

Identification and Characterization of a Novel Retroviral-Like Aspartic Protease Specifically Expressed in Human Epidermis

D. Bernard,* B. Méhul,† A. Thomas-Collignon,* C. Delattre,* M. Donovan,* and R. Schmidt*

*L'Oréal Recherche, Clichy, France; †Galderma R&D, Sophia-Antipolis, France

Proteases play a pivotal role in epidermal differentiation and desquamation. Separation of a total protein extract from human reconstructed epidermis by two-dimensional gel electrophoresis and subsequent peptide analysis of a specific protein spot identified a new protein exhibiting similarities with the retroviral aspartic protease family. Cloning of the corresponding full-length cDNA revealed an open reading frame encoding for a new protease of 343 amino acids, containing a putative aspartic protease catalytic domain. We named this protein Skin ASpartic Protease (SASPase). RT-PCR and northern blot analysis of various human tissues revealed that SASPase was specifically expressed within the epidermis. Immunohistochemical analysis showed a particularly intense expression restricted to the granular layers, whereas in diseased skin, its expression was changed. Western blot analysis, using a monoclonal antibody, revealed the expression of two forms of the enzyme: a 28 kDa putative proform and the active 14 kDa form. Recombinant truncated SASPase (SASP28) was generated from a prokaryotic expression system in *Escherichia coli* as a fusion protein with GST. SASP28 degraded insulin and to a lesser extent casein with a pH optimum of 5. As seen for retroviral proteases, an auto-activation processing was evidenced, generating a 14 kDa protein (SASP14). Site-directed mutagenesis inhibited auto-activation of the enzyme. Indinavir, a potent HIV protease inhibitor used in AIDS therapy, had a significant inhibitory effect on rSASPase auto-activation, which could explain its side effects on skin.

Key words: epidermis/HIV protease inhibitors/proteases/proteomics/retropepsin/skin

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The epidermis is a multilayered squamous epithelium, composed mainly of keratinocytes that migrate from the basal layer to the skin surface. During migration, they undergo what is generally described as epidermal differentiation, i.e., keratinocytes are transformed into corneocytes. These cornified cells form the uppermost layer of the skin, the stratum corneum, which provides the protective barrier between our organism and the environment. Corneocytes are eventually eliminated by a process called desquamation. Throughout the process of keratinocyte differentiation and particularly during the desquamation process, proteases are important players.

Various proteases have been associated with the differentiation-related processing of pro-filaggrin to filaggrin and eventually its degradation to amino acids (aa), also known as natural moisturizing factors (NMF), which contribute to the self-hydration capacity of the stratum corneum. Calpain I, a well-known calcium-activated neutral cysteine protease, is known to process pro-filaggrin (Resing *et al*, 1993). Two recent papers identified keratinocyte Matriptase/MT-SP1, a type II trans-membrane serine protease, as being an essential component of the pro-filaggrin-processing pathway and a key regulator of terminal epidermal differentiation (List

et al, 2002, 2003). The further processing of filaggrin has been attributed to a serine protease of unknown identity termed pro-filaggrin endopeptidase 1 (Resing *et al*, 1995). During filaggrin proteolysis, an NH₂-terminal S-100 protein is released, possibly under the control of furin, belonging to the pro-protein convertase family of Ca²⁺-dependent serine proteases (Pearton *et al*, 2001). Keratinocyte differentiation has been assimilated to a particular apoptosis where the recently discovered and tissue-specific caspase, caspase 14, plays a part in differentiation and skin barrier formation (Van de Craen *et al*, 1998; Lippens *et al*, 2000; Rendl *et al*, 2002; Mikolajczyk *et al*, 2004). Another characteristic feature of keratinocyte terminal differentiation is the formation of the cornified envelope by the cross-linking of precursor proteins under the action of transglutaminases. Two of these require proteolytic activation for their activity. Transglutaminase 1 is activated by Calpain (Kim and Bae, 1998) and transglutaminase 3, which exists as a pro-enzyme, by an unknown protease (Kim *et al*, 2001).

Desquamation requires the degradation of cornodesmosomes, modified desmosomal structures that account for the adhesion of corneocytes in the stratum corneum (Serre *et al*, 1991). Two serine proteases of the Kallikrein family, Kallikrein 5 and Kallikrein 7, have been described to play key roles in this event (Egelrud and Lundstrom, 1991; Hansson *et al*, 1994; Brattsand and Egelrud, 1999; Simon *et al*, 2001; Kuwae *et al*, 2002; Komatsu *et al*, 2003; Caubet *et al*, 2004). Recently, proteases

Abbreviations: aa, amino acid; HERV, human endogenous retroviruses; MALDI TOF MS, matrix-assisted laser desorption ionization-time-of-flight mass spectrometry; rSASPase, recombinant SASPase; SASPase, Skin ASpartic protease or DDI-rp

with an acidic pH optimum were identified in the stratum corneum and associated with desquamation. They belong either to the aspartic acid protease family, like cathepsin D (Horikoshi *et al*, 1998), or to the cysteine protease family, like cathepsin L2 and like the partially characterized "stratum corneum cathepsin L-like" (Bernard *et al*, 2003). The lysosomal cysteine proteases, cathepsin L (Tobin *et al*, 2002) and cathepsin C, also named dipeptidyl aminopeptidase I (Toomes *et al*, 1999), are also described as being related to the process of keratinocyte differentiation. Although these three main proteases families are constitutively expressed in the epidermis, the expression of metalloproteinases is more related to inflammation and to injured tissue. An exception is the expression of MMP19 in normal epidermal layers (Sadowski *et al*, 2003). Finally, another important role of proteases in epidermal physiology is exemplified by trypsin-like protease in the activation of Proteinase-activated receptor-2, which has been demonstrated to be an essential step for melanosome transfer from melanocytes to keratinocytes (Steinhoff *et al*, 1999; Scott *et al*, 2001).

We applied a proteomic strategy to further improve our understanding of epidermal differentiation and the specific role of proteases in this process. Here, we describe the identification and characterization of a new member of the aspartic proteases family, a unique epidermal retroviral-like protease, which we named Skin ASpartic Protease (or SAS-Pase).

Results

Epidermal SASPase: sequence analysis and putative 3D structure Analyzing differentiation-related protein expression, a protein spot with an apparent MW of 14 kDa and an isoelectric point of 5.8 was sequenced (Fig 1). Each of the

four peptide sequences obtained had a 100% match with the sequence of a putative protein identified as a product of the gene "munu", also known as protein FLJ25084 (Fig 2A), a gene product that maps to chromosome 2 at 2p13.3 at locus 151516. This gene is predicted to cover 2356 bp and to contain no intron. The putative "complete" protein is 343 aa long (37 kDa, pI 5.3). An alternative "short" form starting at methionine 85 is also proposed in the databases (259 aa, 28.5 kDa, pI 5.22). Using prosite search (<http://us.expasy.org/prosite/>), an aspartyl protease with a retroviral-type signature was found, with aspartic acid 212 as the putative active aa site. Prosite search predicted a putative N-myristoylation domain (aa 1–7) and a putative dileucine site (aa 50–51). Some putative N-glycosylation, sulfatation, phosphorylation, myristoylation, and amidation sites were also detected (Fig 2A). From aa 56 to 77, a putative transmembrane domain was detected with the SOSUI algorithm (<http://sosui.proteome.bio.tuat.ac.jp/>). Based on a BLAST search, orthologous genes were found in *Mus musculus* and *Rattus norvegicus*. Alignment with the mouse ortholog (data not shown) demonstrated a high degree of conservation during evolution. The homology at the aa level was 87% using the complete sequence and even higher in the putative active site region.

The program 3D-PSSM (Kelley *et al*, 2000) was used to calculate the putative 3D structure of SASP14 (Fig 2B, C). The equine anemia virus (EIAV) protease structure was defined as the best template for the simulation (d1fmb pdb file). The percentage of identity between SASP14 and EIAV PR was 23% and this increases to 47% in the 15 aa surrounding the active site. The calculated percentage of certainty for the structure was 80%.

SASPase mRNA is expressed exclusively within epidermis Northern blot analysis of SASPase expression with a commercial mRNA blot from Origene (Rockville, Maryland) revealed that human SASPase was expressed mainly as a transcript of 1550 bp (Fig 3) that was different from the size deduced from the genome (2356 bp), but similar in size to clone AK057813 (isolated from cerebellum, 1571 bp) from a cap-selected library that appears to be 5' incomplete. Its expression was clearly detected in the skin and to a lesser extent in the brain. SASPase expression was undetectable in the eleven other tissues. Similar results were obtained in a PCR study with a Rapid-scan cDNA kit from Origene (data not shown).

SASPase expression of normal and diseased human skin Western blot analysis of extracts obtained from normal and reconstructed human epidermis with the monoclonal antibody SASP7H9 revealed the presence of the 28 kDa and 14 kDa form of SASPase in both extracts (Figs 4A, 1ab and 2ab). Whereas the 14 kDa represents the activated form of SASPase, the 28 kDa might be considered as the epidermal SASPase pro-form. The fact that in the uppermost layers of the epidermis the pro-form no longer exists (Fig 4A, 4ab) indicates that its activation process seems to be related to keratinocyte differentiation. The incomplete processing observed in psoriatic epidermis, where the inactivated form persists throughout the stratum corneum (Figs 4, 3ab), supports this hypothesis.

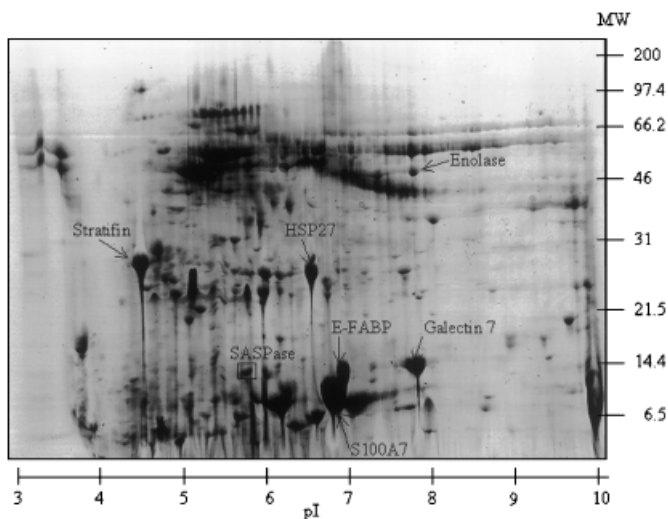


Figure 1
Location of Skin ASpartic Protease (SASPase) in a 2D electrophoresis gel of protein extracts of reconstructed human epidermis. Extracts (4 mg protein) from reconstructed epidermis were separated by 2D electrophoresis. Proteins were stained with Amido Black. The newly discovered human protease "SASPase" has a calculated MW of 14 kDa and an isoelectric point (pI) of 5.8 (spots representing known proteins are listed with their respective names).

All sections of normal human skin showed a strong localized expression of SASPase in the granular layer of the epidermis (Fig 4B). SASPase was also detected in the inner root sheath of hair follicles (Fig 4C). In the psoriatic sample (Fig 4D, E), SASPase expression persisted throughout the stratum corneum, confirming indirectly its high expression level detected by western blot analysis of psoriatic squames. In skin with ulceration, a strong positive staining was observed in the stratum granulosum of intact epidermis (Fig 4F) as in healthy skin. Expression was, however, almost absent in the ulcerated part (Fig 4G).

SASPase is an auto-activated aspartic protease Due to the ambiguity of the translation initiation codon used *in vivo*, two recombinant GST fusion proteins were produced, corresponding to the 37 kDa form, SASP37 and to the 28.5 kDa form, SASP28. SASP37 production gave a poor yield (data not shown), probably due to the known difficulties in producing proteins with trans-membrane domains in *Escherichia coli*. Therefore, we used SASP28 as the recombinant SASPase in our experiments.

We used a highly sensitive, fluorescence-based protease assay to investigate rSASPase catalytic activity. This assay

A

M	G	S	P	G	A	S	L	G	I	K	K	A	L	Q	S	E	Q	18
ATG	GGG	AGC	CCA	GGG	GCC	AGC	CTA	GGC	ATC	AAA	AAG	GCT	CTG	CAG	AGT	GAA	CAG	54
A	T	A	L	P	A	S	A	P	A	V	S	Q	P	T	A	P	A	36
GCC	ACA	GCA	CTG	CCT	GCC	TCT	GCC	CCA	GCA	GTC	AGC	CAG	CCG	ACC	GCG	CCT	GCT	108
P	S	C	L	P	K	A	G	Q	V	I	P	T	L	L	R	E	A	54
CCC	TCC	TGC	TTG	CCC	AAG	GCC	GGA	CAA	GTC	ATC	CCC	ACT	CTG	CTT	CGA	GAG	GCC	162
P	F	S	S	V	T	A	P	T	L	L	C	G	F	L	F	L	A	72
CCG	TTT	TCC	AGC	GTG	ATT	GCG	CCG	ACA	CTG	CTC	TGT	GGG	TTT	CTC	TTC	TTG	GCG	216
W	V	A	A	E	V	P	E	E	S	S	R	M	A	G	S	G	A	90
TGG	GTT	GCT	GCT	GAG	GTT	CCA	GAG	GAG	AGC	AGC	AGG	ATG	GCC	GGG	AGC	GGA	GCC	270
R	S	E	E	G	R	R	Q	H	A	F	V	P	E	P	F	D	G	108
AGG	AGT	GAG	GAA	GGC	CGC	CGG	CAG	CAT	GCC	TTC	GTC	CCG	GAA	CCT	TTT	GAT	GGG	324
A	N	V	V	P	N	L	W	L	H	S	F	E	V	I	N	D	L	126
GCC	AAT	GTC	GTC	CCA	AAC	CTC	TGG	CTG	CAC	AGC	TTT	GAA	GTC	ATC	AAT	GAC	CTC	378
N	H	W	D	H	I	T	K	L	R	F	L	K	E	S	L	R	G	144
AAC	CAT	TGG	GAC	CAT	ATC	ACC	AAG	CTA	AGG	TTC	CTG	AAA	GAG	TCC	CTC	AGA	GGA	432
E	A	L	G	V	Y	N	R	L	S	P	Q	D	Q	G	D	Y	G	162
GAG	GCC	CTG	GGT	GTC	TAC	AAT	AGG	CTC	AGT	CCC	CAG	GAC	CAG	GGA	GAC	TAT	GGG	486
T	V	K	E	A	L	L	K	A	F	G	V	P	G	A	A	P	S	180
ACT	GTG	AAA	GAG	GCC	CTC	CTG	AAG	GCC	TTT	GGG	GTC	CCT	GGG	GCT	GCC	CCC	AGC	540
H	L	P	K	E	I	V	F	A	N	S	M	G	K	G	Y	Y	L	198
CAC	CTG	CCC	AAA	GAG	ATC	GTC	TTT	GCC	AAC	AGC	ATG	GGT	AAG	GGC	TAC	TAT	CTC	594
K	G	K	I	G	K	V	P	N	R	E	L	N	D	S	G	A	Q	216
AAG	GGG	AAG	ATT	GGC	AAA	GTG	CCC	GTG	AGG	TTC	CTG	GTG	GAC	TCT	GGG	GCC	CAG	648
V	S	V	V	H	P	N	L	W	E	E	V	T	D	G	D	L	D	234
GTC	TCT	GTG	GTC	CAC	CCA	AAC	TTG	TGG	GAG	GAG	GTC	ACT	GAT	GGC	GAT	CTG	GAC	702
T	L	Q	P	F	E	M	N	V	K	V	A	M	G	A	E	M	K	252
ACC	CTG	CAG	CCC	TTT	GAG	AAT	GTG	GTA	AAG	GTG	GCC	AAT	GGT	GCT	GAA	ATG	AAG	756
T	L	G	V	N	D	T	A	V	S	I	G	K	L	K	L	K	A	270
ATC	CTG	GGT	GTC	TGG	GAT	ACA	GCG	GTG	TCC	CTA	GGC	AAG	CTG	AAG	CTG	AAG	GCA	810
Q	F	L	V	A	N	A	S	A	E	E	A	I	I	G	T	D	V	288
CAG	TTC	CTA	GTG	GCC	AAT	GCG	AGT	GCC	GAG	GAA	GCC	ATC	ATT	GGC	ACT	GAT	GTG	864
L	Q	D	H	N	A	I	L	D	F	E	H	R	T	C	T	L	K	306
CTC	CAG	GAC	CAC	AAT	GCT	ATC	CTG	GAC	TTT	GAG	CAC	CGC	ACA	TGC	ACC	CTG	AAA	918
G	K	K	F	R	L	L	P	V	G	G	S	L	E	D	E	F	D	324
GGG	AAG	AAG	TTT	CGC	CTT	CTG	CCT	GTG	GGA	GGG	TCC	CTG	GAA	GAT	GAG	TTT	GAC	972
L	E	L	I	E	E	D	P	S	S	E	E	G	R	Q	E	L	S	342
CTG	GAG	CTC	ATA	GAG	GAG	GAC	CCC	TCC	TCA	GAA	GAA	GGG	CGG	CAG	GAG	CTA	TCC	1026
H																		343
CAC																		1029

Figure 2

Skin ASpartic Protease (SASPase) sequence and putative 3D structure. (A) Sequence. Nucleotide sequence coding for human SASPase aligned with its deduced amino acid (aa) sequence. Dotted lines underline aa indicating the prosite signature of a retroviral-type aspartyl protease. Active site aspartic acid is in bold. Sequences obtained from Edman sequencing after trypsin digestion of the isolated 2D spot are boxed with double lines. A putative di-leucine domain and transmembrane region are underlined with double lines or a single bold line, respectively. A single nucleotide polymorphism has been detected at aa position 49 replacing threonine (T) by Alanine (A). Two methionines are highlighted with bold letters and correspond to two putative start codons for SASPase. (B-C) Modelization. The program 3D-PSSM was used to calculate the putative 3D structure of SASP14. The equine anemia virus protease structure was calculated as being the best template for the simulation (d1fmb pdb file). The calculated certainty of the structure was 80% and evaluated to be "good" following the software criteria. The structure was represented with the swiss pdb viewer v3.7 software (B) (<http://www.exspasy.ch.spdbv>) and 3D rendering was improved with the Pov ray software (C). The active site aspartic acid (D24) is highlighted with an arrow.

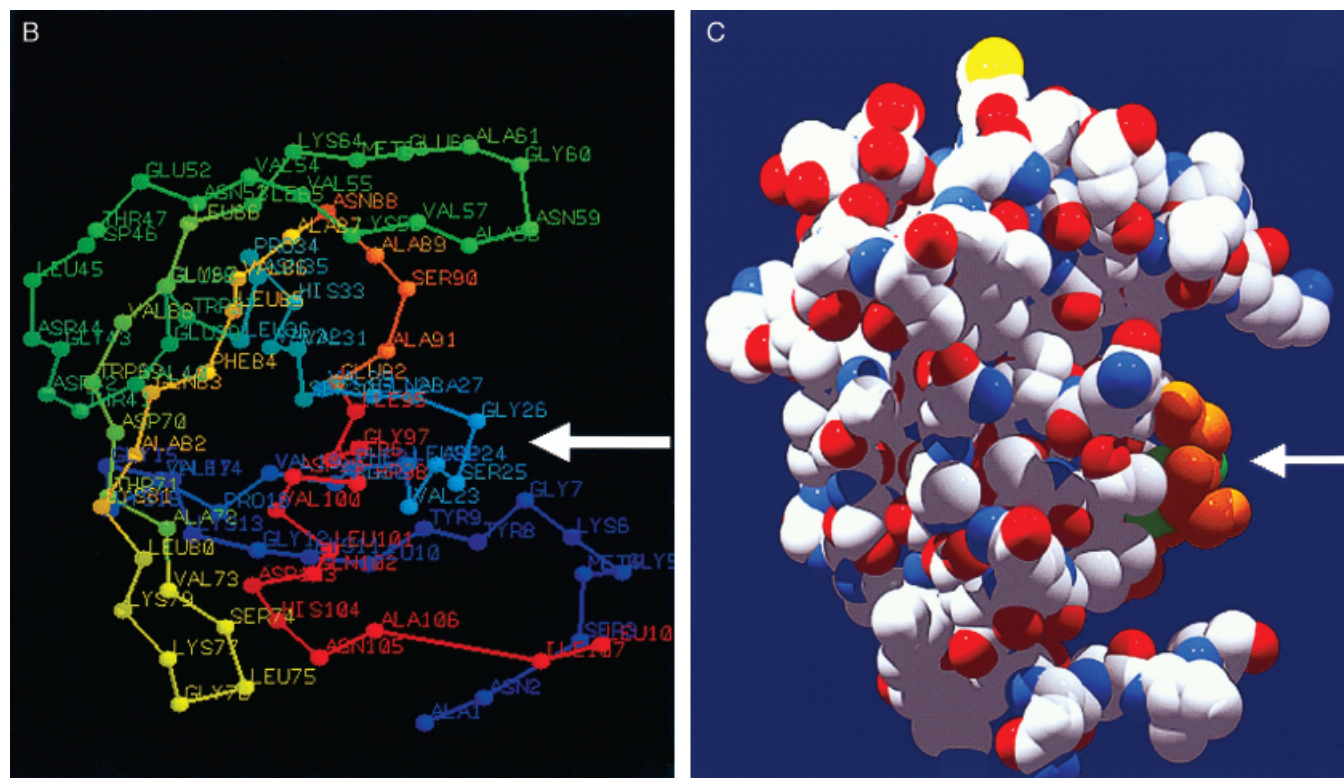


Figure 2
Continued.

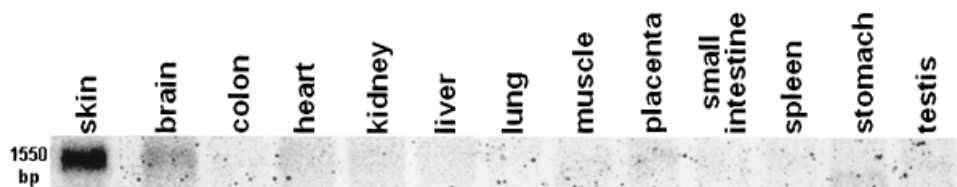


Figure 3
Skin ASpartic Protease (SASPase) Northern blot study in different human tissues. Human multiple tissue mRNA blot (Origene), 2 μ g of poly(A) $\sim$ per lane from tissues indicated above each lane. The calculated mRNA size of SASPase is indicated on the left. The blot was hybridized with a human 1.026 kb radiolabeled SASPase probe.

allowed continuous, real-time measurement of protease activity by using the intra-molecular quenched BODIPY FL-labeled casein as the substrate. Incubation of rSASPase with the quenched casein substrate (data not shown) results in a time-dependent increase of fluorescence at a pH optimum of 5, indicating that rSASPase hydrolyzed casein. Further analysis revealed that rSASPase primarily hydrolyzed one bond in the oxidized B chain of insulin at Glu13+Ala14. Minor cleavages were noted at Leu15+Tyr16 and Tyr16+Leu17 (data not shown).

SDS-PAGE of rSASPase fusion protein performed after its incubation at pH 5, and 37°C, resulted in a time-related generation of a low-molecular-weight protein (Fig 5A). Edman sequencing and mass spectrometry of this auto-catalytically generated SASPase fragment identified a protein composed of 136 aa, an MW of 14.8 kDa, and an isoelectric point of 5.2 (SASP14). The major cleavage sites were at Asn190+Ser191 and Glu326+Leu327. Interestingly, after the removal of the N-terminal auto-activation site, the enzyme used a new cleavage site at Ala167+Leu168 (data not shown). The fact that active site-directed mutations (replacement of active site aspartic acid by either alanine or

glutamic acid) resulted in the loss of auto-activation capacities (Fig 5B) indicated that SASPase was a true member of the aspartic proteases family, and that aspartic acid in position 212 (D212) was the key aa of its active site. The different putative SASPase forms are schematically presented in Fig 5C.

Indinavir inhibits SASPase auto-activation Among the five distinct HIV protease inhibitors tested on the auto-activation capacity of SASPase, only indinavir was able to inhibit this process considerably (Fig 6). Interestingly, pepstatine, the classical inhibitor of the aspartic proteases, did not affect the process of auto-activation at all even when used at 1 mM. DAN and EPNP, two other classical aspartic protease inhibitors (Chang *et al*, 1976), did not inhibit the autoactivation process even when used at 0.1 mM concentrations (data not shown).

Discussion

After 2D electrophoresis of protein extracts of reconstructed human epidermis, we identified a 14 kDa protein spot

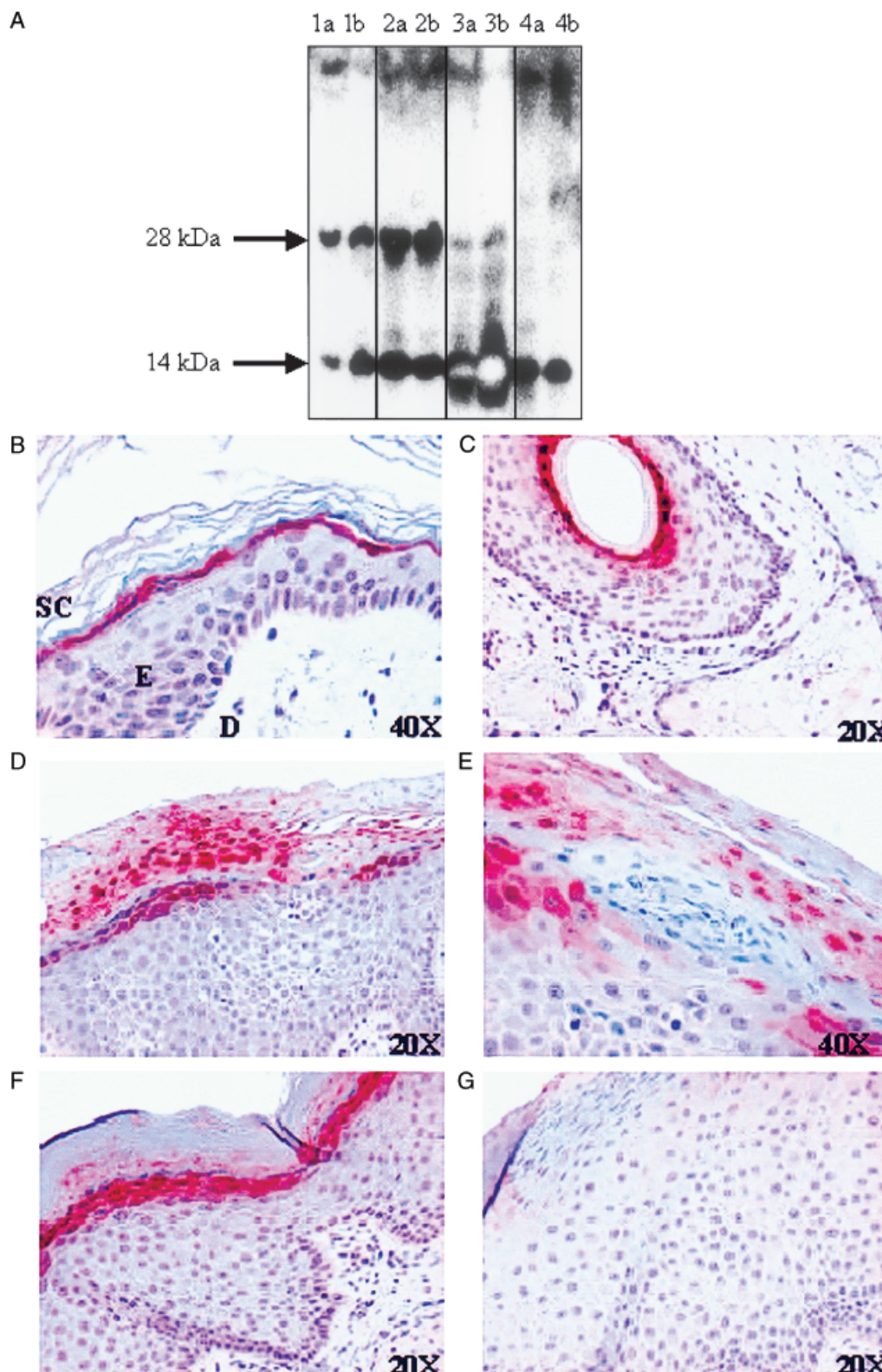


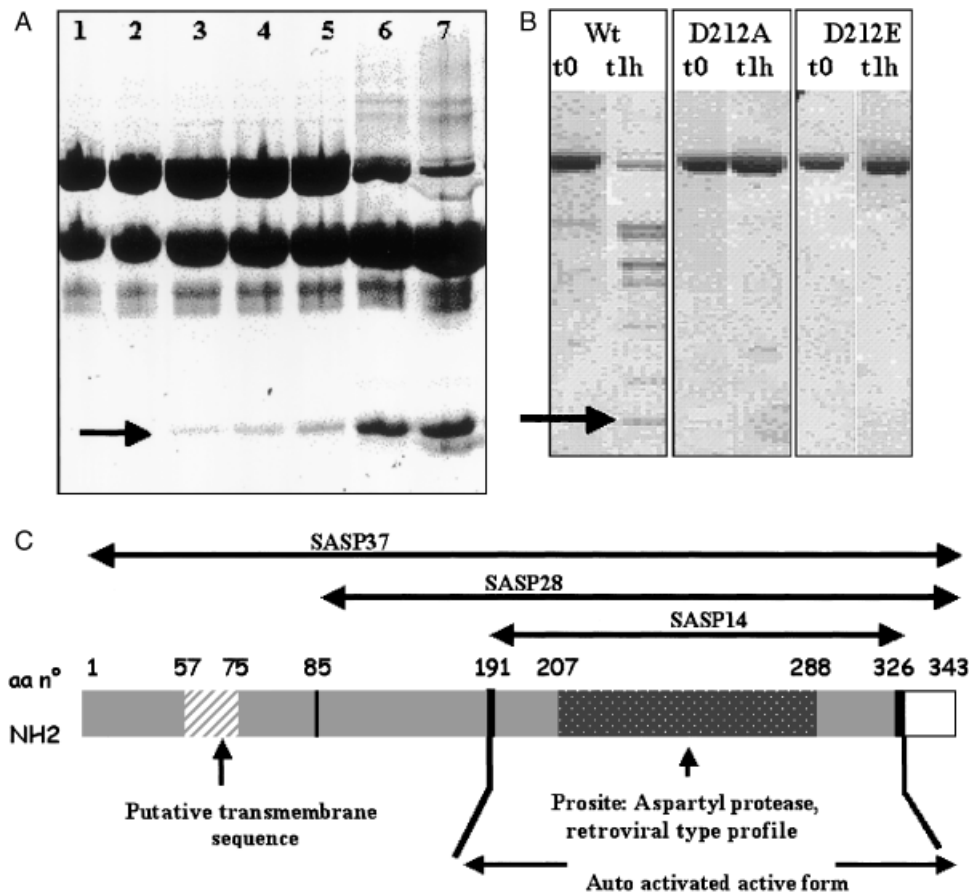
Figure 4
Immunodetection of Skin Aspartic Protease (SASPase) in normal and diseased skin. Western blot (A): Two repetitions (a, b) were made for each sample. 1, normal human epidermis extract; 2, reconstructed epidermis 13 d; 3, psoriatic stratum corneum extract; and 4, normal non-plantar stratum corneum extract. Immunohistochemistry (B–G) was performed on paraffin sections of normal and diseased skin using the monoclonal antibody SASP7H9 as the primary antibody. The positive staining observed was mostly cytoplasmic and occasionally nuclear in the stratum granulosum of normal skin (B) and in the inner root sheath of hair follicles (C). In psoriasis sample (D, E), increased staining was present in the stratum corneum. In skin with ulceration (F, G), strong positive staining was observed in the stratum granulosum of the intact area (F) but was markedly reduced immediately adjacent to the ulcer (G). SC, stratum corneum; E, epidermis; D, dermis.

corresponding to an unknown human aspartic protease, which we named Skin Aspartic Protease due to its abundant expression in keratinocytes. The peptide sequences obtained matched with the sequence of a putative protein identified as a product of gene “munu” that mapped to chromosome 2 at 2p13.3 at locus 151516. An aspartyl protease with a retroviral-type signature could be predicted

from the protein sequence. Production of a recombinant 28 kDa form of the enzyme permitted us to demonstrate that this particular recombinant form undergoes an auto-activation process at pH 5. This auto-activation process is a common feature of retroviral aspartic proteases and thus SASPase is related to this family. Site-directed mutagenesis of the putative-active site aspartic acid definitively demon-

Figure 5

Recombinant Skin Aspartic Protease (rSASPase) auto-activation of wild-type and mutated forms. The auto-activation reaction was performed in 0.1 M acetate pH 5 at 37°C and monitored by SDS-PAGE. (A) Lanes 1–7 incubation time 0, 5, 10, 20, 30, 80, and 120 min for wild-type (wt) SASPase. Note: In this experiment, rSASPase fusion protein batch is contaminated with an equivalent quantity of free GST possibly due to an uncontrolled auto-activation occurring during the preparation process. (B) Comparison of auto-activation of wt with mutated SASPase (Active site aspartic acid (D) was either replaced by alanine (A: D212A) or glutamic acid (E: D212E)) after 60 min of incubation. The mutated forms have lost the autocatalytic property. Arrow on the left indicates the 14 kDa activated form. (C) A proposed model of SASPase structure. The putative complete protein (SASP37 343 aa, 37.0 kDa, pI 5.3) is represented with the location of its putative transmembrane sequence, the predicted retroviral aspartyl protease signature, and the primary auto-activation sites. The alternative short form is named SASP28 (259 aa, 28.5 kDa, pI 5.22). The major auto-activation protease product is called SASP14 (136 aa, 14.8 kDa, pI 5.2).



strated that SASPase was a true new member of the aspartic protease family. Another common feature of retroviral-like proteases is the necessity of a homodimeric association for the formation of the active site pocket. Complementary experiments demonstrated that the low MW SASPase (14 kDa) was probably able to form homodimeric-active associations (data not shown). The use of the monoclonal antibody in healthy and diseased human skin revealed a strong SASPase expression restricted to the granular layer of normal epidermis and the inner root sheath of the hair follicle, whereas in psoriatic epidermis, expression persists throughout the parakeratotic stratum corneum. In ulcerated skin, SASPase expression was normal in the adjacent area of the ulcer and absent in the affected part. Its absence and modified expression pattern in certain skin pathologies will be further investigated and might contribute toward the elucidation of the physiological role of SASPase in the epidermis.

The above results and the detection of SASPase (14 kDa form) by western blot analysis in the stratum corneum extracts of normal human skin might be contradictory, however, since it is well known that many antigens are masked within the lipid environment of the stratum corneum. Labeling of SASPase within psoriatic stratum corneum could reflect an impaired maturation of the enzyme within the stratum corneum.

Further western blot analysis during the reconstruction of epidermis revealed that SASPase expression is related to keratinocyte differentiation (data not shown), in particular,

its activation, i.e., the conversion of the 28 kDa proform into the active 14 kDa protein. Thus, our monoclonal antibody seems to label preferentially, if not exclusively, the activated form of SASPase. The putative 37 kDa form, predicted from the database DNA sequence, could not be detected in epidermal extracts.

Aspartic proteases are suggested to have evolved by gene duplication and fusion of an ancestral protein with the size of a pepsin lobe (Tang *et al*, 1978). Retroviruses, e.g., HIV, are known to express a "half-sized" aspartic protease, which becomes activated after dimerization (Wlodawer and Gustchina, 2000). Although the hypothesis exists that retroviral proteases might be fossils of ancestral dimeric aspartic proteases (Pearl and Taylor, 1987), it is generally more accepted that aspartic proteases have evolved by gene duplication and fusion (Tang *et al*, 1978; Lin *et al*, 1992). The discovery of SASPase with its unique properties of auto-activation and subsequent dimerization to form an active enzyme provides new arguments in favor of an ancestral eucaryotic protease being at the origin of retroviral proteases.

Most of the human endogenous retroviral (HERV) genome has been acquired 10–100 million years ago and constitutes between 0.1% and 1% of our germ line DNA (Lower *et al*, 1996). HERV genomes are transmitted as part of the host's normal genetic material. Thus, the selective pressure to maintain functional open reading frames (ORF) for viral replication no longer applies. The maintenance of an HERV

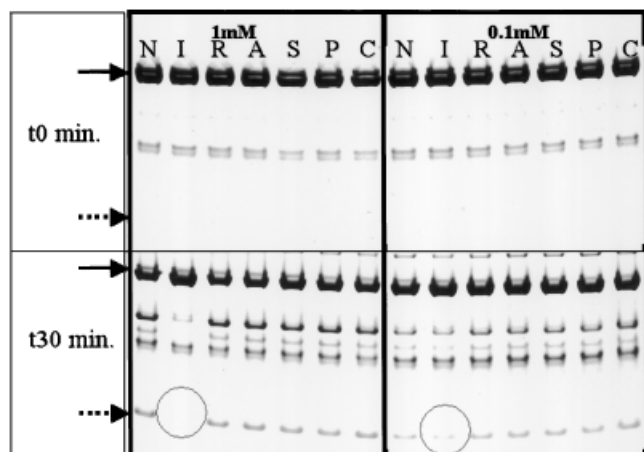


Figure 6
Indinavir as an inhibitor of Skin ASpartic Protease (SASPase) auto-activation. Recombinant fusion protein (1 mg per mL in Tris buffer saline) was initially acidified to pH 5 with 1 M acetate buffer pH 4.5. Stock solutions of 10 mM inhibitors in DMSO (N, norvir; R, ritonavir; A, amprenavir; P, pepstatine; S, saquinavir) or in water (I, indinavir) were used to bring the test solution to 0.1 or 1 mM final concentrations. A control (C) was run without inhibitors. The final concentration of DMSO was 10% in each case. Samples from the 0 and 30 min timepoints after the initial acidification step were run on Nu-PAGE 12% gels in MES buffer. The black arrow indicates the fusion protein, which is progressively degraded to activated SASPase or SASP14 (dotted arrow).

ORF over a significant evolutionary time period suggests a biological function (Patience *et al*, 1997) and indeed, mammalian proteins containing HERV sequences have been described (Johansen *et al*, 1989; Lower *et al*, 1996; Benit *et al*, 1999). SASPase, a protease with a very high expression level in human skin, is unique in the sense that it contains several of the above-described features: (i) SASPase is likely to be an HERV-derived protein based on its sequence homology with viral proteases (data not shown) and (ii) SASPase auto-catalyzes the generation of proteolytic fragments from a precursor fusion protein, which, after dimerization, generates the active enzyme. This mechanism is strikingly similar to that described for the HIV protease (Meek *et al*, 1989). Proteases sequences, highly conserved from rodents to humans, served as a basis for Puente *et al* (2003) to perform a bio-informatic search within the mouse and human genome. By this approach, five new members of the human aspartic protease family were identified, some of them presenting in addition to the retroviral aspartic protease signature, a DNA damage-inducible (DDI) signature, responsible for SASPase designation in this study as DDI-related proteins (DDI-rp). Moreover, we can consider that the high conservation between mouse and human SASPase orthologs implies that this protein is likely to have an indispensable function.

It was also interesting to observe that the classical aspartic protease inhibitor pepstatine, even at a concentration of 1 mM, did not affect SASPase auto-activation, indicating that SASPase is not only a new but also a very particular member of the aspartic protease family.

The similarities described above between SASPase and HIV protease and the fact that therapeutics target HIV protease (Kohl *et al*, 1988; Kay and Dunn, 1990; Dunn *et al*, 2002) prompted us to investigate possible interactions of these inhibitors with SASPase. Among the four evaluated

therapeutic HIV protease inhibitors, only indinavir inhibited SASPase auto-activation. It is interesting to note that indinavir is reported to have severe cutaneous side effects (Ward *et al*, 2002) like paronychia, cheilitis, xerosis (Garcia-Silva *et al*, 2000), dermatitis, dry lips, hair loss, and nail dystrophy (Carr *et al*, 1998; Calista and Boschini, 2000; Harry *et al*, 2000). Recently, an indinavir-calpain interaction was proposed to explain the skin manifestations induced by indinavir (Ghibelli *et al*, 2003); however, inhibition of SASPase might also account for some of the observed adverse effects and even shed some light on its physiological role in skin which has yet to be identified.

The adverse *in vivo* effects of indinavir have also been described as "retinoid-like" effects (Bouscarat *et al*, 1998; Toma *et al*, 2001; Garcia-Silva *et al*, 2002) and apparently, indinavir is able to stimulate retinoid signaling *in vitro* (Lenhard *et al*, 2000). Carr and collaborators (Carr *et al*, 1998) observed that the catalytic region of HIV-1 PR, to which protease inhibitors bind, has about 60% homology with regions located within two proteins known to regulate epidermal differentiation and lipid metabolism, the cellular retinoic acid-binding protein 1 (CRABP-1), and the low density lipoprotein-receptor-related protein (LRP), respectively. It is noteworthy that a comparison of the complete HIV-1 PR and SASPase sequence reveals a much better alignment than that observed between the viral protease and CRABP-1 or LRP. This observation reinforces interest in studying how far indinavir-SASPase interactions might be responsible for side effects observed during antiviral therapies with this agent.

Another open question that remains is the identification of SASPase substrates. Based on a certain homology between SASPase and retroviral proteases, substrates for the latter enzyme family might give some indication of endogenous SASPase targets. Among the non-viral protein substrates described for retroviral proteases are vimentin, desmin (Shoeman *et al*, 1990), lactate dehydrogenase (Tomaszek *et al*, 1992), calmodulin (Tomasselli *et al*, 1991), actin, troponin C, Alzheimer amyloid precursor protein, and pro-interleukin 1B (Tomasselli *et al*, 1991).

To speculate on the possible physiological role of SASPase, we performed a screen within the MEROPS database to identify proteases cleaving the same peptide bonds as SASPase (data not shown). Besides retroviral-type proteases, proteases with well-described physiological functions in the skin were extracted from the database, e.g., SCCE implicated in desquamation, MMP1, 3 (affecting wound healing), thrombin (regulating the activation of the protease-activated receptors) and convertase 3 (processing filaggrin and other differentiation-related processes).

All these observations strengthen the idea that SASPase might play an important role in cutaneous physiology. The highly conserved status of SASPase throughout mammalian and rodent evolution, its tight transcription regulation, and elevated expression level in skin are good reasons for us to further investigate its physiological role in the skin. The indication that SASPase could represent an undesired target of some HIV protease inhibitors as well as the possibility that this human-retroviral chimeric protein could have other as yet undiscovered interactions with the viral world makes it an interesting target for further investigations.

Material and Methods

Materials Isopropyl-1-thio- β -D-galactopyranoside, CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propane-sulfonate), sinapinic acid, BS3, DAN, EPNP, glutathione-Sepharose matrix, and Taq polymerase were purchased from Sigma (L'Isle d'Abeau, France). Immobililine pH gradient (IPG) solution, IPG strips, pre-made SDS-PAGE gels, Rainbow molecular weight markers, silver staining kit, first-strand synthesis kit, *E. coli* strain BL21, pGEX-4T3 plasmid and ECL advance kit, premade SDS-PAGE gelbond PAG film-supported gels for horizontal electrophoresis, HiTrap and gel filtration G75 HR10/30 column were from Amersham Pharmacia Biotech (Saclay, France). RNase, DNase, thrombin, Taq polymerase, pfu polymerase, and restriction enzymes were from Promega (Lyon, France). The RNA preparation kit RNeasy was obtained from Qiagen (Courtaboeuf, France). Oligonucleotides were synthesized by Genset (Evry, France), and PCR-ready cDNA kits were from Maxim Biotech. Site-directed mutagenesis kit was obtained from Stratagene. Gelstar fluorescent stain was from Cambrex (Verviers, Belgium). The HIV protease inhibitors norvir, ritonavir, amprenavir, saquinavir, and indinavir were obtained from the NIH AIDS Research and Reference Reagent Program.

2D electrophoresis and protein sequencing Reconstructed epidermis after 13 d of culture (RE13D), obtained from Episkin (Lyon, France) was used to prepare the epidermal protein extract (30 samples of RE13D, 1 cm² each). Samples were immersed in 2.5 mL buffer (28 mM Tris-HCl, 22 mM Tris-base, containing 0.3% SDS). The mixture was homogenized and boiled for 10 min. After centrifugation (10,000 $\times$ g for 10 min), the supernatant (2 mL) was recovered and passed through a 0.22 μ m membrane filter. Proteins were precipitated by adding 10 mL of ice-cold acetone. After 20 min incubation, precipitated proteins were recovered by centrifugation (9400 $\times$ g for 10 min), dried at room temperature for 20 min, and solubilized in 300 μ L 2D buffer (8 M urea, 2% (wt/vol) CHAPS, 20 mM DTT and 0.5% (vol/vol) IPG solution pH 3-10).

Isoelectric focusing was performed using a Pharmacia Multiphor II system. One IPG strip, 18 cm, pH 3-10 was used according to the manufacturer's instructions. Horizontal SDS electrophoresis was performed on pre-fabricated gelbond PAG film-supported gels (12%-14% gradient) according to the manufacturer's instructions. For the second dimension, pre-stained molecular weight markers were included after a 1:4 dilution. The apparent MW were calculated using Quantity one software from Bio-rad (Marnes la Coquette, France). After staining the 2D gel with Amido Black, the stained spots were recovered by performing 3 mm punch biopsies. For protein identification, Edman sequencing was generally used as previously described (Mehul *et al*, 2000). When necessary, matrix-assisted laser desorption ionization time of flight (MALDI TOF) or mass spectrometry (MS)/MS Q-TOF mass spectrometry analyses were performed on samples desalted with Zip Tip C4 columns (Millipore, Guyancourt, France) on a Voyager STR MALDI TOF apparatus from Perkin-Elmer (Courtaboeuf, France) or on an ESI Q-TOF apparatus from Micromass (Waters, St Quentin-en-Yvelines, France) at ESPCI (Ecole Supérieure de Physique et de Chimie Industrielle de la ville de Paris).

SASPase putative 3D structure The program 3D-PSSM (<http://www.sbg.bio.ic.ac.uk/~3dpssm/>) was used to calculate the putative 3D structure of SASP14. The EIAV protease structure was used as a template for the simulation (d1fmb pdb file).

Northern blot analysis An mRNA blot for human adult tissues was purchased from Origene (HB-1102, HB-2010) and used according to the instructions. The selected probe was a 1026 bp (human) SASPase cDNA fragment, which was digested from the expressed sequence coding for SASP37 by using restriction enzymes *EcoRI* and *BsmBI*. Labeled cDNA probes were synthesized by using the Rediprime random primer labeling kit (Amersham Pharmacia) and 25 ng of the purified insert was used according to

the manufacturer's instructions. ³²P-labeled nucleotides were purchased from Amersham Pharmacia.

Preparation of antibodies, immunohistochemistry, and western blot The monoclonal antibody SASP7H9 was developed at Covalab (Lyon, France). Briefly, mice were immunized with both a gel filtration-purified SASP14 form and a denatured form within acrylamide gel obtained after its separation by SDS-PAGE. The antibody was finally purified from culture supernatants using protein A Sepharose.

Immunolocalization studies were conducted on thin paraffin-embedded sections (5 μ m) from normal and diseased skin at Life-Span BioSciences (Seattle, Washington). Antibody SASP7H9 was used as the primary antibody (1.25 μ g per mL), and the principal detection system consisted of a Vector (Burlingame, California) anti-mouse secondary (BA-2000) and a Vector ABC-AP kit (AK-5000) with a Vector Red substrate kit (SK-5100), which was used to produce a fuchsia-colored deposit. Tissues were also stained with positive control antibodies (CD31 and vimentin) to ensure that the tissue antigens were preserved and accessible for immunohistochemical analysis. Only tissues that were positive for CD31 and vimentin staining were selected for the study. The negative control was sections incubated in the absence of the primary antibody.

For tissue expression analysis, samples of normal human epidermis (obtained after plastic surgery), reconstructed epidermis (day 13), and stratum corneum from normal and psoriatic skin, obtained as described before (Mehul *et al*, 2000), were homogenized in 1 $\times$ Laemmli buffer containing 2% SDS and 200 mM DTT. The buffer/sample wt/vol ratios were 500 μ L of buffer for 10 mg of wet tissue weight and 500 μ L of buffer for 5 mg of dry tissue weight. Homogenization was performed with a polytron for 1 min, and then the suspension was boiled for 10 min before centrifugation (10,000 $\times$ g for 10 min). Proteins in the supernatant were quantified using a Bio-Rad protein assay and samples were adjusted to a protein concentration of 1 mg per mL. Twenty microliters (diluted 1:1 in 2 $\times$ Laemmli buffer) were run on 12.5% Criterion SDS-PAGE gels following the manufacturer's instructions. Proteins were transferred to a PVDF membrane (Immobilon P, Millipore) and the rest of the western blot was run using a standard procedure with the primary monoclonal antibody SASP7H9 diluted 1:2000 in buffer containing 1% milk. The bands were visualized using the ECL advance substrate system (Amersham Pharmacia Biotech) on a FluorSmax imager (Bio-Rad).

Isolation of SASPase cDNA from human keratinocytes and expression of rSASPase

cDNA was cloned by RT-PCR. Total RNA was obtained from reconstructed human epidermis. RNA was prepared using an RNeasy kit (Qiagen). Briefly, two samples of reconstructed epidermis (day 13) were homogenized in 300 μ L of lysis buffer (included in the kit) and the cell debris were removed using a QIA shredder column. cDNA was produced from purified total RNA (2 μ g) using the First-Strand cDNA Synthesis Kit. One to 5 μ L of generated first-strand cDNA, with *NotI*-d(T)₁₈ as primer, was directly used for PCR. cDNA fragments of 1135 bp coding the full-length SASPase (SASP37: methionine 1—end) was amplified by PCR using pfu polymerase and oligonucleotides SC140: 5'-taggatccatggggagcccaggggc-3' and SC131: 5'-ttgaattctcagtg-gatagctcctgccgc-3', under the following conditions: 1 cycle for 2 min at 95°C, 35 cycles (94°C for 30 s, 65°C for 30 s, 72°C for 2 min), and 1 cycle at 72°C for 7 min. A cDNA fragment of 883 bp coding for the truncated form (SASP28: methionine 85—end) was amplified by PCR using pfu polymerase and oligonucleotides SC131 and SC130: 5'-gatagatccatgccgggagcggagccaggag-3', under the same conditions as described for primers SC131/140. These primers contained restriction sites (*Bam*H1/*Eco*RI) to facilitate the incorporation of cDNA into a pGEX-4T3 plasmid. This construct, containing an in-frame coding region for SASPase and glutathion S-transferase, was used to transfect the *E. coli* strain BL21(DE3). Expression and purification of rSASPase were performed according to manufacturer's instructions. Purified proteins

were pooled, and buffer was changed using G25 gel filtration to 50 mM Tris-HCl, pH 8, containing 75 mM NaCl, 50% glycerol (SB). Protein was maintained at 4°C before use or at -20°C for long-term storage. If not indicated otherwise, the term rSASPase refers to the GST-SASP28 fusion protein.

Autocatalytic properties of SASPase rSASP (3 mg per mL) in SB was rapidly acidified at pH 5 by 1 M acetate buffer pH 4.5 and then incubated at 37°C. 10 μ L aliquots were recovered at different times during the incubation step and the reaction was stopped by addition of 10 μ L Laemmli buffer. All the aliquots were analyzed by SDS-PAGE on 12.5% acrylamide criterion gels following the manufacturer's instructions. Pre-stained Rainbow molecular weight markers served as standards and molecular weights were calculated using the Quantity-one software (Bio-Rad).

Site-directed mutagenesis The active site aspartic acid (D212) was replaced either by glutamic acid (E: mutant D212E) or by alanine (A: mutant D212A) using Site-directed Mutagenesis with the quick change kit from Stratagene. The primers used for amplification of the mutated SASPase were 5'-gaggttctctggtgagctctgggcccaggtctctg-3' forward primer and 5'-cagagacctgggcccagactcaccaggaacctca-3' reverse primer for mutant D212E and 5'-gaggttctctggtggtctctgggcccaggtctctg-3' forward primer and 5'-cagagacctgggcccagagccaccaggaacctca-3' reverse primer for mutant D212A. The template used for PCR was the rSASPase construct (SASP28) in pGEX-4T3. Competent cells (XL1 blue bacteria) were transformed with the PCR product and selected clones were sequenced to control for the presence of the mutation. The production and purification of mutant proteins were then conducted following the same protocols as for the wild-type form. The auto-activation test reactions of wild-type and mutated forms of SASPase were performed and monitored by SDS-PAGE as described above.

HIV protease inhibitors and SASPase rSASPase (1 mg per mL in SB) was initially acidified to pH 5 with 1 M acetate buffer pH 4.5. Norvir, ritonavir, amprenavir, pepstatine, saquinavir in DMSO, and indinavir in MilliQ water (10 mM stock solutions) were used to bring the test solutions to either 0.1 mM or 1 mM. A control was run without inhibitors. The final concentration of DMSO in each sample, including indinavir sample, was 10%. Nu-PAGE 12% gels (Invitrogen) were run with MES buffer just after the initial acidification step and after 30 min incubation. The gel was finally stained with a pre-made Coomassie solution (Bio-Rad) following the manufacturer's instructions.

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Address correspondence to: D. Bernard, Centre Charles Zviak, 90 rue du Général Roguet, 92583 Clichy, France. Email: dbernard@rd.loreale.com

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