7. RT-PCR (25 cycles) was performed with primers specific for *SLURP1* and *β-ACTIN* transcripts. cDNA was size-fractionated on 1.5% agarose gel and stained with ethidium bromide. (**b**) Whole-cell extracts or cell culture medium (30 μl each), prepared in parallel with RNA (see above), were separated on 15 or 6 % SDS-PAGE and blotted onto nitrocellulose membrane. Membranes were incubated with anti-SLURP1, anti-keratin 10, and anti-collagen 17 Ab.

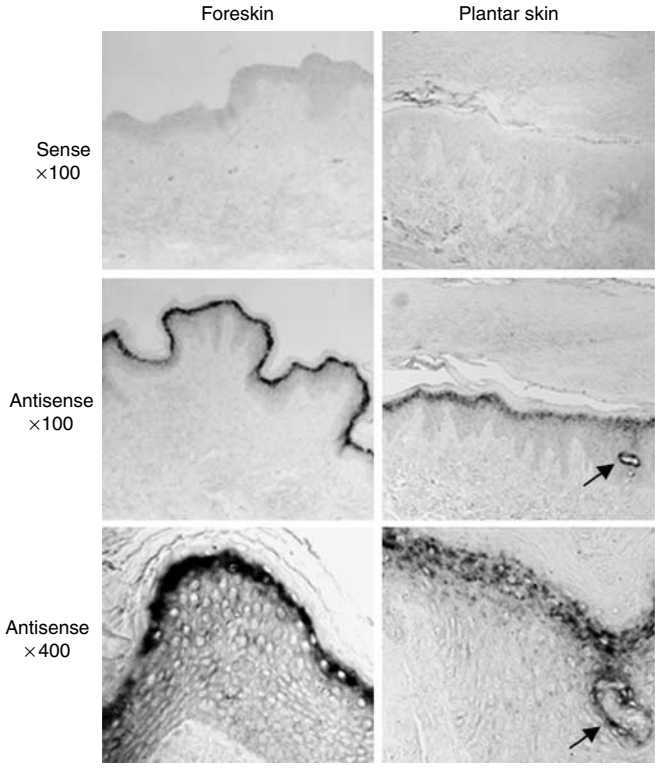

Figure 2. *SLURP1* is transcribed in the granular layer of human skin. ISH analysis on human foreskin and plantar skin tissue sections was performed with sense and anti-sense cRNA probes. After immunodetection of digoxigenin-labeled probes, the granular layer of both tissues was specifically stained, as well as eccrine sweat gland conducts in plantar skin (arrow).

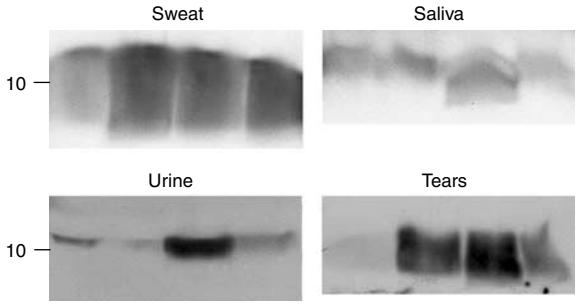

Figure 3. SLURP1 is present in biological fluids. The presence of SLURP1 in sweat, saliva, tears, and urine (20 μl each) from four healthy volunteers was analyzed after 15% SDS-PAGE by WB with anti-SLURP1 Ab.

Identification of a new mutation in *SLURP1* underlying MDM
Genomic DNA was isolated from a patient suffering from compact, yellow hyperkeratosis and desquamation of palms and soles, accompanied by onychodystrophy of all nails on both hands and feet, a clinical pattern compatible with MDM. Sequencing of PCR-amplified *SLURP1* gene revealed indeed the existence of a new, homozygous, single-point mutation in the open reading frame, 238G>A (NM_020427) leading to the amino-acid substitution R71H. Within the primary protein sequence of SLURP1, four stretches of amino acids can be distinguished. The amino-terminus polypeptide corresponds to the signal sequence for secretion, which is cleaved by the signal peptidase complex. The three other stretches constitute the three loops characteristic for the three-dimensional structure of LY6/PLAUR members (Table 1) (Casey *et al.*, 1994; Ploug and Ellis, 1994). The mutation R71H is located in between loops 2 and 3. Table 1 recapitulates all mutations found so far in *SLURP1* gene in relation to MDM phenotype. So far no obvious correlations between genotype and phenotype could be identified in MDM patients (Bouadjar *et al.*, 2000; Fischer *et al.*, 2001; Bakija-Konsuo *et al.*, 2002; Charfeddine *et al.*, 2003; Eckl *et al.*, 2003; Marrakchi *et al.*, 2003; Ward *et al.*, 2003).

SLURP1 and MDM
Several mutations found in *SLURP1* gene in MDM patients theoretically affect the integrity of the protein (see Table 1). As the effect of MDM mutations had never been investigated at the protein level, we analyzed the expression of SLURP1 in some MDM patients. We had four plantar biopsies from MDM patients at disposal with two mutations: (1) the early stop codon R96X (three patients) and (2) a splicing site mutation in intron 2 (178+1G→A) (Fischer *et al.*, 2001), altering the protein sequence after residue 59. WB analysis of proteins extracted from biopsy cryosections (5 μm thick) revealed that SLURP1 was absent in all four studied MDM samples when compared to two normal tissues (Figure 4a). Because the size of the biopsies was not identical, the upper part of the membrane was blotted with anti-keratin 14. This analysis revealed that the differences in SLURP1 amount were not correlated with that of keratin 14. Our results indicate the absence of immunogenic peptide in tissue extracts from the MDM patient bearing these types of mutation.

The amount of SLURP1 is high and quite constant in sweat collected from normal people by the pilocarpine method; therefore, we analyzed sweat from three MDM patients carrying the following mutations: the homozygous amino-acid substitution R71H, the homozygous early stop codon R96X, and the heterozygous mutations 82delT and W15R. In parallel, we analyzed by dot blotting the amount of IgA in sweat (Metze *et al.*, 1989). In all sweat samples, IgA was clearly present, whereas SLURP1 could not be detected in two out of three MDM patients, and very little immunogenic peptide was present in sweat from the third MDM patient with the R71H amino-acid substitution (Figure 4b). The mutation R71H is localized in the peptide used to raise the most sensitive Ab 17 and thereby could affect the immuno-reactivity of the protein towards this Ab. Therefore, we analyzed the level of R71H-SLURP1 in sweat with Abs 11 and 17 separately. With both Abs the amount of R71H-SLURP1 in sweat was much less than in that from a normal volunteer (data not shown).

Apart from R71H, other mutations in SLURP1 from MDM patients lead to a single amino-acid substitution (Table 1). We analyzed the effect of mutations W15R, located in the signal sequence responsible for secretion of the protein, and G86R, situated in the mature part of the protein, on the properties of SLURP1. Human embryonic kidney 293T cells were transfected with three constructs coding for wild-type SLURP1-MycH_6 (wt-SLURP1-MH), W15R-SLURP1-MH, and G86R-SLURP1-MH. All of them expressed *SLURP1* transcripts (Figure 4c). WB analysis of both whole-cell extracts and culture medium revealed that wt-SLURP1-MH and G86R-SLURP1-MH were expressed and secreted, although the latter to a lower extend than the former (Figure 4d). In contrast, W15R-SLURP1-MH was undetectable even in cell extracts (Figure 4d). So *in vitro*, MDM point mutations in *SLURP1* have also a negative impact on the expression of the protein.

DISCUSSION

In this study, we have demonstrated that SLURP1 is a late marker of epidermal differentiation and is present in all analyzed biological fluids, sweat, saliva, tears, and urine. In connection with MDM, we have found a new point mutation R71H in SLURP1 causing the disease and analyzed for the first time at the protein level the impact of several mutations. Our results show that SLURP1 is almost undetectable in MDM skin or sweat. Therefore, most MDM cases can be detected by a simple immunologic test.

According to our results, *SLURP1* is predominantly expressed in the granular layer of the epidermis. Mastrangeli et al. (2003) conclude from a set of slightly divergent data (*SLURP1* transcription in culture keratinocytes was not dependent on calcium-shift) that *SLURP1* expression might be associated with the mid-to-late differentiation of human keratinocytes. However, their immunohistochemical staining of fixed human skin with anti-SLURP1 Ab in the spinous layer was weak and uneven in comparison with the strong and

Table 1. Mutations in *SLURP1* associated with MDM

Mutation[1]	Consequence on the protein	References
27A>C	No translation initiation codon M1L	(Eckl *et al.*, 2003)
69T>C	Point mutation W15R	(Eckl *et al.*, 2003; Marrakchi *et al.*, 2003)
108delT[2]	Frame shift after T27 and premature stop codon	(Fischer *et al.*, 2001; Marrakchi *et al.*, 2003; Ward *et al.*, 2003)
204+1G>A	Abnormal protein sequence after residue A59	(Charfeddine *et al.*, 2003; Marrakchi *et al.*, 2003)
238G>A	Point mutation R71H	This study
255T>C	Point mutation C77R	(Charfeddine *et al.*, 2003; Eckl *et al.*, 2003; Marrakchi *et al.*, 2003; Chao *et al.*, 2005)
282G>A	Point mutation G86R	(Eckl *et al.*, 2003)
282G>C	Point mutation G86R	(Eckl *et al.*, 2003)
312C>T	Point mutation R96X (stop codon)	(Fischer *et al.*, 2001; Eckl *et al.*, 2003; Marrakchi *et al.*, 2003)
319T>C	Point mutation L98P	(Yerebakan *et al.*, 2003)
322G>A	Point mutation C99Y	(Charfeddine *et al.*, 2003; Marrakchi *et al.*, 2003)

Signal sequence[3] Loop 1 Loop 2 Loop 3

MASRWAVQLLLVAA**W**SMGCGEALKCYTCKEPMTSAS*CRTITRCKPEDTAC*MTTLVTV*EAEYPFNQSPVVT***R***S*C*SSS**C**VATDPDSI**G**AAHLIFCCF**R**D**LC**NSEL

MDM, Mal de Meleda.
[1]Counting is based on Genbank NM_020427.
[2]The most frequent mutation (48% of the number of reported MDM kindreds), probably because of its location within a consensus deletion hot spot sequence CCTG surrounded by repeats (Charfeddine *et al.*, 2003).
[3]Structural regions along SLURP1 sequence are indicated in white, position of single MDM mutations in Bold, and targets of Ab 11 and 17 are Italic.

homogenous staining of the granular layer, whose pattern is identical to our ISH results. Arredondo *et al.* (2005) observe a progressively increasing staining of the upper layers of the epidermis on unfixed human foreskin sections with their monoclonal anti-SLURP1 peptide Ab. However, based on the weak expression of SLURP1 in foreskin and its high solubility in unfixed tissue sections (see Figure S2b), it is surprising that SLURP1 could be easily localized in this tissue. Analysis of MDM skin sections would be a way to test the specificity of this mAb.

Mastrangeli *et al.* (2003) found that IFN, epidermal growth factor, and all-*trans* retinoic acid repressed the expression of *SLURP1* in cultured keratinocytes. Therefore, *SLURP1* transcription appears to be tightly regulated during epidermal differentiation and can be modulated by several external stimuli.

SLURP1 was first identified in blood and urine (Adermann *et al.*, 1999). Here, we demonstrate that SLURP1 is also present in sweat, saliva, and tears. In relation to these results, positive immunoreactive cells were also observed in human gingiva, vagina, and ectocervical mucosa (Mastrangeli *et al.*, 2003), suggesting that SLURP1 should also be present in vaginal secretion. If SLURP1 has a specific function in these biological fluids is unknown. Actually, SLURP1 is a positive modulator of nicotinic acetylcholine receptor α7 (Chimienti *et al.*, 2003). In skin, SLURP1 localization correlates well with that of nicotinic acetylcholine receptor α7 (Grando *et al.*, 1995; Hagforsen *et al.*, 2002; Kurzen *et al.*, 2004). Moreover, the same receptor is also present in eccrine sweat ducts and glands (Hagforsen *et al.*, 2002), whose secretions are regulated by the cholinergic system (Kurzen *et al.*, 2004). Interestingly, MDM patients usually suffer from hyperhydrosis and have hypertrophic sweat glands (Bakija-Konsuo *et al.*, 2002). It was reported that recombinant SLURP1 induces apoptosis on cultured keratinocytes by increasing the activity of caspases 3 and 8, and could upregulate the expression of transglutaminase 1, keratin 10, p21, and caspase 3 (Arredondo *et al.*, 2005). Although these new results are interesting, it remains to be demonstrated by physico-chemical analysis (Almeida *et al.*, 2001) that this recombinant SLURP1, whose M_r 22,000 (Arredondo *et al.*, 2005) is higher than that of mature SLURP1 (see Figure S1d), is properly folded, a process requiring the formation of five disulfide bridges.

For the first time, the consequences of some mutations found in MDM patients were investigated with anti-SLURP1 antibodies either directly from biological material or *in vitro* from transfected cells. Mutations affecting the integrity of the protein result in the absence of immunogenic peptide. Premature termination codon caused by 108delT and the splicing site alteration 201+1G>A (Table 1), situated upstream the last exon (before residue 60 in SLURP1), will induce the nonsense-mediated mRNA decay (Maquat, 2004) and also alter the protein sequence recognized by Abs 11 or 17 (see Table 1). The nonsense mutation R96X, located in the third and last exon, prevents the translation of the last Cys residue of the protein, part of the LY6/PLAUR CCX_4CN motif, and thereby the formation of the fifth disulfide bridge. This shortening probably destabilizes the whole structure and particularly the third loop of the three-finger fold. The highly deleterious effect of the amino substitution W15R is more surprising. This mutation introduces a positively charged residue in the signal peptide sequence (Table 1), which negatively affects the probability for the presence of a signal

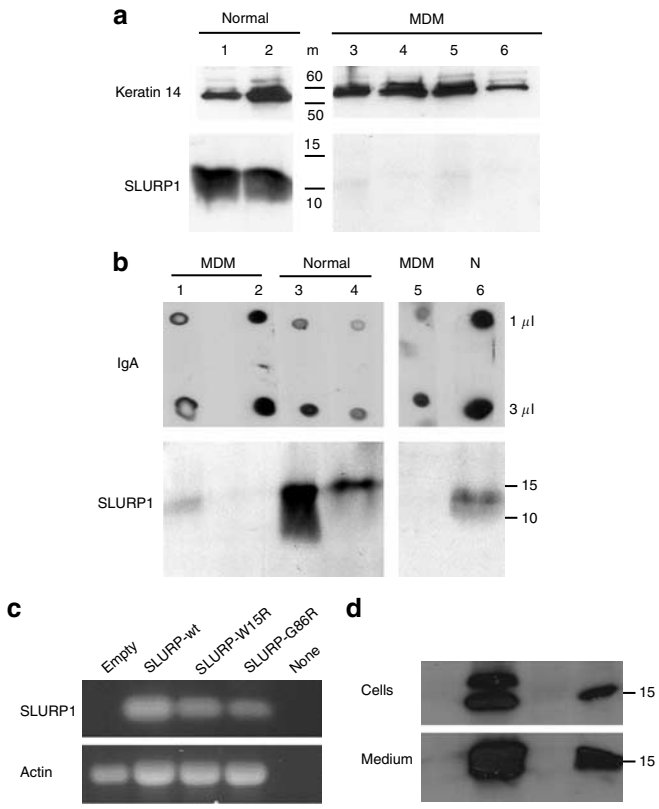

Figure 4. Most MDM mutations in *SLURP1* alter the expression or stability of the protein. (**a**) WB analysis on whole SDS-extracts from 5 μm plantar sections, separated on 15% SDS-PAGE, from normal volunteers (lanes 1 and 2) or MDM patients with mutations R96X (lanes 3–5) or splice site mutation 204 + 1G > A (see Table 1, lane 6) with anti-SLURP1 Ab. (**b**) Sweat from MDM patients carrying the mutation R71H (lane 1), or the heterozygote mutations 82delT and W15R (lane 2), or R96X (lane 5) or from healthy volunteers (lanes 3, 4 and 6) was either spotted (upper panel) or size fractionated (20 μl) on 15% SDS-PAGE (lower panel). The upper membrane was incubated with anti-IgA Ab, the lower with anti-SLURP1 Ab. (**c**) RT-PCR analysis on RNA isolated from cells transfected with pBudCE4, or pBud-SLURP1-MH, -W15R-SLURP1-MH, or -G86R-SLURP1-MH, a control without RNA was also included. All cells transfected with the SLURP1 constructs transcribed the encoded gene. (**d**) WB analysis on whole-cell extracts and culture medium from the same cells as in (**c**) with anti-SLURP1 Ab. No immunoreactive band could be detected in cells transfected with pBud-W15R-SLURP1-MH, in contrast to the other SLURP1 constructs. The second upper band in the second lane of cell extracts, not systematically observed, could be dimeric SLURP1.

peptide predicted by the program SignalP 3.0 (Bendtsen *et al.*, 2004), without however annihilating it. The absence of immunogenic peptide even in cell extracts nevertheless indicates that the W15R-SLURP1 is not correctly processed and probably degraded by the post-translational quality control (Wickner *et al.*, 1999). The single substitution R71H, located at the end of loop 2, has also a dramatic negative impact on the concentration of the protein in sweat. Even the mutation G86R, situated in the middle of loop 3, negatively affects the expression level of the protein by human embryonic kidney 293T cells. All these results suggest that SLURP1 stability is very sensitive to primary amino-acid sequence alterations, probably because of the degradation of the mutated protein by the post-translational quality control (Wickner *et al.*, 1999), by the reticulum endoplasmic quality control (Hirsch *et al.*, 2004) or by extracellular proteases, depending on the degree of misfolding of the protein.

Several amino-acid substitutions found in MDM patients are located towards the C-terminus of the protein (Table 1), suggesting that the third loop could be involved in the binding to its receptor, the nicotinic acetylcholine receptor α7. This conclusion is strengthened by the alignment of all SLURP1 sequenced so far, which reveals a higher conservation of the C-terminal part than the rest of the molecule (Figure S3).

The presence of SLURP1 in several biological fluids, for example, saliva, sweat, tears, and urine, suggests that an immunologic MDM pre-diagnosis could be performed without any invasive procedures. Based on our results, sweat contains the highest concentration of SLURP1 among the biological fluids tested and WB analysis from sweat gave the cleanest results (no major cross reaction with other sweat proteins of higher molecular mass), suggesting that a dot blot assay is feasible. Moreover, sweat can be collected with the standardized pilocarpine procedure, used for the diagnosis of cystic fibrosis (Beauchamp and Lands, 2005). This could explain the small variations observed among the tested samples. On the other hand, both urine and saliva are easier to collect. For example, we systematically detected SLURP1 in 17 saliva samples analyzed from healthy volunteers aged from 3 to 50 years. Further work is required to develop and optimize such tests. Nevertheless, molecular screening will remain the gold standard for the diagnosis of genetic diseases.

MATERIALS AND METHODS

Plasmids

Sequence corresponding to mature SLURP1 protein sequence, Res. 23–103 (Genbank NP_065160) with the carboxy extension SRGSEQKLISEEDLNMHTGH$_6$-COOH, identical to pBud-SLURP1 (Chimienti *et al.*, 2003), bearing myc (underlined) and His$_6$ tags, was cloned into pGEX-4T1 (GE Healthcare, Otelfingen, Switzerland) in frame with glutathione *S*-transferase sequence. Cloned *SLURP1* in pET-28a(+)-amp^R (kanamycin-resistance gene in pET-28a(+) (Novagen, Luzern, Switzerland) was replaced with a β-lactamase gene) leads to the expression of SLURP1 Res. 22–103 (Genbank NP_065160) flanked by Res. M and EH$_6$ at the N- and C-terminus, respectively. LYPD2 and LYNX1-B were also cloned into pET-28a(+)-amp^R so that the expressed protein corresponds to Res. 22–102 (Genbank NP_991108) and Res. 23–97 (Genbank NP_803253), respectively, with the extra Res. M at the N-terminus and LEH$_6$ at the C-terminus.

Expression and purification of recombinant protein in *E. coli*

Origami strain (Novagen, Luzern, Switzerland) was used as recipient bacteria for expression of recombinant proteins, induced by the addition of 0.1 mM isopropylthiogalactoside in the cultures for 4 hours at 37°C (independent on the conditions tested, all recombinant LY6/PLAUR proteins were completely insoluble). To prepare whole bacterial extracts, centrifuged bacteria, corresponding to an OD600 nm of 0.6, were lyzed in 1/10 volume of

2 × SDS-sample buffer. H_6-tagged proteins were purified to apparent homogeneity according to a procedure described in Chimienti *et al.* (2003) in the presence of 8 M urea.

Antibodies, dot, and WB

Rabbit polyclonal Ab nos. 11 and 17 were raised against the synthetic peptides (1) ^{37}CRTITRCKPEDTA and (2) ^{58}EAEYPFNQ SPVVTRSC (Genbank NP_065160), respectively, and purified by affinity chromatography at Eurogentec (Seraing, Belgium). Their specificity was exhaustively investigated (Figure S1). Both Abs were usually mixed to increase the sensitivity of WB. Rabbit polyclonal anti-cornulin 1:2,000 (Contzler *et al.*, 2005), anti-keratin 10 AF96 1:200, anti-loricrin AF62 1:2,000, anti-myc 1:2,000 (Santa Cruz Biotechnology, Nunningen, Switzerland), anti-human IgA 1:500 (DakoCytomation, Baar, Switzerland), guinea pig polyclonal anti-keratin 14 AF29 1:1,000, mouse monoclonal anti-collagen-17 233 1:500, anti-polyhistidine (clone HIS-1) 1:1,000 (Sigma, Buchs, Switzerland) were used in WB analysis. After size-fractionation of proteins on SDS-PAGE, they were transferred onto Hybond-ECL (GE Healthcare, Otelfingen, Switzerland) for WB (Contzler *et al.*, 2005).

For the analysis of IgA in sweat, 1 μl and 3 × 1 μl sweat were spotted onto nitrocellulose membrane. After drying at room temperature, membranes were processed as a WB. Collection and use of human samples were performed with the informed consent of all patients and healthy donors, and the approval of the respective institutions. This study was conducted in accordance to the Declaration of Helsinki Principles.

Preparation of foreskin extracts

Freshly excised foreskins were washed 3 × with phosphate-buffered saline, pieces of 0.8 g were homogenized in 3 ml of SDS-sample buffer supplemented with protease inhibitor mix without EDTA with an Ultra Turrax T18 basic (Sigma, Buchs, Switzerland). Homogenates were boiled for 5 minutes, centrifuged, and the supernatant saved.

Immunoprecipitation

Ab 11, Ab 17, and rabbit polyclonal anti-myc Ab (Santa Cruz Biotechnology, Nunningen, Switzerland) 10 μg each were loaded onto 25 μl of Protein G Sepharose (GE Healthcare, Otelfingen, Switzerland) in a final volume of 100 μl consisting of phosphate-buffered saline supplemented with 1% BSA, 0.1% Tween 20 and protease inhibitor mix without EDTA (Roche, Rotkreuz, Switzerland) for 1 hour at 4°C. A control without Ab was also included. After thorough washing, the beads were split in two and incubated with 100 μl of Protein G-precleared SLURP1-conditionned medium (see below) or control medium from mock-transfected human embryonic kidney 293 cells for 2 hours in the cold. After extensive washes, proteins bound to the beads were dissolved in SDS-sample buffer and analyzed by WB.

Cell culture

In vitro differentiation of human keratinocytes and cell extracts preparation were performed as described in Contzler *et al.* (2005). RNA was isolated with GenElute mammalian total RNA miniprep kit (Sigma, Buchs, Switzerland). Human embryonic kidney 293T cells were cultured, transfected, and cloned according to Chimienti *et al.* (2003). When transfected cells were about 90% confluent, they were rinsed and cultured for 72 hours in medium deprived of fetal calf serum. Medium was then collected and cells harvested as described previously (Contzler *et al.*, 2005). Proteins in the medium were concentrated by precipitation with 5% trichloroacetic acid for 30 minutes on ice and centrifuged for 30 minutes at 4°C. Pellets were then dissolved in SDS-PAGE loading buffer.

RT-PCR

RT was done with ThermoScript RT-PCR system (Invitrogen, Basel, Switzerland) following manufacturer's instructions and PCR was performed with *β-ACTIN* primers (Contzler *et al.*, 2005) or SLURP1-F: 5′-AAGCTTGGAGCAATGGCCTCTCGCTGG and SLURP1-R: TCTAG AGAGTTCCGAGTTGCAGAGGTC.

In situ hybridization

For ISH experiments, *SLURP1* (NM_020427, nt 21-509) was cloned in pGEM-T Easy (Promega, Wallisellen, Switzerland). ISH was performed on plantar and foreskin tissue samples, fixed in 4% paraformaldehyde, and embedded in paraffin, exactly as described previously (Contzler *et al.*, 2005), except for the hybridization temperature which was raised to 63°C.

Laser-captured microdissection

Sections (5 μm) were cut from optimum cutting temperature (OCT) compound-embedded skin biopsies with a cryostat (Kryostat 1720 digital, Leitz, Lenzburg, Switzerland), transferred onto membrane-mounted metal frame slide, briefly fixed either with methyl green or hematoxylin (without eosin) (Shekouh *et al.*, 2003), cut with MMI Cell cut (Molecular Machines & Industries, Volketswil, Switzerland), and captured with MMI IsolationCaps. Tissue sections were extracted with 3 × 7 μl SDS-sample buffer, which were pooled and boiled before being loaded onto 15% SDS-PAGE.

Identification of mutation in *SLURP1*

DNA was isolated from blood with the Nucleon BACC1 kit (GE Healthcare, Otelfingen, Switzerland). Sequencing of PCR-amplified *SLURP1* was performed as described in Fischer *et al.* (2001).

Collection of biological fluids

Saliva was obtained by spitting into plastic tubes. First morning urine was collected. Tears were obtained by inducing tearing with onion exhalations, and sweat was induced and collected by pilocarpine iontophoresis using the Macroduct sweat stimulation and sweat collection system (Wescor, Wetzikon, Switzerland). All biological fluids were immediately put on ice, transferred into Eppendorf tubes and centrifuged for 10 minutes at 4°C. Supernatants were saved, 0.25 volume of 5 × reducing SDS-sample buffer added, and samples boiled for 5 minutes.

Site-directed mutagenesis

Site-directed mutagenesis was performed using Quickchange Site-Directed Mutagenesis Kit (Stratagene, Basel, Switzerland) following manufacturer's instructions. Forward mutagenic primers for W15R and G86R were: 5′-CTCGTGGCAGCCCGGAGCATGGGCTG and 5′-CCGACAGCATCAGGGCCGCCCACCTG, respectively. They were used on pBud-SLURP1 (Chimienti *et al.*, 2003) to produce pBud W15R-SLURP1-MH and pBud G86R-SLURP1-MH.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Figure S1. Characterization of the specificity of anti-SLURP1 peptide Ab 11 and 17.

Figure S2. SLURP1 is present in the upper layers of human skin epidermis.

Figure S3. The protein sequence of SLURP1 from different species is highly conserved towards the C-terminus.

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