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Disulfide-bond-mediated cross-linking of corneous beta-proteins in lepidosaurian epidermis

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ABSTRACT

Corneous beta-proteins (CBPs), formerly referred to as beta-keratins, are major protein components of the epidermis in lepidosaurian reptiles and are largely responsible for their material properties. These proteins have been suggested to form filaments of 3.4 nm in thickness and to interact with themselves or with other proteins, including intermediate filament (IF) keratins. Here, we performed immunocytochemical labeling of CBPs in the epidermis of different lizards and snakes and investigated by immunoblotting analysis whether the reduction of disulfide bonds or protein oxidation affects the solubility and mobility of these CBPs. Immunogold labeling suggested that CBPs are partly co-localized with IF-keratins in differentiating and mature beta-cells. The chemical reduction of epidermal proteins from lizard and snake epidermis increased the abundance of CBP-immunoreactive bands in the size range of CBP monomers on Western blots. Conversely, in vitro oxidation of epidermal proteins reduced the abundance of putative CBP monomers. Some modifications in the IF-keratin range were also noted. These results strongly indicate that CBPs associate with IF-keratins and other proteins via disulfide bonds in the epidermis of lizards and snakes, which likely contributes to the resilience of the cornified beta- and alpha-layers of the lepidosaurian epidermis in live animals and after shedding.

1. Introduction

The epidermis of reptiles contains intermediate filament keratins (IF-keratins), also known as alpha keratins, that are associated with specialized corneous proteins (CPs) and give rise to corneous layers of different texture referred to as beta- and alpha-layers (Baden and Maderson, 1970; Maderson et al., 1998; Alibardi and Toni, 2006). The beta-layer forms the thick corneous layer of the shell (carapace, bridge and plastron) in turtles and in crocodilian scutes. In lizards, snakes, amphisbenids, and sfenodontids (lepidosaurians), the beta-layer is thin and pliable but still constitutes the external and hardest layer of the scales. The thickness of the beta-layer determines the stiffness and resistance to mechanical insults and the wearing of scales in these reptiles.

Most of the proteins associated with IF-keratins are small proteins of 10–24 kDa, formerly referred to as beta-keratins, produced and packed in the hard corneous layers of the scales referred to as oberhautchen, alpha- and beta-layers (Baden and Maderson, 1970; Wyld and Brush, 1979, 1983; Alibardi and Toni, 2006; Alibardi et al., 2009). Beta-keratins are very different from IF-keratins; they are corneous beta-proteins (CBPs) (Alibardi et al., 2009; Calvaresi et al., 2016) containing a central region of 34 amino acids and conformed in 4–5 anti-parallel beta-pleated sheets that produce filaments of 3.4 nm in thickness (Fraser and Parry, 1996, 2011). These small CBPs likely associate with IF-keratins during the differentiation of the oberhautchen and beta-cells in the epidermis but CBPs have also been found in the alpha-layer. The beta-layer contains a higher proportion of CBPs and a lower quantity of IF-keratins while the alpha-layer contains a lower proportion of CBPs and a higher proportion of IF-keratins (alpha-keratins) (Alibardi et al., 2012; Alibardi, 2013, 2015).

Differently from IF-keratins, CBP genes are encoded in the epidermal differentiation complex (EDC), a locus where other genes coding for numerous types of corneous proteins, such as loricrin, cornulin, trichohyalin-like proteins etc., are present (Vanhoutteghem et al., 2008; Strasser et al., 2014; Holthaus et al., 2016, 2017). Differently from CBPs, the other corneous proteins of the EDC, mainly present in the alpha-layers of the lepidosaurian epidermis (Alibardi and Toni, 2004; Mlitz et al., 2014; Strasser et al., 2015; Alibardi, 2016), do not possess a central beta-sheet region. It is believed that during the formation of the beta-layer, CBPs are deposited over an IF-keratin meshwork. Proof for this association has been derived from the isolation of IF-keratins and CBPs from the beta-layer of various reptiles (Wyld and Brush, 1979, 1983; Alibardi and Toni, 2006; Toni et al., 2007), from the co-localization of IF-keratins and CBPs in the beta-layer of numerous

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reptiles found by immunolabeling (Alibardi, 2013, 2015, 2016), and from detailed X-ray diffraction studies that showed some IF-keratin components mixed with the prevalent CBP components in the beta-layers of snakes (Ripamonti et al., 2009). Also, the gradual change of mechanical properties in the snake epidermis from hard and inflexible to soft and stretchable, as detected by micro-nanoindentation and chemical analysis, suggests that the tougher layers (oberhautchen and beta-layer) mainly contain CBPs (beta-keratins) that decrease and are replaced by IF-keratins and lipids in the inner (alpha-) layers (Klein et al., 2010; Klein and Gorb, 2012; Torri et al., 2014).

Standard biochemical methods for separating epidermal proteins and for showing protein interactions are generally not applicable to epidermal proteins present in the cornified layer due to the highly cross-linked, resilient nature of this structure. Despite various treatments, proteins often remain bound, forming insoluble complexes, and require harsh conditions for separation that can negatively influence further sample processing. In the present study, we applied additional treatments and attempted an alternative approach to this problem.

Anomalies of Western blot labeling for CBPs (some labeled bands above the beta-keratin range) have previously been noted and were interpreted as being due to the formation of polymers or to the presence of strong and stable associations between IF-keratins and CBPs that were not cleaved in conventional preparations for electrophoresis, thus giving rise to immunolabeled bands outside the expected molecular weight (MW) (Alibardi and Toni, 2006; Toni et al., 2007). In order to provide further proof for the existence of direct interactions between IF-keratins and CBPs responsible for the different mechanical properties of the beta- and alpha-layers in lepidosaurian epidermis, we utilized electrophoretic separation of epidermal proteins extracted under different chemical conditions to determine cleavage and separation as well as bonding between these two types of proteins.

In the following text we will use the term corneous beta proteins (CBPs) instead of beta-keratins to distinguish these proteins from the IF-keratins that belong to a separate gene family.

2. Materials and methods

2.1. Tissue collection and embedding

The samples were collected, fixed and embedded as indicated in previous studies (Toni et al., 2007; Alibardi, 2013, 2014, 2015), following the Italian Guidelines for Animal Care and Handling (Art. 5, DL 116/92). In the present study we utilized fresh epidermis from the lizards *Podarcis sicula* and *Tarentola mauritanica*, whole fresh skin from *Anolis carolinensis* and molts from *Pogona vitticeps,* molts from the snakes *Morelia bredli* and *Agkistrodon contortrix* and fresh skin from *Python bivittatus*, *Liasis fuscus,* and *Natrix natrix*. For immunocytochemical analysis, samples (2 mm x 3 mm) were immediately fixed in 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4 (Sigma, St. Louis, MO, USA) at 0–4 °C for 5–8 h, dehydrated in ethanol and embedded in bioacryl resin for 3–4 h at 0–4 °C. This resin was made following the instructions of Scala et al. (1992), and the embedded tissues were finally embedded in gelatin capsules for polymerization under UV light at 0–4 °C for 3 days. Using an ultramicrotome (Ultrotome III; LKB, Bromma, Sweden), semi-thin sections of 2–4 μm were made, collected on glass slides and dried for the histological examination. The sections were stained on a hot plate at 40–45 °C using a 1% toluidine blue solution. Additional sections were collected on chromoallume- and gelatin-coated slides, stained on a hot plate at 40–45 °C, dried and later utilized for immunocytochemical analysis.

2.2. Immunocytochemistry

The semi-thin sections were pre-incubated for 30 min at room temperature with 2% BSA in 0.05 M Tris/HCl buffer at pH 7.6 containing 3% normal goat serum. Afterwards, the sections were incubated for 8 h at room temperature with the primary antibody (rabbit pre-core box antibody; Alibardi, 2015), and with rabbit IF-keratin AK2 antibody (Alibardi, 2013) diluted in Tris buffer (1:100). In controls, the primary antibody was omitted. Sections were rinsed in buffer and incubated with secondary anti-rabbit fluorescein isothiocyanate (FITC)-conjugated antibody (Sigma, Steinheim, Germany) at a dilution of 1:100, and were observed using a fluorescence microscope (Euromex, Arnhem, The Netherlands) equipped with a fluorescein filter.

Immunogold labeling was used on thin sections of the epidermis collected with an ultramicrotome on nickel grids (200 mesh). The sections were incubated for 5 h at room temperature with the above primary antibodies (pre-core box and AK2, dilution 1:50–100 in buffer), and rinsed in the buffer. Some sections were double-immunolabeled with a beta-protein antibody raised in goat (beta-keratin G30 antibody, a gift form Dr. R.H. Sawyer, University of South Carolina, Columbia, SC, USA) and with the AK2 antibody raised in rabbit against IF-keratins. In control sections, the primary antibodies were omitted in this incubation step. Subsequently, the sections were incubated with secondary antibodies (anti-rabbit 5–10 nm gold-conjugated IgG; anti-goat 20 nm gold-conjugated; Sigma, Steinheim, Germany) diluted in buffer (1:80), for 1 h at room temperature. The sections were finally stained for 4 min at room temperature with 1% uranyl acetate, rinsed in distilled water, and dried. The grids were studied using a 10C/CR Zeiss transmission electron microscope operating at 60 kV (Zeiss, Jena, Germany).

2.3. Protein extraction and reductive or oxidative treatments

Fresh skin, epidermis and molt samples from various lepidosaurian species as listed above were collected. In the case of *P. sicula* three different samples were used (numbered 1–3). Samples were homogenized in a solubilization buffer (modified from Sybert et al., 1985) with 8 M urea (Sigma, Germany), 50 mM Tris-HCl (pH 7.6; Sigma, Germany), 0.1 M 2-mercaptoethanol (Sigma, USA), 1 mM dithiothreitol (DTT; Biorad Laboratories, Hercules, CA, USA) and a protease inhibitor (Sigma, Germany). The particulate material was removed by centrifugation at 10,000 × g for 10 min. Using the Bradford protein assay (Biorad Laboratories, München, Germany) with bovine serum albumin as the standard, the sample protein concentration was determined. The obtained solutions underwent different treatments before electrophoresis was performed to further reduce the disulfide bonds or to oxidize them in order to detect variations in the electrophoretic pattern; the control solution remained without any treatment.

In the reduction treatment, samples were treated with a reduction and alkylation protocol normally used for two-dimensional electrophoresis (Görg et al., 1987) to enhance the breaking of the disulfide bonds. The protocol consisted of adding first a reduction and alkylation buffer (pH 9), then a reducing solution with DTT (final concentration 50 Mm) and leaving the samples in agitation for 1 h at room temperature. These first steps were followed by incubation in the dark in agitation for 1 h at room temperature with an alkylation solution containing iodioacetamide (final concentration 50 mM; GE Medical Systems, Chalfont St Giles, UK). After this pre-treatment, Laemmli buffer was added and samples were denatured for 5 min at 100 °C. Standard samples contained 40 μg of protein (unless the protein concentration was too low) and were loaded onto a SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel) at 12 or 15% and separated using a Biorad apparatus with wide-range molecular weight markers (MW 10–250 kDa). Gels at 15% were only used to check for the presence of bands below 10 kDa. A sample which had not undergone the reduction and alkylation treatment was used as a control.

For its well-known reducing capacity, tributylphosphine (TBP) was tested as an alternative to DTT, but results did not yield more signs of reduction (not shown). We also experimented with both longer exposure times and higher concentrations of the reduction and alkylation agents, but this did not lead to better results.

In the oxidative treatment, we instead attempted to enhance the