Transglutaminase-3 Enzyme: A Putative Actor in Human Hair Shaft Scaffolding?

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The family of transglutaminases (TGase) is known to be involved in terminal differentiation processes in the epidermis. These enzymes contribute also to the physical resistance and the preservation of the hair follicle structure. Our particular interest in hair fiber keratinization led us to focus on the TGase 3, exclusively expressed in the hair shaft. To date its function is still to be elucidated, thus we have developed a multidisciplinary approach in order to define the localization, activity, and substrates of TGase 3. The hair fiber is characterized by the expression of specific proteins essentially consisting of keratin intermediate filaments and keratin-associated proteins (KAPs), which are essential for the formation of a rigid hair shaft through their extensive disulfide cross-links. Gel electrophoresis combined with mass spectrometry experiments revealed an unexpected protein migration pattern, suggesting the existence of covalent interactions other than disulfide bonds. Western blot and amino-acid analysis revealed the presence of γ-glutamyl-ε-lysine isopeptide linkages that could constitute this second covalent network. Our hypothesis is that TGase 3-driven specific isopeptide bonds between intermediate filaments and KAPs participate to the progressive scaffolding of the hair shaft.

INTRODUCTION

During the last stages of the epidermis differentiation, various insoluble proteins are assembled at the cell periphery of the stratum corneum, and generate the cornified cell envelope (CCE) (Steven and Steinert, 1994; Nemes and Steinert, 1999). This structure consists of sequential incorporation of precursor proteins including involucrin, loricrin, and small proline-rich proteins, followed by covalent attachment of extracellular lipids. Skin barrier function is conferred by the stratum corneum and CCE (Hardman *et al.*, 1998), which seems to be the essential factor contributing to the physical resistance of this most superficial epidermal layer. Involucrin is synthesized in abundance during terminal differentiation of keratinocytes and is one of the early structural proteins to be expressed and cross-linked into CCE (Eckert and Green, 1986; Eckert *et al.*, 1993). Indeed, involucrin is a substrate for transglutaminases (TGases) and one of the precursors of the cross-linked envelopes present in the corneocytes of the epidermis and other stratified squamous epithelia. TGases are

Ca^{2+}-dependent enzymes that catalyze the formation of an isodipeptide cross-link between the ε-NH2 of a protein-bound lysine residue and a γ-carboxamide of a protein-bound glutamine residue, generating an insoluble macromolecular aggregate (Folk and Finlayson, 1977). Their involvement in terminal differentiation events in the epidermis has been supported by many observations, many studies of coexpression with CCE structural proteins (Greenberg *et al.*, 1991; Michel *et al.*, 1992; Reichert *et al.*, 1993) and *in vitro* cross-linking (Candi *et al.*, 1995).

Although four TGase isoforms are expressed during terminal differentiation in stratified squamous epithelia such as the epidermis, the role of each transglutaminase in the fate of differentiating epidermal cells has not been fully characterized. The 92 kDa membrane-associated TGase 1 (Rice and Green, 1977; Thacher and Rice, 1985; Schmidt *et al.*, 1988) is essential for CCE formation. Inactivation of this TGase by mutation in the TGM1 gene (encoding for TGase 1) is the cause of lamellar ichthyosis and leads to disorder of the cornification (Russell *et al.*, 1994). TGM1 transcription was shown to be regulated negatively by retinoids and induced by 12-*O*-tetradecanoylphorbol-13-acetate and calcium (Lichti and Yuspa, 1988). TGase 1 is colocalized on the plasma membrane of migrating keratinocytes with involucrin but not with loricrin (Inada *et al.*, 2000), TGase 1 is expressed in the granular layer of the epidermis. The ubiquitous soluble tissue type TGase 2 (80 kDa; Chung, 1972) is expressed in the basal layer of the epidermis and has been shown to be implicated in apoptosis, cell adhesion, and signal transduction (Greenberg *et al.*, 1991; Reichert *et al.*, 1993). TGase 2 is expressed in the basal layer of the epidermis. The soluble proenzyme

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TGase 3 (77 kDa; Chung and Folk, 1972) is widely expressed and is important for epithelial barrier formation. It is a zymogen, requiring activation by proteolytic cleavage, and supposed to be responsible for the later stages of the forming of the epidermis (Kim et al., 1993). In the epidermis, TGase 3 is present only in the upper granular layer (Candi et al., 2001). The recently characterized TGase 5 is very efficient in using specific epidermal substrates (involucrin, loricrin, and small proline-rich protein 3). It is induced during the early stages of keratinocytes differentiation and is differently regulated in comparison with the other epidermal TGases. In cultured keratinocytes, this isoform is retained with insoluble proteins and is also colocalized with vimentin, which suggests a role related to this association with cytoskeleton components (Candi et al., 2001). TGase 5 is expressed in epidermis following a gradient of concentration from the basal layer to stratum corneum (Candi et al., 2002).

In the human hair follicle, the CCE formation is known to occur in the hair shaft, hair cuticle, and outer root sheath (ORS) in the isthmus region (Hashimoto, 1988). Indeed, TGase and major CCE precursor proteins, such as involucrin, loricrin, small proline-rich proteins 1 and 2, have been described to be coordinately expressed in developing human hair follicle (Akiyama et al., 1999). Involucrin and loricrin CCE precursors were found expressed in the hair canal, inner root sheath (IRS), and distal ORS of the bulbous hair peg and the differentiated lanugo hair follicle (Akiyama et al., 2002). TGases could thus be involved in these terminal differentiation events in the hair follicle and contribute to the physical resistance and preservation of this structure. In the bulbar and suprabulbar portions of human anagen hair follicles, TGase 1 was detected in the three layers of the IRS and found associated with terminal keratinization process (Tamada et al., 1995; Yoneda et al., 1998). Immunohistochemical analysis of tissue remodeling during the anagen–catagen transition indicated that this compartment was an early target in this process, suggesting a key role for the IRS and TGase 1 in the maintenance of hair follicle homeostasis (Commo and Bernard, 1997). TGase 2 has been recently observed in the hair germ, as the first active transglutaminase, and in the IRS of terminal anagen follicles (Akiyama et al., 2002). TGase 3 was thought to be required for the cross-linking of the structural protein trichohyalin and keratin intermediate filaments to form a rigid structure within the IRS cells, and participated in shape determination (Lee et al., 1996). In mouse hair follicle, this isoform was expressed in the IRS and the hair medulla but was absent from the hair cortex. Its pattern of expression in human hair follicle however remained to be established. Similarly, though TGase 5 was recently identified in the epidermis (Candi et al., 2001, 2002), its role and distribution in human hair follicle remained unknown. In our previous study (Thibaut et al., 2005), we had compared TGases 1, 3, and 5 distributions in the human hair follicle. TGases 1 and 5 were detected in the three layers of the IRS and their respective distribution in the complete pilosebaceous unit suggested that they probably had a complementary and sequential role in the hair CCE formation. On the other hand, we observed that TGase 3 was unexpectedly and exclusively expressed in the cortex and cuticle of human hair fiber. These latter results conferred a specific function to TGase 3, which we further investigated in this study.

RESULTS

TGase 3 expression and activity in the hair fiber

Indirect immunofluorescence experiment showed that TGase 3 did not codistribute with involucrin, which is a specific marker of IRS (Figure 1a). Both proteins were exclusive from each other and TGase 3 was specific to the hair shaft. In situ TGase activity in the different compartments of the hair follicle was revealed by monodansyl-cadaverine incorporation and a strong activity was found in IRS and in the hair shaft (Figure 1b). We thus concluded that the activity detected in the hair fiber was due to TGase 3. In the presence of 5 mM EDTA a very strong decrease in monodansyl-cadaverine detection was noted (Figure 1c). The TGase activity was totally inhibited in the keratogenous zone, in Henle's and Huxley's layers, but residual monodansyl-cadaverine signal remained detectable in the IRS cuticle, the hair cuticle and the hair cortex. As 5 mM EDTA was generally used to inhibit TGase activity in in vitro experiments, one cannot exclude that dansyl-cadaverine had chemically reacted with accessible free aldehydes, for example, generating some background. The immunohisto-chemical observation was then confirmed by western blot experiments. Hair fibers have been separated from IRS/ORS as evidenced by using a mix of K35 and involucrin antibodies. K35 was detected in hair shaft extract (Figure 1d, lane 3) but not in IRS/ORS extract (Figure 1d, lane 5). Inversely, involucrin was detected in IRS/ORS extract but not in hair fiber extract. Considering the high content of involucrin in IRS, there is no doubt that an IRS contamination would have been detected. When hair shaft proteins were blotted with primary antibodies specific for the N-terminal and C-terminal sites of the 77 KDa TGase 3 zymogen, the results revealed the two bands of proteolyzed TGase 3 (Figure 1d, lanes 1 and 2). Activation of the human TGase 3 zymogen indeed required a proteolytic cleavage to release two fragments of 47 and 30 KDa which noncovalently joint together to form the active enzyme, as previously described (Hitomi et al., 2003; Cheng et al., 2006). On the opposite, when blotted with the same antibodies, inner- and outer root sheaths protein did not reveal any TGase 3 peptides (Figure 1d, lane 4). Thus, we concluded that the active form of TGase 3 was indeed specific to the hair fiber.

Presence of isopeptide bonds in the hair fiber

After 2D separation of total hair shaft proteins under reducing conditions, K33-b keratin was detected by western blot experiment. We observed three clusters of spots, at 46, 58, and 92 KDa (Figure 2a). These results suggested that we also have extracted heterodimers (92 KDa) and that this 46-KDa hair keratin was covalently linked to small proteins (about 12 KDa). In the same way, K85 was detected in several spots ranging from 40 to 100 kDa (Figure 2b). Furthermore, anti-Nε-(γ-L-glutamyl)-L-lysine antibody revealed a strong signal in this range of molecular weight (Figure 2c). Indeed, several
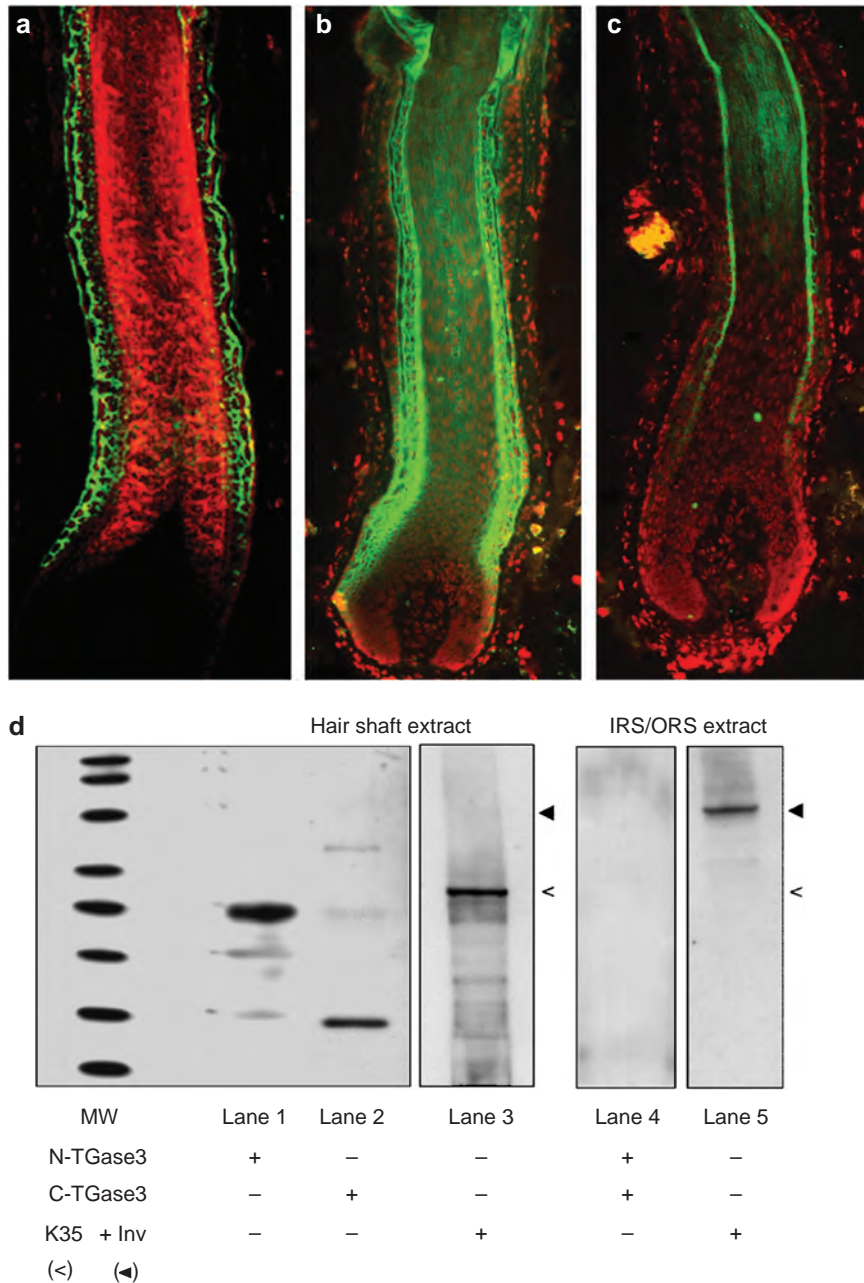
Figure 1. **TGase 3 expression and activity in the hair fiber.** (**a**) Unlike involucrin (green), TGase 3 immunolabeling (red) was detected in the hair cuticle and cortex. (**b**) *In situ* transglutaminase activity was detected by incorporation of dansyl-cadaverine in the different compartments of the hair follicle: a strong activity in the IRS corresponds to TGase 1 and TGase 5, whereas it corresponds to TGase 3 activity in the hair fiber. (**c**) Negative control, in presence of EDTA (5 mM). (**d**) Western blot experiments on hair shaft and IRS/ORS extracts. The two 47 kDa (lane 1) and 30 kDa (lane 2) subunits of activated TGase 3 were revealed in the hair shaft extract by primary antibodies specific for N terminal and C terminal, respectively. They were not detected in IRS/ORS extract (lane 4). Purity of each protein extract was controlled by using a mix of K35 and involucrin antibodies. K35 was evidenced in hair shaft extract (lane 3) but not in IRS/ORS extract QJ;(lane 5). Inversely, involucrin was detected in IRS/ORS extract (lane 5) but not in the hair shaft extract (lane 3). Scale bar: 100 µm.

bands were detected between 45 and 100 KDa, which led us to hypothesize that isopeptide bonds were involved in the intermediate filaments network. A control western blot was been performed with the commercial biotinylated secondary antibody with the expected negative signal (Figure 2d).

In a first attempt to confirm these hypotheses, 1D gel separation was performed on the total hair shaft extract and four bands from 75 to 250 kDa (Figure 3a) were excised, digested by trypsin and analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MS). A peptide mass fingerprinting obtained from each bands allowed the identification of several keratins listed in Table 1. The relevance of these identifications was based on the number of matching peptides (four peptides at least) combined with the mass measurement accuracy (15 p.p.m.).

The identification in a same band of K31 and K81 keratins, as illustrated for the band number 2 and K31 and K86 for band number 4 suggested the presence of heterodimers. The other bands showed a more complex distribution as we identified K31, K33-a, K83, and K85 for band 1 and K31, K33-a, K34, and K83 for band 3. Nevertheless these results confirmed the western blot observations and brought new evidences for the existence of a covalent network other than the disulfur network between the intermediate filaments. Moreover some nonassigned masses suggested the presence of other proteins that could participate to this network. As indicated before, the identification criteria required a minimum of 4 peptides matching, which excluded the identification of small proteins and particularly the keratin-associated protein (KAP) family proteins that generated fewer numbers of peptides after tryptic digestion. In order to fully interpret the mass data we manually compared the nonassigned masses to the predicted tryptic digestion peptides of KAP proteins. It appeared that two masses (common to band 1 and 2 mass lists, data not

shown) at m/z 976.455 and 1,049.468 could correspond to peptides from the KAP 1 family.

In order to complete this first gel-MS approach, we choose to improve gel resolution by performing 2D gel separation of the total hair shaft extract combined with nano-LC-MS/MS analyses to gain sequence information on identified proteins. Unfortunately, in the 250–75 kDa mass range, only faint spots appeared that did not allow us to obtain good MS/MS data. But surprisingly in the region below the intermediate filaments signature spots (annotated in Figure 4), we clearly identified in a same spot, acidic and basic hair keratins with five KAPs from four different KAP families (2, 3, 11, and 13). In this paper, we specifically focused on spots 1–3 as illustrated on Figure 4, whereas the full proteome by 2D-gel approach combined to nano-LC-MS/MS will be published elsewhere (paper in preparation). According to the position of these spots on the gel, the experimental molecular weight was too low for intermediate filaments suggesting the cleavage or degradation of these proteins, whereas the identification of KAP largely above their experimental molecular weight (10–18 kDa) suggested that KAPs were trapped into a macromolecular aggregate, likely stabilized by isopeptide bonds, considering that the extraction was performed under reducing conditions. The MS/MS sequence information were clear providing unambiguous identification of 12 different proteins—K81, K85, K86, K31, KAP2.n, KAP 3.1, KAP 3.3/3.2, KAP11.1 KAP 13.1, and S100A9/8. As indicated in Table 2, keratin proteins were clearly identified with more than one peptide sequences. In the case of KAP and S100A proteins, because of their particular small size, we accepted the identification with only one sequenced peptide but after manual examination and validation of the MS/MS spectra. An example of collision-induced dissociation fragmentation spectrum of a common peptide to the KAP 2 family is shown in Figure 4. Very good quality of the MS/MS spectra
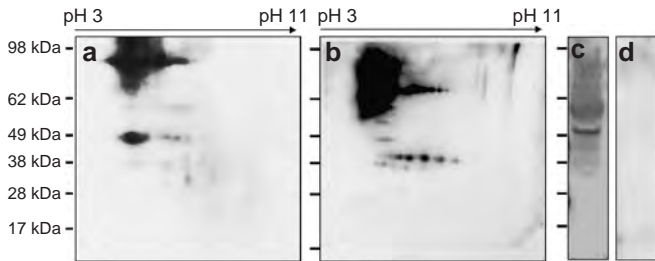

Figure 2. **Presence of isopeptide bonds in the hair fiber.** (**a**) Under reducing conditions, K33-b keratin migration revealed three areas at 46, 58, and 92 kDa, and (**b**) K85 was detected in several spots between 40 and 100 kDa, which suggested covalent cross-links with other keratins and small proteins. (**c**) Hair fiber extract was blotted with anti-Nε-(γ-L-glutamyl)-L-lysine antibody, and a strong signal was detected between 45 and 100 kDa. (**d**) Negative control western blot with commercial antiserum.
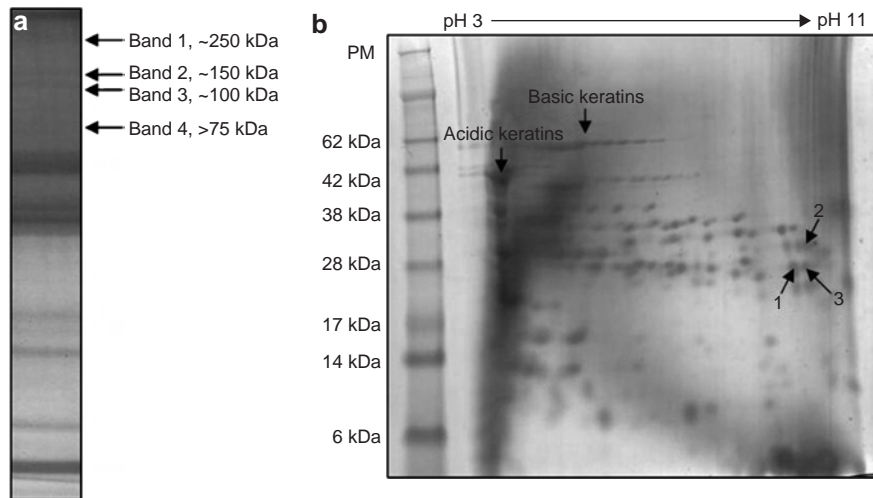

Figure 3. **1D and 2D gel electrophoresis of the hair shaft.** (**a**) 1D SDS PAGE of the total hair shaft extract. Arrows indicate four bands from 250 to 75 kDa. These bands were cut and digested with trypsin before MALDI-TOF MS analysis leading to the identification of KIF proteins. Band 1 MS analysis revealed K31, K33A, K83, and K85 proteins; band 2 K31 and K81 proteins; band 3 K31, K33A, K34, and K83 proteins; band 4 K31 and K86 proteins. (**b**) 2D SDS-PAGE of the total hair shaft extract. Arrows 1–3 represent the analyzed spots by nano-LC-MS/MS after tryptic digestion. In addition to KIF proteins, S100A9 and KAP 13.1 were identified in spot 1; S100A8, KAP 2.n, KAP 3.1, KAP 3.3, and KAP 11.1 in spot 2; and S100A8, KAP 2.n, KAP 3.1, KAP 3.3, KAP 11.1, and KAP 13.1 in spot 3.

was obtained, where b and y ions are observable as a minimum of 10 continuous amino-acid series, and the fact that the amino-acid sequences identified were specific to KAP peptides, permitted us to be confident with the identification results.

Finally, we performed amino-acid analysis on these same spots as above, but after pronase E digestion in order to detect the γ-glutamyl-ε-lysine dipeptide (Popescu and Hocker, 2007). Pronase E is a nonspecific enzyme that cleaves peptide bond and thus digests proteins into individual amino acids except for amino acids linked by an isopeptide bond or linked to proline (X-Pro; Tsao and Otter, 1999). In a first step, a standard solution of γ-glutamyl-ε-lysine dipeptide was injected on the instrument in order to determine the dipeptide retention time. The chromatogram revealed a peak at the retention time 53 minutes between the tyrosine and phenylalanine (data not shown). As depicted in Figure 5, the amino-acid analysis result of the 2D gel spot digested by pronase E revealed the presence of a small peak between the tyrosine and phenylalanine suggesting the presence of the γ-glutamyl-ε-lysine dipeptide in these spots and hence of isopeptide links at least between the identified proteins. Nevertheless, as proline-containing X-Pro dipeptides have been described to be resistant to pronase E digestion (Tsao and Otter, 1999), one cannot exclude that some of these dipeptides could have eluted at a similar location than Gln-Lys isodipeptide in the chromatogram. However, as all prolyl dipeptides were normally well separated in a high-pressure liquid chromatogram (Inoue *et al.*, 1999) and none of these X-Pro dipeptides were detected in our sample, a single X-Pro dipeptide, if present, could thus not have masked the Gln-Lys peak, detected between tyrosine and phenylalanine.

Cross-linking of intermediate filaments and KAPs with TGase 3
Considering all these observations, we next studied the *in vitro* reaction between TGase 3 and two TGase 3 potential substrates that are known to be expressed in the same cortical cells than
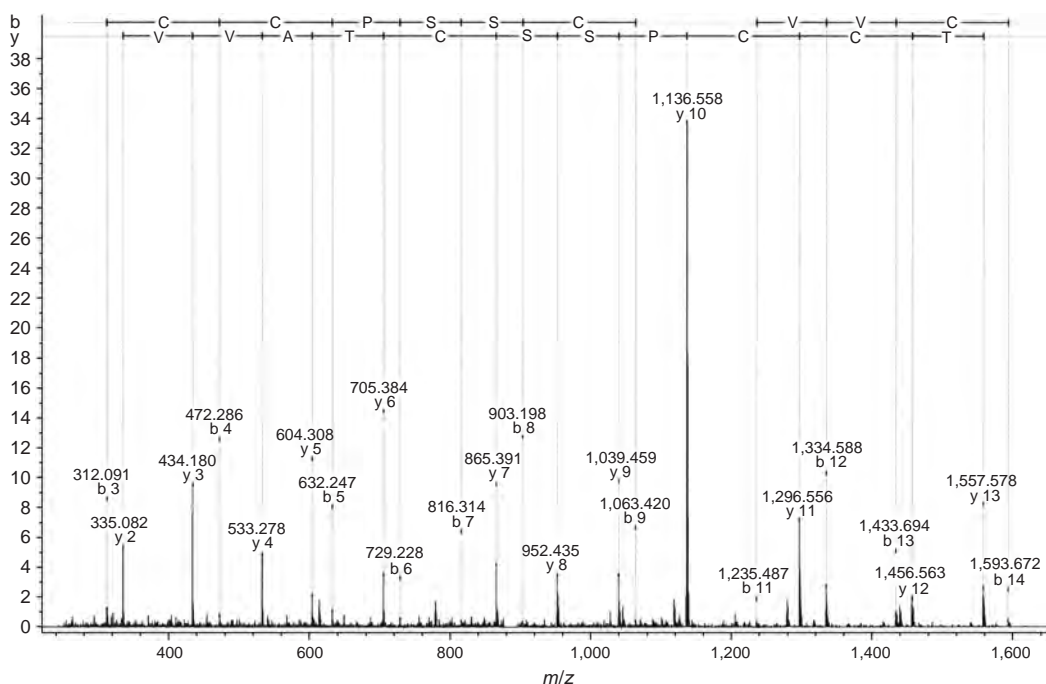

Figure 4. Nano-LC-MS/MS Sprectrum. Product ion spectra obtained by nano-LC-MS/MS after CID of the doubly charged molecular ion with m/z 884.44. The peptide sequence is shown above the spectra with the numbers corresponding to the b and y ions marked in the spectra. The fragmentation spectrum was identified as the common peptide to KAP 2 family: CCPSSCTAVVC.

Table 2. Identified proteins on 2D gel by nano-LC-MS/MS

2D spot no.	Precursor ion (charge)	Peptide number or peptide sequence	Identified protein	Coverage %	MW theoretical (kDa)	MW experimental (kDa)	MS/MS Mascot scores
1		4 Peptides	K81 (hHb1)	11	54.9	28	281
		4 Peptides	K85 (hHb5)	8	55.8		265
	807.907 (2+)	QLSFEEEFIMLMAR	S100A9	11	13.2		111
	704.351 (3+)	SLGYGGCFPSLGYGVGFCR	KAP 13.1	11	18.3		50
2		4 Peptides	K86 (hHb6)	14	53.5	28	382
		4 Peptides	K85 (hHb5)	12	55.8		341
		2 Peptides	K81 (hHb1)	5	54.9		168
		3 Peptides	K31 (hHa1)	9	47.2		210
	1196.089 (2+)	ELDINTDGAVNFQEFLILVIK	S100A8	22	10.8		104
	884.442 (2+)	PITCCPSSCTAVVCR	KAP 2.n	11	13.5		119
	931.464 (2+)	SCSVPTGPATTFCSFDK	KAP 3.1	17	10.5		89
	869.435 (2+)	GCSVPTGPATTICSSDK	KAP 3.3	17	10.4		84
	888.441 (2+)	QTTCISNPCSTTYSR	KAP 11.1	9	17.1		77
3		7 Peptides	K85 (hHb5)	17	55.8	35	622
		6 Peptides	K86 (hHb6)	19	53.5		591
		5 Peptides	K81 (hHb1)	14	54.9		363
		2 Peptides	K31 (hHa1)	6	47.2		128
	1196.089 (2+)	ELDINTDGAVNFQEFLILVIK	S100A8	22	10.8		57
	884.442 (2+)	PITCCPSSCTAVVCR	KAP 2.n	11	13.5		105
	931.464 (2+)	SCSVPTGPATTFCSFDK	KAP 3.1	17	10.5		86
	869.435 (2+)	GCSVPTGPATTICSSDK	KAP 3.3	17	10.4		60
	888.441 (2+)	QTTCISNPCSTTYSR	KAP 11.1	9	17.1		68
	712.359 (2+)	SCYSVGCGSSGFR	KAP 13.1	7	18.3		53

MW, molecular weight.
The precursor ions m/z and charges are specified when identifications were achieved with only one peptide as it was the case for KAP and S100A proteins. The sequenced peptides allowing the identification are also indicated. When more than one peptide was sequenced, only the number of peptides is specified. Mascot scores >50 are significant.

the enzyme (Langbein *et al.*, 1999; Thibaut *et al.*, 2003; Rogers *et al.*, 2006), that is, the hair keratin K33-b and the high-sulfur-protein KAP 3.1, here used as a rhodamine-conjugated synthetic peptide (rhodamine–FSDKSCRCGVCLPSTCPHEISLLQPI, rhodamine-KAP3.1-SP). When these two cystein-rich proteins were incubated with no enzyme (Figure 6a, lane 3) or with inactivated TGase 3 (Figure 6a, lane 2), we only observed two bands corresponding to K33-b and KAP3.1-SP peptides (35 and 2.5 KDa, respectively). However, in the presence of activated TGase 3 (recombinant zymogen was cleaved with dispase I), we produced covalent links that were stable under reducing conditions (Figure 6a, lane 1). We indeed noted that rhodamine-peptides migrated at 2.5 KDa (free rhodamine-labeled KAP 3.1) and at 35 KDa (Figure 6b, lane 1). TGase 3 thus produced at least one covalent cross-link between K33-b and KAP3.1, which supported our hypothesis.

DISCUSSION

TGase 3 revealed a strikingly different distribution from other isoforms in human hair follicle, as it was specifically expressed in the cortex and cuticle of growing hair fiber. Akiyama *et al.* (2002) recently studied the CCE formation in fetal hair follicle and observed that TGase 3 was expressed, as TGase 1, during the bulbous hair peg, in the hair canal and IRS. This pattern was maintained in differentiated lanugo hair follicle. Their results were however not in agreement with ours. This discrepancy might be related to the developmental stage studied, namely fetal hair versus adult hair. In this study, TGase 3 specific distribution was confirmed by laser confocal microscopy: it was found clearly exclusive of those of involucrin, TGase-1 and -5 (Thibaut *et al.*, 2005) and strictly restricted to the hair shaft. This TGase 3 expression pattern has recently been confirmed by western blot experiments, the active form of TGase 3 being specific of hair fiber extract (Figure 1d). The *in situ* activity detection revealed TGase activity in both IRS and hair shaft. Taken together, these results showed that TGases 1 and 5 were likely involved in the IRS CCE formation whereas TGase 3 could participate in hair shaft keratinization.

The hair shaft is characterized by the expression of specific proteins, consisting of a set of acidic and basic keratins
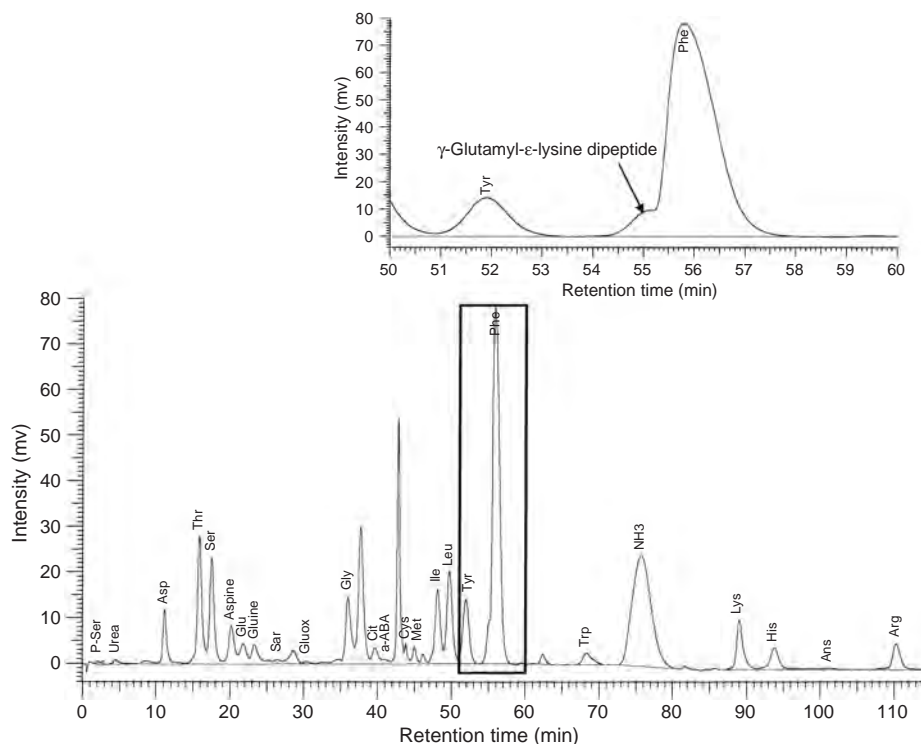
Figure 5. **Amino-acid analysis of the 2D gel spots.** Chromatogram obtained after pronase E digestion of 2D gel spots. The peaks are labeled by the three-letter code of amino acids, recognized from their specific retention times. The upper panel shows a part of the chromatogram zoomed between the RT 50–60 minutes. This region revealed a new peak between tyrosine and phenylalanine. The injection of the γ-glutamyl-ε-lysine dipeptide alone indicated that its retention time was around 53 minutes (between tyrosine and phenylalanine) and thus could be the new peak magnified in the upper panel.

(Langbein *et al.*, 1999, 2001) and KAPs (Rogers *et al.*, 2001, 2002; Shimomura *et al.*, 2002a, b). Indeed, in the hair cortex, hair keratin intermediate filaments are embedded in an interfilamentous matrix, consisting of hair KAPs, which are essential for the formation of a rigid and resistant hair shaft through their extensive disulfide bond cross-linking with abundant cysteine residues of hair keratins (Powell *et al.*, 1991; Powell and Rogers, 1997). To date, it is still not clear how intermediate filaments of keratins and KAPs interact with each other. These proteins are believed to interact mainly through disulfide bonds and hydrophobic connections (Shimomura and Ito, 2005). In this study, we propose a new model of interactions between keratins and KAPs.

The TGase 3 specificity and the transglutaminase activity in the hair shaft led us to hypothesize a similar function to that in the stratum corneum (Kim *et al.*, 1993; Candi *et al.*, 2001). In the epidermis, TGase 3 is specific for the upper granular layer and supposed to be responsible for the later stages of the CCE formation. Its mechanism and various substrates have been studied *in vitro* and the production of isopeptide cross-links between the ε-NH2 of protein-bound lysine residue and the γ-carboxamide of protein-bound glutamine residue generated insoluble macromolecular aggregates (Ahvazi and Steinert, 2003; Ahvazi *et al.*, 2004). Here, by combining 2D gel electrophoresis of reduced-alkylated human hair fiber extracts, western blot analysis and MS, we demonstrated the presence of KAP-keratin macro-molecular aggregates and the signature of γ-glutamyl-ε-lysine

isopeptide bonds. Even though isopeptide linkages can thus be suspected in hair extracts, we did not provide the definite demonstration that a given isopeptide bond held together one given keratin and one given KAP. Indeed, as only spots from 2D gels were digested and analyzed, the high sequence homology which characterized both hair keratins and KAPs, made it impossible to discriminate which keratin was connected to which KAP, and in which position. As a matter of fact, it is not certain that such a bond could be identified, as Tgase might be involved in a stochastic reticulation process, as suggested by the "dustbin" hypothesis (Michel *et al.*, 1988). This isopeptide network was without any doubt quantitatively less important than the disulfide network in the hair shaft, as the analyzed peaks were rather weak. Nevertheless, considering the reduction-alkylation procedure we used for the preparation of MS samples, we do believe that isopeptide bonds indeed exist between keratins and KAPs and/or keratins–keratins and/or KAPs–KAPs.

To verify this hypothesis, we studied the *in vitro* reaction between TGase 3 and two potential substrates, namely recombinant keratin K33-b and KAP 3.1, that are known to be expressed in the same cortical cells as the enzyme (Langbein *et al.*, 1999; Thibaut *et al.*, 2003; Rogers and Schweizer, 2005). The hair keratin K33-b carries 33 glutamine and 12 lysine residues, and the KAP 3.1 mimetic rhodamine-conjugated peptide, we synthesized, contained one glutamine and one lysine residue. When these two substrates were incubated with activated TGase 3, we
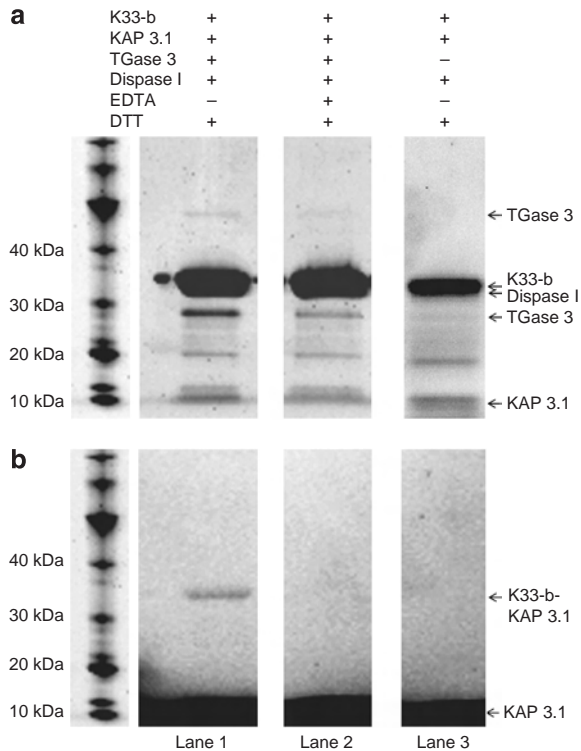
a

K33-b	+	+	+
KAP 3.1	+	+	+
TGase 3	+	+	−
Dispase I	+	+	+
EDTA	−	+	−
DTT	+	+	+

← TGase 3

K33-b
← Dispase I
← TGase 3

← KAP 3.1

40 kDa
30 kDa
20 kDa
10 kDa

b

← K33-b-KAP 3.1

← KAP 3.1

40 kDa
30 kDa
20 kDa
10 kDa

Lane 1   Lane 2   Lane 3

Figure 6. *In vitro* cross-linking of keratins and KAPs by TGase 3. Lane 1: K33-b and KAP 3.1-synthetic peptide (KAP3.1-SP) were incubated with activated TGase 3 and electrophoresed under reducing conditions; lane 2: K33-b and KAP3.1-SP were incubated with inactivated TGase 3 (with EDTA); lane 3: K33-b and KAP3.1-SP in dispase I buffer. Reaction products were electrophoresed, and then (**a**) total proteins were revealed with sypro-ruby staining whereas (**b**) free and cross-linked rhodamine-KAP3.1-SP was visualized by UV transillumination. In the presence of activated TGase 3 only, rhodamine-KAP3.1-SP (2.5 kDa) migrated as a 35 kDa peptide under reducing condition.

observed the emergence of covalent, nonreducible, TGase 3 catalyzed isopeptide cross-links between recombinant hair keratin and KAP.

The demonstration of TGase3 activity in the hair shaft led us to examine carefully the amino-acid sequences of human hair keratins and KAPs. First, when analyzing the most abundant amino acids in K81 compared with K31 and specifically for the rod part, we found that for both keratins the three most abundant amino acids were glutamic acid, leucine, and alanine and interestingly, the fourth most abundant amino acid was glutamine for the acidic keratin whereas it was lysine for basic keratin. Secondly, when looking at KAP sequences it appeared that some families had a ''lysine rich'' profile and others were ''glutamine rich''. In this respect, KAP 5 and KAP 10 families, as cuticle markers (Rogers *et al.*, 2006), exhibit the highest levels of glutamine and lysine in their primary sequences. As it is now admitted that cuticle contains isodipeptide reticulation by homology with CCE (Popescu and Hocker, 2007), it is likely that these KAPs are involved in the cuticle reticulation.

All these lysine- and glutamine-rich sequences in human hair keratins and KAPs could thus represent as much potential targets for naturally occurring TGase 3-mediated cross-

linking. Our results were in total agreement with these observations and thus suggested that the hair fiber was composed by a keratin network with additional covalent cross-links aside from disulfide bonds. Indeed, western blot experiments under reducing conditions revealed that hair keratin was cross-linked with a number of small proteins. These results were confirmed by 1D and 2D gel electrophoresis combined with MS by the unambiguous identification of keratin and KAP partners in common spots. Taken together, these results led us to propose the existence of an isodipeptide network in human hair fiber implicating keratins and KAPs, even though the interacting partners are yet not clearly defined. To note, this isopeptide network is probably not strictly restricted to keratin and KAP interactions because, for example, S100A8/9 proteins were also identified and could thus participate to this network. Our hypothesis was further supported by a recent publication of the human hair shaft proteome (Lee *et al.*, 2006) where TGase 3 was identified by LC-MS/MS as a major component of the hair fiber, highly involved in the hair fiber keratinization.

All these data indicate that TGase3 may indeed have a role in hair shaft keratinization and scaffolding. This enzyme could also contribute to the rigidity of the hair fiber, as revealed by Tarcsa *et al.* (1997) in the mouse anagen hair. The statement that TGase3 was involved in hair shaft scaffolding would be strengthened by a functional study, for example, on human hair follicle grown *in vitro*. Specific inhibitors for TGase 3 would provide relevant investigation but none has ever been described so far. In addition, for technical and ethical reasons, we could not look at any hair follicles that have fiber defects, such as Nethertons. Indeed, according to Raghunath *et al.* (2004), TGase 3 activity was absent in Netherton syndrome and this differential TGase expression was linked to a marked alteration of epidermal differentiation and hair synthesis, somehow supporting our hypothesis of TGase3 being involved in hair fiber scaffolding. The mechanisms of interaction between keratins and KAPs are not completely identified (Shimomura and Ito, 2005) and these proteins are believed to interact mainly through disulfide bonds and/or hydrophobic connections. We propose that TGase 3-driven specific isopeptide bonds between keratins and KAPs participate in the progressive scaffolding of hair shaft and reinforce these interactions.

MATERIALS AND METHODS
Immunohistochemistry
Human scalp biopsies were obtained from facelift surgery in healthy caucasian volunteers after written consent, and according to the principles of the declaration of Helsinki. Institutional approval was not required for experiments.

Face-lifting biopsies were cut into small pieces containing about six intact follicles. Unfixed tissue samples were embedded in Tissue-Tek OCT compound (Miles, Naperville, IL, USA), quick frozen over dry ice (−80 °C), and stored at −80 °C until used for immunohistochemistry. Longitudinal and transverse frozen sections of hair follicles were prepared as previously described (Thibaut *et al.*, 2003). Anti-TGase 3 primary antibody (provided by Dr D Bernard, L'OREAL; Thibaut *et al.*, 2003), was diluted 1:200 in phosphate-

buffered saline, containing 0.05% Tween 20, and its distribution was compared to that of involucrin (Sigma, St Quentin-Fallavier, France; dilution 1:40). Fluorescent species-specific secondary antibodies were then applied for 30 minutes at room temperature. Stainings were analyzed with a Zeiss Axioscop microscope (Carl Zeiss, Oberkochen, Germany).

In situ total TGase assay
Longitudinal hair follicle sections were first incubated in 100 mM Tris-HCl pH 8 containing 1% goat serum for 30 minutes. 12 μM dansyl-cadaverine (Sigma) and 5 mM $CaCl_2$ were then added for 1 hour incubation, followed by immunolabeling with an anti-dansyl antibody (Interchim, Montluçon, France). Dansyl-cadaverine incorporation revealed TGase activity in the different compartments of the hair follicle. The reaction was stopped with 10 mM EDTA. For negative control, 5 mM EDTA was added during the incorporation of dansyl-cadaverine.

In vitro TGase 3 assay
To prepare the proteolyzed TGase 3 form, 2.5 μg zymogen (Immundiagnostik AG, Bensheim, Germany) was treated with 20 mU dispase I (Roche Diagnostics, Mannheim, Germany) in the presence of 5 mM $CaCl_2$ at 37 °C for 30 minutes, as described by Zeeuwen *et al.* (2001). Then the activated TGase 3 was incubated with two potential substrates: recombinant K33-b (hHa3-II) hair keratin (Tebu-Bio, Le Perray en Yvelines, France) and a rhodamine-conjugated synthetic peptide of the high-sulfur-protein KAP 3.1 (rhodamine–FSDKSCRCGVCLPSTCPHEISLLQPI, rhodamine-KAP3.1-SP; NeoMPS, Strasbourg, France) in 100 mM Tris-HCl pH 7.5, 0.1 M $CaCl_2$ for 2 hours. Reaction products were electrophoresed on 4–12% SDS-PAGE gels under reducing conditions, in the presence of DL-Dithiothreitol (DTT; Sigma). Proteins were revealed with sypro-ruby staining (Invitrogen, Cergy Pontoise, France) and rhodamine-peptides were detected with a CCD camera (BioRad, Marnes La Coquette, France).

One- and two-dimensional gel electrophoresis for western blot
Human hair follicles were microdissected (Philpott *et al.*, 1990) and dermal compartment was removed. Hair follicles were then immersed in phosphate-buffered saline and IRS/ORS were separated from hair fiber as previously described (Heid *et al.*, 1986). The ORS/IRS layers were removed by manual dissection in 1 D buffer (10 mM Tris-HCl, 1% triton X-100, 2% SDS) and separated by electrophoresis on NuPAGE 4–12% Bis-Tris gel (Invitrogen). Alternatively, hair swatches were cut into small pieces, and crushed in a Laemmli–β-mercaptoethanol (5%) buffer for 1 hour at 90 °C. The filtrate was precipitated with acetone containing 10% trichloroacetic acid and 20 mM DTT, centrifuged 2 minutes at 10,000 *g* and the pellet was resuspended in 2D buffer (8 M urea, 2 M thiourea, 2% SDS, 100 mM DTT, 100 mM Tris-HCl, pH 8.0). Proteins were then quantified and separated by 2D gel electrophoresis. Isoelectric focusing was performed using an Invitrogen system. Immobilized pH gradient strips, pH 3–10, were incubated overnight at room temperature with 50 μg of the solubilized proteins plus 80 μl of 2D buffer, then focused at 20 °C at 200 V for 20 minutes, 450 V for 15 minutes, 750 V for 15 minutes, and 2,000 V for 4 hours. Immobilized pH gradient were re-equilibrated following the manufacturer's instructions and alkylated with iodoacetamide. Horizontal SDS electrophoresis was performed on NuPAGE 4–12% Bis-Tris gel at 200 V for 1 hour. For western blot analysis, proteins were transferred onto Hybond-ECL nitrocellulose membranes (Amersham Pharmacia Biotech, Buckinghamshire, England). After blocking, membranes were incubated with primary antibodies specific for N-terminal and C-terminal TGase 3 (Dr D Bernard, L'OREAL, France), involucrin (Sigma), K35 and K33-b hair keratins (provided by Dr J Schweizer, DKFZ, Heidelberg, Germany), and Nε-(γ-L-glutamyl)-L-lysine (mab0009; Covalab, Villeurbanne, France). After incubation with biotinylated secondary antibodies, detection was carried out using the ECL-plus chemiluminescence reagent (Amersham Pharmacia Biotech).

Hair fiber protein extraction, 1D and 2D gel electrophoresis for mass spectrometry and amino-acid analysis
After delipidation of the hair shafts with ethanol followed by cyclohexane, proteins were extracted with 200 mM Tris-HCl, 200 mM DTT, 0.1% Triton X100, and 2% CHAPS, for 16 hours at 37 °C. The protein extract was then alkylated with a solution of 1 M iodoacetamide, 3 M Tris-HCl, pH 8.4 for 10 minutes in the dark at room temperature, dialyzed in 3,500 MWCO dialysis cassettes (Pierce, Rockford, IL, USA) against water over a period of 48 hours before being freeze dried.

For 1D gel, the extract was solubilized in Laemmli buffer (BioRad). After 5 minutes at 95 °C, the protein sample was run on the mini protean II electrophoresis system at 200 V for 40 minutes.

For 2D gel, the proteins were suspended in the rehydration buffer composed of 7 M urea, 2 M thiourea, 2% CHAPS, 0.5% ampholytes pH 3–11 and DeStreak rehydration solution (GE Healthcare, Uppsala, Sweden) and incubated with IPG strips pH 3–11 from GE Healthcare, in a PROTEAN isoelectric focusing cell (BioRad) for 16 hours. Isoelectric focusing was performed by a stepwise voltage increase until reaching 14,400 VH. Then after the strips were equilibrated in 6 M urea, 2% SDS, and 0.5 M DTT and loaded on NuPAGE 10% Bis-Tris gels (Invitrogen). The electrophoresis was carried out at 200 V for 40 minutes on the Novex mini cell system from Invitrogen. All the gels were stained by Coomassie blue (SimplyBlue, Invitrogen). The spots of interest were excised from both gels and one half subjected to trypsin digestion for further MS analysis and the other half was digested by pronase E for amino-acid analysis.

In-gel protein digestion
All chemical treatments were performed using an automated protein digestion system, MassPREP station (Waters, Milford, MA, USA). Buffers and reactants were prepared with water purified using a Direct-Q from Millipore. The gel spots were washed twice with 50 μl of 25 mM NH_4HCO_3 (Sigma, Steinheim, Germany) and 50 μl of acetonitrile (Carlo Erba Reactifs-SDS, Val de Reuil, France). The cysteine residues were reduced at 60 °C for 1 hour by 50 μl of 10 mM DTT prepared in 25 mM NH_4HCO_3 and alkylated at room temperature for 20 minutes by 50 μl of 55 mM iodoacetamide (Sigma) prepared in 25 mM NH_4HCO_3.

Trypsin digestion: after dehydration of the gel spots with acetonitrile, the proteins were digested overnight in gel by adding 10 μl of 12.5 ng μl^{-1} modified porcine trypsin (Promega, Madison, WI, USA) in 25 mM NH_4HCO_3 at room temperature. The generated peptides were extracted with 35 μl of 60% acetonitrile in 5% formic acid (Riedel-de Haën, Seelze, Denmark) followed by removing acetonitrile excess and were subjected to nano-LC-MS/MS.

Pronase E digestion: The spots of interest were pooled together and digested with 40 µl of pronase E (Sigma) at 0.5 mg ml^{-1}, in 0.5 M Tris-HCl buffer pH 6.8 containing 2 mM CaCl$_2$ for 72 hours at 37 °C.

The supernatant containing amino acids was taken and injected for the amino-acid analysis after adding 80 µl of LiOH buffer.

Protein identification by mass spectrometry

Matrix-assisted laser desorption ionization time-of-flight MS.

The analysis was carried out on the Ultraflex matrix-assisted laser desorption ionization time-of-flight/time-of-flight MS (Bruker Daltonics, Bremen, Germany) operating in the positive ion reflectron mode at 25 kV accelerating voltage. Ionization was accomplished with the 337-nm beam from a nitrogen laser with a repetition rate of 5 Hz. Tryptic digests were deposited on a MALDI probe and cocrystallized with an α-cyano-4-hydroxycinnamic acid matrix prepared in water/acetonitrile (1/1). A camera allowed visualization of the sample crystallization homogeneity before measurements. Internal calibration was performed using the two monoisotopic trypsin autolysis peaks at m/z 842.50 and 2211.10. Matrix-assisted laser desorption ionization time-of-flight MS data collected were searched against the SwissProt database with a mass tolerance of 50 p.p.m. using a local Mascot server (Matrix Science, London, UK).

Nano-LC-MS/MS.

The tryptic digests were analyzed by nano-LC-MS/MS using an Agilent 1100 series HPLC-Chip/MS system (Agilent Technologies, Palo Alto, CA, USA) coupled to an HCT Ultra ion trap (Bruker Daltonics). Peptides were separated on a reversed phase C18 column (Zorbax 300SB-C18, 75 µm × 43 mm, 5 µm i.d.) using an acetonitrile gradient going from 8 to 40% solvent B in 7 minutes with A: H$_2$O + 2% acetonitrile + 0.1% formic acid and B: acetonitrile + 2% H$_2$O + 0.1% formic acid. Solvents used were of highest quality. Water was purified using a Direct-Q from Millipore and ACN HPLC grade was purchased from Carlo Erba Reactifs-SDS (Val de Reuil, France). The voltage applied to the capillary cap was optimized to −1,850 V. For tandem MS experiments, the system was operated with automatic switching between MS and MS/MS modes. The three most abundant peptides and preferentially doubly charged ions, were selected on each MS spectrum for further isolation and fragmentation. The MS/MS scanning was performed in the ultrascan resolution mode at a scan rate of 26,000 m/z per second. A total of 6 scans were averaged to obtain a MS/MS spectrum. The complete system was fully controlled by ChemStation (Agilent Technologies) and EsquireControl (Bruker Daltonics) softwares.

In order to verify the nano-LC-MS/MS system performances, 200 fmol of a bovine serum albumin tryptic digest was injected. Criteria as peptide retention times, resolution, sensibility, and mass precision were verified.

Mass data collected during nano-LC-MS/MS analysis were processed, converted into *.mgf files and searched against the SwissProt database using a local Mascot server (Matrix Science). Database search criteria included the possibility to have one misscleavage and tryptic peptides with a proline as N-terminal amino acid. Mass tolerance for the MS and MSMS ions was fixed at 0.3 Da. Methionine oxidation and cysteine carbamidomethylation were allowed as variable modifications. For proteins identified with one peptide, MS/MS spectra were manually inspected and validated in the case when interpretable successive y- and b-ions series were observed, permitting to unambiguously attribute an amino-acid sequence to a protein.

Amino-acid analysis

The proteins digested by pronase E were analyzed by the L-8800 High Speed Amino Acid Analyzer (Hitachi, Tokyo, Japan) equipped with an autosampler, an ammonia filter column (4.6 mm ID × 40 mm L), a precolumn (4.6 mm ID × 20 mm L), and a column (4.6 mm ID × 60 mm L) packed with the Hitachi custom ion exchange resin. The column oven was set to 50 °C. The amino-acid separation was performed by the stepwise increase of the ionic force of the LiOH buffer. After separation the amino acids were detected by postcolumn derivatization with ninhydrin, with a photometer at a wavelength of 570 nm. The calibration of the retention times was done by injection of a standard amino-acid mixture sample and the γ-glutamyl-ε-lysine dipeptide at 0.25 µmol ml^{-1}. The sample was analyzed in duplicate.

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