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Ordered Structure Acquisition by the N- and C-Terminal Domains of the Small Proline-Rich 3 Protein

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Abstract The cell envelope (CE) is a vital structure for barrier function in terminally differentiated dead stratified squamous epithelia. It is assembled by transglutaminase (TGase) cross-linking of several proteins, including hSPR3 in certain specialized epithelia normally subjected to mechanical trauma. Biochemical studies show that hSPR3 serves as a complete substrate for TGase1, TGase2, and TGase3. Multiple adjacent glutamines and lysines of only head-and-tail domain sequences are used by each enzyme for cross-linking. Structural data suggest that the hSPR3 central repeats, as well as hSPR1 and hSPR 2, are highly flexible and mobile; thus, the TGases might not be able to recognize the residues localized on the repeats as adequate substrate. To investigate this hypothesis further and to complete the structural investigation of hSPR3, we performed circular dichroism (CD) studies on peptides corresponding to the N- and C-terminal domain. CD spectra have also been carried out in the presence of different concentrations of the structure-promoting agent cosolvent trifluoroethanol (TFE), which mimics a partial hydrophobic environment found in vivo in or next to the membrane. In fact, this agent increases the dielectric constant of water proportionally, depending on its concentration, and confers structuring properties to the solution, to peptides and proteins that have a structuring propensity. The results indicate that in both the N-terminal and C-terminal, peptides acquire a more ordered structure as a function of the TFE concentration in water. This ability of both N- and C-terminal domain to acquire a more stable ordered conformation might be relevant for SPR3 to act as substrate of TGases. Indeed, only the N- and C-terminus is cross-linked by TGase1 and 3. J. Cell. Biochem. 77:179–185, 2000. © 2000 Wiley-Liss, Inc.

Key words: barrier function; small proline-rich protein; cornified envelope; circular dichroism

Stratified squamous epithelia undergo a complex terminal differentiation program that begins from the basal proliferate layer and ends with the horny layer, formed by dead epithelial cells that are eventually lost from the epithelial surface by desquamation [Melino et al., 1998] (Fig. 1A). These layers of dead epithelial cells function largely to prevent water loss and as a physical barrier against the environment. A major component of this physical barrier is a specialized structure termed the cell envelope (CE), a thick layer of highly insoluble protein just beneath the plasma membrane (Fig. 1B) [Hohl, 1990; Reichert et al., 1993; Robinson et al., 1997; Simon, 1994; Steinert, 1995; Steinert and Marekov, 1995, 1997; Steinert et al., 1998; Nagy et al., 1997]. The CE is assembled by cross-linking several defined structural proteins (Fig. 1) by both disulfide and N^{ϵ}-(γ-glutamyl)lysine isopeptide bonds formed by the action of transglutaminases (TGases) [Hohl, 1990; Reichert et al., 1993; Simon, 1994]. Several TGase enzymes are likely to be involved, including the membrane-bound TGase 1 enzyme and its various highly active isoforms, and the cytoplasmic TGase2, TGase3, and TGaseX enzymes [Aeschlimann et al., 1998; Candi et al., 1995, 1998; Steinert and Marekov,

Abbreviations used: CE, cornified envelope; SPR, small proline-rich (protein); TGase, transglutaminase; TFE, 2,2,2-trifluoroethanol; CD, circular dichroism.

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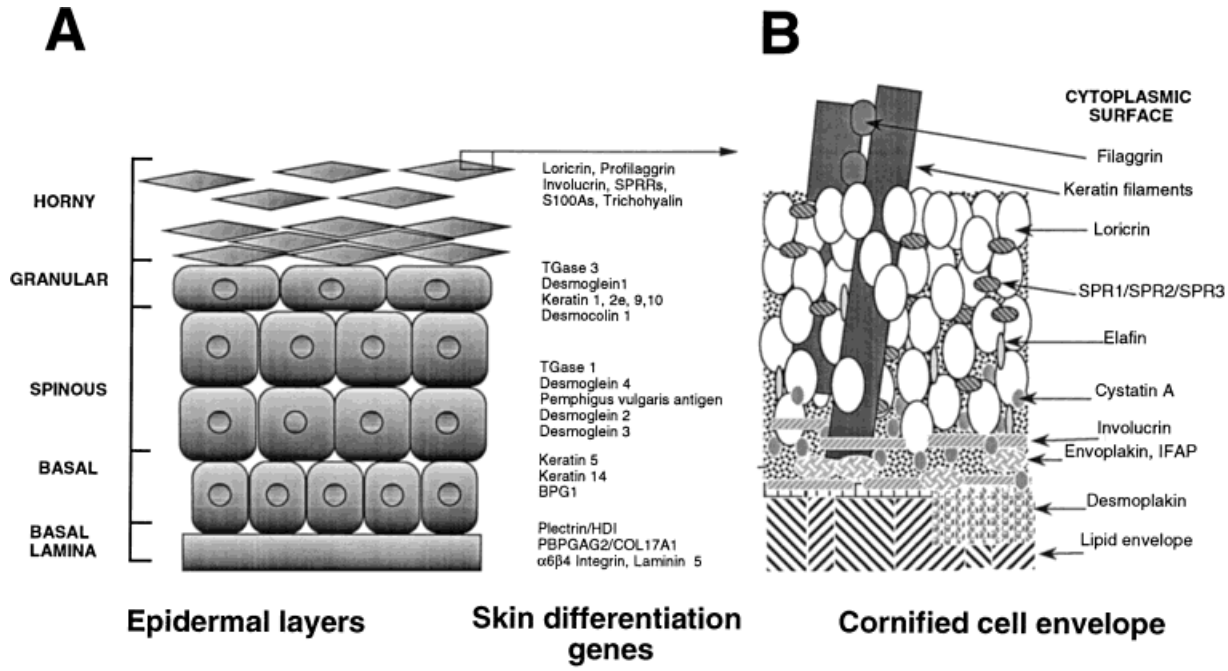

Fig. 1. Schematic representation of the epidermal differentiation. **A:** Epidermal layers, from the basal layer, inner, to the horny layer, outer. Genes expressed in the specific layers during epidermal differentiation are also indicated. **B:** Theoretical model of the cornified cell envelope of human foreskin epidermis. The model has been done by microsequencing peptides directly extracted from cornified envelope of human foreskin epidermis.

1997; Tarcsa et al., 1997, 1998]. Among the structural proteins involved in the CE assembly, the SPR family represents the most complex group of constituents (Fig. 1B).

The SPR family consists of human and mouse of 11–12 members divided into three families: SPR1 (two members), SPR2 (8–11 members), and SPR3 (1 member) [for review, see Gibbs et al., 1993; Kartasova et al., 1996; Song et al., 1999; Steinert et al., 1998]. All members are built according to a common plan of amino (N) (head) and carboxy (C) (tail) domains containing several adjacent glutamine and lysine residues, spanning a central domain composed of a series of peptide repeats of 8–9 residues that are highly enriched in prolines. The precise sequence of these repeats permits distinction into the three families. Various individual members of SPR proteins are expressed in widely differing amounts in different epithelia, whose expression is generally upregulated in response to epithelial injury or disease. The SPR3 protein, for example, is widely expressed in a variety of terminally differentiated, dead stratified squamous epithelia that are normally subjected to significant mechanical trauma, such as the esophagus, filiform ridges of the tongue, inner root sheath of the hair follicle, and rodent forestomach [Austin et al., 1996; Fujimoto et al., 1997; Hohl et al., 1995; Song et al., 1999; Steinert et al., 1998].

We have shown that for the SPR1 [Candi et al., 1999], SPR2 [Tarcsa et al., 1998], and SPR3 [Steinert et al., 1999] proteins, each TGase enzyme preferentially cross-links certain glutamine and lysine residues with high specificity, from which we could conclude that multiple enzymes are required to cross-link them in vivo. In addition, we have shown that glutamine and lysine residues on only head-and-tail domains are used in cross-linking SPRs both in vivo and in vitro [Candi et al., 1999; Tarcsa et al., 1998; Steinert and Marekov, 1995; Steinert and Marekov, 1997; Steinert et al., 1998a,b]. The data imply that the SPRs serve as cross-bridging proteins by adjoining themselves or other proteins of the CE through the use of multiple adjacent residues of the end domains [Steinert et al., 1998]. So far, detailed investigation of the structural features of human SPR3 protein have been carried out only

on a peptide corresponding to three repeats of the central domain. These data suggest that the hSPR3 central repeats are highly flexible and mobile; thus, the TGases might not be able to recognize the residues localized on the repeats as adequate substrate. To investigate this hypothesis further and to complete the structural investigation on hSPR3, we performed circular dichroism (CD) studies on peptides corresponding to the N- and C-terminal domain. CD spectra have also been carried out in the presence of different concentrations of the structure-promoting agent cosolvent trifluoroethanol (TFE). The increase in the hydrophobicity of this cosolvent exerts a large structuring effects on peptides or proteins with an intrinsic propensity for adopting a secondary structure. The results indicate that small structuration occurs in both the N- and C-terminal peptides as a function of the TFE concentration in water, suggesting that a very low intrinsic propensity toward a classical secondary structure is present in the protein under study. In particular, both in destructuring or structuring conditions such as high temperature or high concentration of TFE, the protein does not undergo very large changes of conformation, but small structuring effects occur (β-turns) locally in some part of the primary structure.

MATERIALS AND METHODS

Circular Dichroism and Peptides

CD spectra were recorded on a Jasco 600 spectropolarimeter. Spectra were recorded from 200–250 nm with readings every 0.1 nm, using a 0.1-cm pathlength curvet. Sample temperatures were maintained at 23°, 30°, 40°, 60°, or 70°C in a thermostatted sample holder, using external circulation for at least 1 h before measurement. The synthetic peptide was synthesized by IDI-IRCCS (Rome, Italy) and purified by high-performance liquid chromatography (HPLC), dissolved into 10 mM phosphate buffer (pH 7.0) and used at a concentration of 1 mM. Solvent spectra were subtracted from those of the samples. A total of three or four scans were accumulated and averaged for each sample.

RESULTS AND DISCUSSION

The purpose of this study is to provide basic information on secondary structure of N- and C-terminal domains of hSPR3 in an attempt to correlate their structure and their possible functional role. The biochemical data on the utilization of the Gln and Lys residues of hSPR3 [Steinert et al., 1999] are summarized in Figure 2. It is shown that Gln and Lys residues in the repeats are not used by TGases, while the same residues in the head-and-tail domain are specifically used by TGase1 and TGase3, the major cross-linking enzymes present in the stratified squamous epithelia.

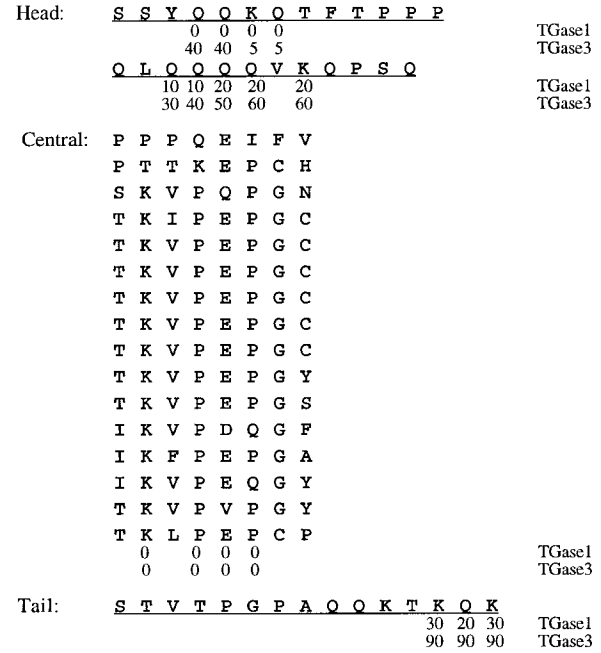

Fig. 2. Complete amino acid sequence of human SPR3. Underlined sequences correspond to the peptides studied. The numbers below the sequence show the percentage of utilization of the residues for TGase1 and TGase3 enzyme. Amino acids are shown in a single letter code.

Secondary Structure Features of SPR3

The CD spectra of N- and C-terminal hSPR3 peptide, corresponding, respectively, to the head-and-tail domain of the protein, show that the most striking feature in this region is the absence of characteristic shapes with either an α-helical or β-sheet conformation. Indeed, the spectrum most closely resembles that of a random coil. However, several lines of evidence suggest the presence of some ordered structure.

Upon increase of temperature, only slight changes are visible in the CD spectra. In particular, the N- and C-terminal peptides show a

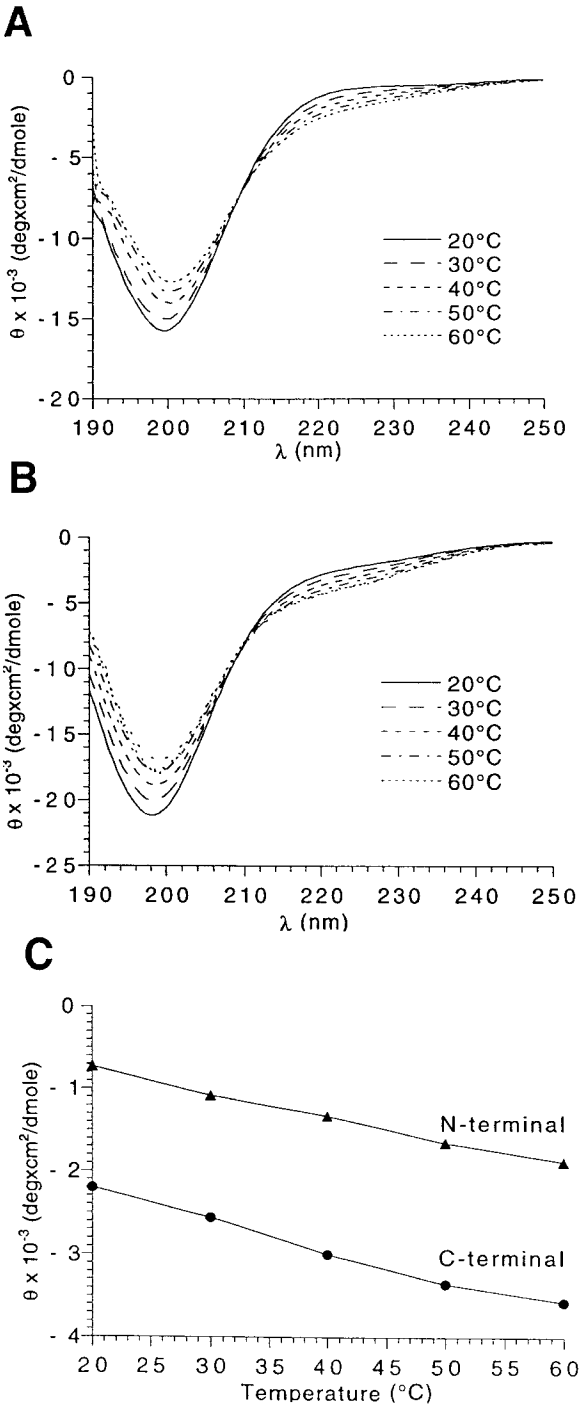

Fig. 3. CD spectra of human SPR3 peptides at different temperature transition. Peptides were HPLC purified and dissolved in 10 mM phosphate buffer (pH 7.0) and used at a concentration of 10 mM. Samples were analyzed at 20°–30°–40°–50°–60°C using a thermostatted bath. **A:** Spectra of N-terminal peptide of human SPR3. **B:** Spectra of C-terminal peptide of human SPR3. **C:** Difference spectra of human SPR3 peptides at 225 nm, obtained at different temperature transition (20°–30°–40°–50°–60°C). A total of three or four acquisitions were accumulated for each sample.

marked increase of ellipticity around 222 nm. Both the N-terminal (Fig. 3A) and C-terminal domain (Fig. 3B) show a marked change centered at 225 nm, indicating the formation of a small amount of α-helical conformation. The thermal behavior in water, the reversibility, and the presence of an isodichroic point at 212 nm suggest that one equilibrium with at least two conformers is detectable by CD. Higher temperatures shift the equilibrium between the conformers. This can be explained as a result of the stabilization by hydrophobic interactions of the secondary structures, which are favored at high temperatures [Tanford, 1970]. The temperature could produce denaturation or a change in the conformation of the proline peptide-bond from *trans* to *cis* as previously suggested [Darrell Fontenot et al., 1993].

Experiments were also performed upon addition of TFE (2,2,2-trifluoroethanol) (Fig. 4A–C). This solvent in fact increases the hydrophobic character of the solvent, enhancing the thermodynamically driving force of the hydrophobic interactions. The environment thus obtained is similar to that natural of the protein. This results in many cases in a very strong structuring effects in those peptides and proteins with a propensity toward a secondary structure. Increasing the concentration of TFE, from 10% to 70% (v/v) in water, resulted in similar changes in the far- and near-ultraviolet (UV) regions as those observed with increasing temperature (Fig. 4A–C). TFE enhances the intramolecular interactions such as hydrogen-bonding and electrostatic salt bridges in proteins and peptides. For both the N- and C-terminal domains, TFE causes a decrease in intensity and a shift in the peak wavelength from 200 to 203–204 nm, and an increase of the shoulder at 225 nm (Fig. 4A,B). Also in this case the presence of an isodichroic point at 212 nm, suggests the existence of a conformational equilibrium. However, the dichroic profile indicates only small changes in conformation with no overall transition toward helix or β conformation of the peptides.

The differences observed in the CD spectrum obtained both by increasing the temperature (Fig. 3D) and by the concentration of TFE (Fig. 4D), correspond to a class B β-turn spectrum, according to the classification scheme of Woody [1976], with a minimum around 225 nm and a maximum in the region 200–205 nm. This class B spectrum is the most common class

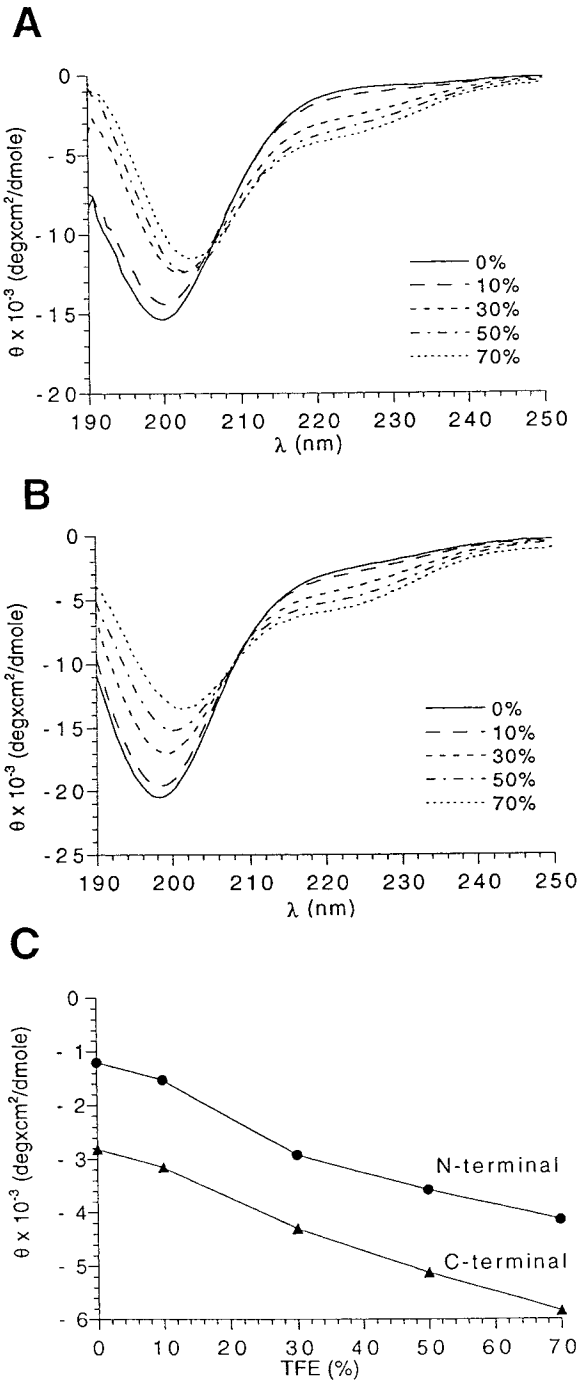

Fig. 4. Effect of TFE on the CD spectra of the human SPR3 peptides, using different TFE concentration. Peptides were HPLC purified and dissolved in 10 mM phosphate buffer (pH 7.0) and used at a concentration of 10 mM. Samples were analyzed in the buffer containing 0%, 10%, 30%, 50%, and 70% of TFE. **A:** Spectra of N-terminal peptide of human SPR3. **B:** Spectra of C-terminal peptide human SPR3. **C:** Difference spectra of human SPR3 peptides at 225 nm, obtained at different TFE concentration (0%, 10%, 30%, 50%, 70%). A total of three or four acquisitions were accumulated for each sample.

associated with any β-turn conformation [Smith and Pease, 1980; Tatham et al., 1985]. The increase in β-turn conformation content with both increasing the temperature and the hydrophobicity of environment by addition of TFE to solvent clearly indicates that a very low propensity toward a classical secondary structure is intrinsic in the primary structure. In fact, many peptides and proteins in these physicochemical conditions either tend to loose structured conformation (temperature) or to assume ordered conformations in the classical secondary structure (TFE). This behavior is to be explained on the basis of solute-solvent interaction and would thus indicate that a conformation rich in β-turn minimizes these interactions favoring the internal hydrogen bonding within the protein. The poor propensity of these proteins toward extensive classical secondary structure led to the limitation of these effects to small tracts of peptidic sequence. This effect confers to this protein a characteristic alternance between structured but still mobile parts (β-turns) and flexible regions (random coil). This alternance is most likely the molecular property, which acts as a source of elasticity. By contrast, one can also speculate that solutions containing TFE, and then with a partial hydrophobic character, probably correspond to the physicochemical environment more close to the natural one (membrane environment; see Fig. 1B), where the SPR proteins are present and cross-linked by TGases. These results explain why only the N-, C-terminus are cross-linked by TGase1 and 3 (see Fig. 2).

Consequences of the Present Structural Data

Biochemical studies on the recombinant hSPR1, SPR2, and SPR3 as well as the in vivo data [Candi et al.1999; Steinert et al., 1999; Tarcsa at al., 1998] indicate that in this family of proteins only the head-and-tail domains glutamines/lysines are used for cross-linking by TGases, even thought these residues are also present in the central repeat domain. This study was performed in order to understand whether the absence of the cross-linking catalyzed by TGases in the central domain is attributable to the conformation adopted by the protein in solution.

Structural studies on the central repeats domain obtained by CD and nuclear magnetic resonance (NMR) spectroscopy [Steinert et al.,

1999] on hSPR3 suggested that the repeats of the central domain are very flexible, with only a low order of organized secondary structure. Previous NMR analysis suggested the presence of a β-turn for a each central repeat. The present CD studies on the N- and C-terminal domains of hSPR3 show that, in both peptides, a small but significant structuration occurs. Similar results have been obtained studying the structural properties in solution of three peptides that cover the entire amino acid sequence of the human SPR2 [Candi et al., 1999]. Thus, the SPR3 protein, as well as the other members of the SPR family, functions as a flexible cross-bridging spacer or filler: the N- and C-terminal domains, cross-linked by TGases, are the anchors with other CE precursor proteins, and the central repeats, which are very flexible and mobile, contribute to the elasticity and flexibility of the CE. Moreover, we suggested that TGases might be unable to recognize the residues localized on the repeats as adequate substrate because of the high degree of mobility in this region.

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