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Cornification of the Beak of *Rana dalmatina* Tadpoles Suggests the Presence of Basic Keratin-Associated Proteins

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Lorenzo Alibardi (2010) Cornification of the beak of *Rana dalmatina* tadpoles suggests the presence of basic keratin-associated proteins. *Zoological Studies* **49**(1): 51-63. An ultrastructural, immunocytochemical, and electrophoretic study on keratinocytes formation in the horny beak of tadpoles of the frog *Rana dalmatina* was conducted. The study showed that hard cornification in amphibians occurs with similar cytological details as the hard corneous structures of amniotes. Dense corneous bundles containing scarce keratin intermediate filaments and mainly inter-keratin (matrix) proteins accumulated in central keratinocytes (cone cells) of the horny beak. Ribosomes appeared to be associated with these bundles indicating that newly synthesized matrix molecules immediately aggregated on the keratin filaments, turning the complex into an amorphous and electron-dense material. In the 2 corneous sheaths surrounding the cone cells, the oral and labial sheaths, tonofilaments made up of individual keratin intermediate filaments were initially present. The filaments were later transformed into an amorphous keratin mass as cornification progressed. Two-dimensional electrophoretic analysis determined that most proteins of the beak are acidic to neutral keratins. However protein spots outside the keratin range were also isolated for the 1st time. These proteins of 16-17 or 30 kDa with a isoelectric point of 9-9.5 are believed to represent the 1st keratin-associated proteins found in amphibian corneous tissue, and are likely responsible for the intense cornification of the horny beak. http://zoolstud.sinica.edu.tw/Journals/49.1/51.pdf

Key words: Frog tadpoles, Horny beak, Cornification, Keratins, Ultrastructure.

The process of cornification in the skin of vertebrates requires 2 main components: a fibrous framework formed by specialized intermediate keratin filaments and a matrix formed by keratin-associated proteins (KAPs) (Matoltsy 1987, Alibardi 2003a 2006, Rogers 2004). The molecular structure and role of KAPs are becoming increasingly important for our understanding of the formation of horny structures in vertebrates (Rogers et al. 2006). This is now well established for amniote skin derivatives (scales, claws, hairs, feathers, horns) but not for skin derivatives of anamniotes, such as breeding tubercles of fish (Whitear 1976a b, Mittal and Banerjee 1977), or nuptial pads, claws, and beaks of amphibians (Fox 1994).

The process of keratinization in the amphibian epidermis usually takes place in the 2 more-external layers of the epidermis, by replacement or the pre-corneous and corneous layers (Budtz 1977, Fox 1994). The replacement layer contains 2 main types of submicroscopic granules: a smaller mucus type of 0.4-0.9 μm in size, and a larger protein type of 0.1-0.2 μm in size (Bani 1966, Ceresa-Castellani 1969, Lodi and Bani 1971, Lavker 1972 1973 1974). Only the 2nd type can contribute some protein material that remains intracellular, which is trapped among the filaments of keratins and forms a type of matrix material.

The presence of a single corneous layer allows the amphibian epidermis to engage in cutaneous respiration, and only some regions of the skin of amphibians undergo an intense form of keratinization that produces horny structures.

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Examples of such horny structures are the claws of some anurans (Maddin et al. 2007), the horny beak of anuran tadpoles (Beaumont and Deunff 1959, Luckenbill 1965, Kaung 1975, Kaung and Kollros 1977, Marinelli and Vagnetti 1988), and the nuptial pads of males in some newts (Forbes et al. 1975) and anurans (Duelman and Trueb 1994). In these structures, keratinocytes rapidly fill up with dense bundles of filaments that transform these cells into dense corneocytes capable of high resistance to friction and mechanical wear. In these skin derivatives, the normal process of keratinization of the stratum corneum of the epidermis is accentuated, but the mechanism that determines local hyperkeratinization remains virtually unknown.

Previous studies indicated that keratins are involved in the formation of the corneous layer in amphibians (Ellison et al. 1985, Nishikawa et al. 1992, Suzuki et al. 2001, Watanabe et al. 2001). Recent studies tried to demonstrate that in addition to keratins, specific matrix molecules are necessary for the formation of hard horny material (Alibardi 2002 2003b, Alibardi et al. 2003 2007, Alibardi and Toni 2004). In the amphibian epidermis and its derivatives (claws, beaks, warts, and nuptial tubercles), these putative proteins are referred to here as amphibian (a)KAPs. The above studies were conducted after administration of tritiated histidine, and attempted to show the presence of histidine-rich proteins (HRPs) in the epidermis of amphibians, since HRPs are known to represent a major component of the KAP matrix in the mammalian epidermis (Resing and Dale 1991).

Despite these attempts, 2 main limitations have hampered the advancement of the search for aKAPs, namely the limited amount of material derived from the mono-layered corneous layer of the amphibian epidermis, and the limited sensitivity of the immunological and autoradiographic methods employed. The study of more heavily cornified skin derivatives, such as the horny beak or claws, can allow the analysis of molecules involved in the process of cornification. In fact, materials derived from these heavily cornified tissues should be present in higher amounts in comparison to the normal unmodified epidermis. In order to pursue the search for aKAPs, in the present study, we selected the horny beak of an amphibian tadpole to conduct an ultrastructural analysis of the process of cornification coupled with the extraction and 2-dimensional (2D) separation of corneous proteins.

MATERIALS AND METHODS

About 200 tadpoles of *Rana dalmatina*, raised in the laboratory from fertilized eggs collected in the countryside around Padova, Italy, were utilized at stages 38-40 (Cambar and Marrot 1954), which correspond to before or at the beginning of the formation of the posterior limbs. Ten tadpoles were sacrificed by decapitation, and the oral beak was fixed for the histological and ultrastructural examination. Tissues samples of 2 × 4 mm were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 5 h, post-fixed in 2% osmium tetroxide, dehydrated in a graded ethanol solution, infiltrated with propylene oxide, and finally embedded in Durcupan resin.

Tissues were sectioned with an ultramicrotome, and 1-2 μm thick sections were stained with 0.5% toluidine blue and studied under a light microscope. Selected sections of 40-90 nm thick were collected with the ultramicrotome on copper grids, stained with 2% uranyl acetate for 40 min and 0.2% lead citrate for 10 min, and observed under a Hitachi-600 electron microscope (Tokyo, Japan) operating at 80 kV.

Other tissue samples from the tadpole's mouth, containing the horny beak were fixed for 3-5 h in Carnoy fluid, dehydrated in 90% ethanol, and embedded in Bioacryl resin under ultraviolet irradiation at 0-4°C (Scala et al. 1992). After sectioning with the ultramicrotome, 2-4 μm thick sections were collected on chromallume-albumin-coated slides, and sections were immunostained for keratins. Immunocytochemistry was performed using the anti-cytokeratin monoclonal antibodies of K10, pan-cytokeratin (Sigma, St. Louis, MO, USA), and loricrin (Abcam, Cambridge, MA, USA). Semithin sections were preincubated for 20 min at room temperature with 1% bovine serum albumin (BSA) in 0.05 M Tris/HCl buffer at pH 7.6 containing 5% normal goat serum. Sections were then incubated for 3 h at room temperature in the primary antibody diluted with Tris buffer (1: 50-1: 100). In the controls, the primary antibody was omitted. Sections were rinsed in Tris buffer and incubated with the secondary anti-mouse FITC-conjugated antibody (Sigma, diluted 1: 50). Sections were studied with a fluorescence microscope using a fluorescein filter.

The remaining tadpoles were sacrificed as described above, and their beaks were rapidly isolated under a stereomicroscope using sharp dissecting tweezers, and stored at -80°C until protein extraction and analysis (Sybert et al. 1985).

The epidermis and horny beak were incubated in 5 mM ethylenediaminetetraacetic acid in phosphate-buffered saline (PBS) for 5 min at 50°C and for 4 min in cold PBS. The epidermis and horny beaks were homogenized in 8 M urea, 50 mM Tris-HCl (pH 7.6), 0.1 M 2-mercaptoethanol, 1 mM dithiothreithol, and 1 mM phenylmethylsulphonyl fluoride.

The particulate matter was removed by centrifugation at 10,000 ×*g* for 10 min, and the protein concentration of the supernatant was assayed by the Lowry method before electrophoresis. For the 2D electrophoresis analysis, 80 μg of protein samples was separated in 7 cm strips by the Ettan IPGphor III IEF System GEHealcare (Chicago, IL, USA). Application of the strips and the running procedure were carried out as described by the manufacturer. The 2nd dimension was carried out in 10%-12% sodium dodecylsulfate-polyacrylamide gels. After electrophoresis, the gels were immunoblotted on nitrocellulose paper (Hybond C-Extra, GE Healthcare) or stained with colloidal Coomassie blue.

After the Western blot analysis, the nitrocellulose membranes were stained with Ponceau red to verify the protein transfer and incubated with primary antibodies directed against the keratins K10 (Sigma, diluted 1: 1000), pan-cytokeratin (Sigma, diluted 1: 2000), and loricrin (Abcam, diluted 1: 5000). Detection was performed using the enhanced chemiluminescence (ECL) procedure developed by Amersham (Buckingamshire, UK). In the electrophoresis experiments, the Wide Range (MW 6500-20,500) molecular weight marker (Sigma) was used.

RESULTS

Longitudinal sections of the beak showed that the dorsal and ventral parts were sustained by rostral cartilages (Fig. 1A). A detailed examination of both the ventral (Fig. 1B) and dorsal (Figs. 1C, D) beak allowed a better understanding of the morphology of cornifying keratinocytes. In comparison to the normal epidermis, the epithelium of both the oral (internal) and labial (external) parts (sheaths, see terminology in Luckenbill 1965) of the beak appeared thicker, due to the thick corneous layer present in these areas. The oral part of the ventral beak showed layers of spindle-shaped cells which flattened as they became part of the corneous layer of the oral sheath (arrow in Fig. 1B). The labial part presented less-evident stratification but made a compact corneous layer that became thicker toward the apex of the beak. However, both the oral and labial sheaths of the beak were derived from flat cells: a different process instead occurs in the central, axial part of the beak.

The central part of the horny beak was characterized by the presence of piled cells, that distally form the cone-piled cells (pre-cone cells; Luckenbill 1965). The outer rim of differentiating pre-cone cells became rapidly cornified forming a piled series of corneous coniform corneocytes (cone cells, Luckenbill 1965).

Antibodies against both pan-cytokeratin and K10 showed that labeling was restricted to the outer cells of the lamina, while cone cells remained unlabeled (Figs. 1E, F). Loricrine immunolabeling showed some reactivity in the upper epidermal layers but not in the corneous material of the beak (Fig. 1G).

A general ultrastructural description of the horny beaks in different species of anuran tadpoles was previously reported (Luckenbill 1965, Marinelli and Vagnetti 1988). The present study describes the specific details of the process of cornification of central cone cells, versus the process that takes place in external oral and labial sheath cells. In general, fully mature cone cells were electron-dense, while labial sheath cells appeared dark but mottled and oral sheath cells were largely vacuolated (Fig. 1G).

Piled central cells were joined by numerous desmosomes in the early stages of keratinization, and their cytoplasm was rich in ribosomes but contained scarce ergastoplasmic reticulum (Fig. 2A). Bundles of keratin filaments were seen, but they appeared short or irregularly elongated. A closer analysis of these filaments showed that some intermediate keratin filaments were present, but most of them were comprised of an amorphous corneous material (Fig. 2B). At a more-distal level, pre-cone cells became wedge-shaped, and their nuclei remained on 1 side of the piled cones (Fig. 2C). Nuclei were euchromatic, and the nuclear membrane often showed deep folds while nucleoli showed a rich granular component. Pre-cone cells were filled with denser bundles of corneous (keratin) material than were cells of the labial and oral sheaths, and were especially concentrated along the cell periphery.

Both the number of corneous bundles and their diameter increased in the last 2 pre-cone cells, before complete cornification into cone

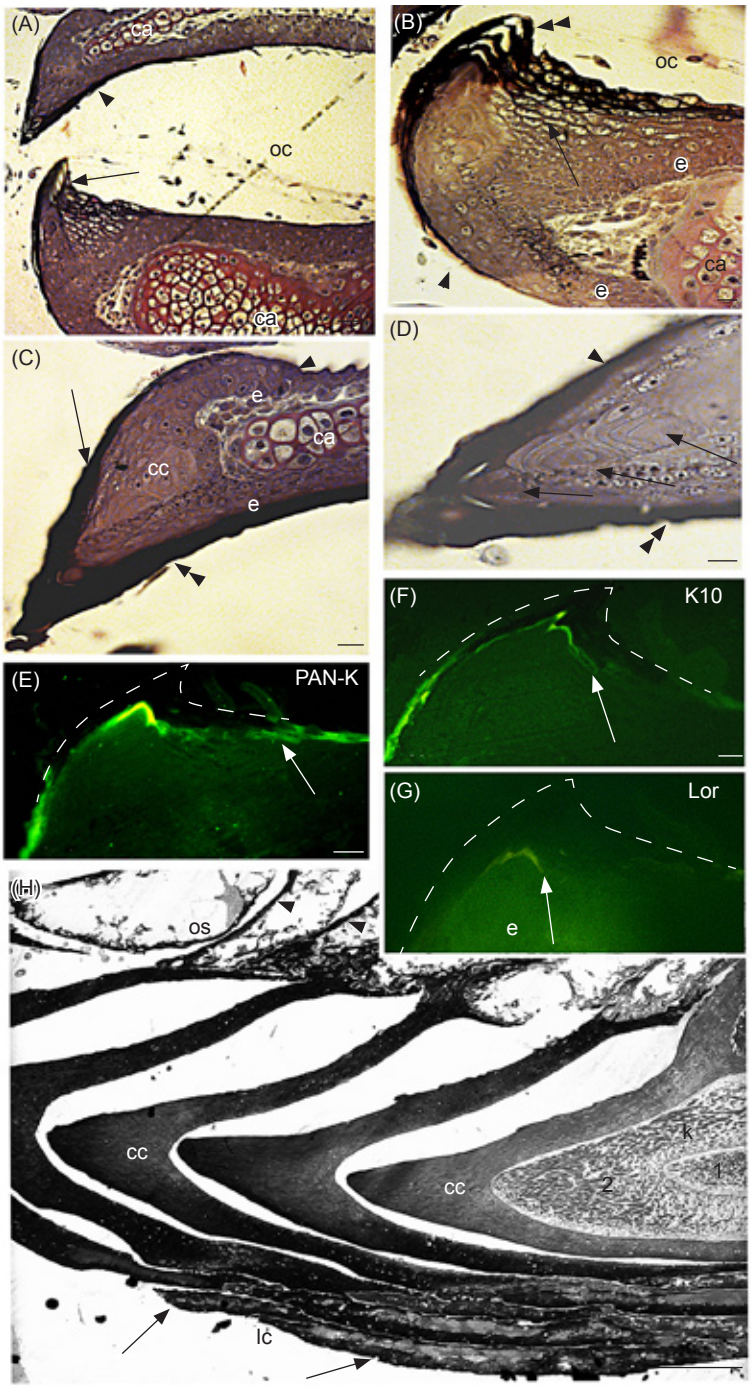

Fig. 1. Light microscopic view of the beak in longitudinal section (A-D), immunocytochemistry for keratins (E, F), and general ultrastructure (G). (A) The upper (arrowhead) and lower (arrow) horny beaks supported by rostral cartilaginous plates. (B) Close-up of the dorsal horny beak featuring central cone cells (double arrowhead), pre-corneous cells of the oral sheath (arrow), and the thickening outer (labial) sheath (arrowhead). (C) Close-up of the ventral beak featuring a differentiating row of cone cells, the oral sheath (double arrowhead), and labial (external) sheath (arrow) which terminates in continuation with the epidermis (arrowhead). (D) Detail showing the piled pre-cone cells (arrows) and spindle-shaped pre-sheath cells (the arrowhead points to the labial sheath; the double-arrowhead to the oral sheath). (E) Pan-cytokeratin immunolabeled sheath (arrow). The dashed line indicates the external horny tissue. (F) K10-immunolabeled sheath (arrow). (G) Loricrine-like immunofluorescence in the upper epidermal layers beneath the corneous layers (arrow) of the beak. (H) Low-power view of keratinizing pre-cone cells (1, 2) and a row of electron-dense cone cells. Arrows indicate the boundary between the cone and outer sheath cells. Arrowheads indicate the corneous wall of oral sheath cells. ca, cartilage; cc, cone cells; e, epidermis; k, keratinizing cone cells; lc, labial (external) sheath corneous cells; oc, oral cavity; os, oral (internal) sheath corneous cells. Scale bars: A = 30 μm; B, D-E = 10 μm; C = 20 μm; H = 5 μm.

cells (Figs. 1G, 2D). The cytoplasm and other cells and organelles, including intermediate keratin filaments, had largely disappeared in the stage of late differentiating pre-cone cells, and mainly ribosomes remained free or attached to bundles of amorphous keratin bundles (Fig. 2E). Earlier bundles appeared more electron-pale than later larger bundles which were surrounded by ribosomes. Electron-dense material was also deposited along the plasma membrane of these cells, forming a thick corneous envelope of 15-20 nm. Amorphous material was occasionally observed in the extracellular space. In mature cone cells at high magnification, the corneous material showed a variegated electron-density, with darker areas more frequent than paler areas (Fig. 2F). The keratin pattern of this material, examined at high magnification, revealed an irregular disposition of electron-lucid filaments of 3-5 nm within an irregularly distributed more-electron-dense matrix.

A different pattern of cornification occurred in cells of the oral and labial sheaths, which are localized on either side of the pre-cone cells (Fig. 2C). Spindle-shaped keratinocytes of the labial sheath contained numerous, typical tonofilaments, among which numerous vesicular bodies were observed (Fig. 3A). The latter organelles contained an amorphous material, but sometimes also smaller vesicles were present. Other common organelles, originating from the Golgi apparatus mainly comprised small mucus granules (Fig. 3B).

Early differentiating cells of the oral side showed an accentuation of features seen in the opposite sheath cells. In particular, numerous vesicles of variable dimensions (0.2-3.0 μm) occupied large areas of the cytoplasm (Figs. 2C, 3C). The detailed analysis of these vesicles, especially of the smaller ones, showed that they were derived from enlargements of regions of the rough endoplasmic reticulum as they were connected to its cisternae (Fig. 3D). The vesicles were also surrounded by ribosomes, and an amorphous material was present inside their lumen. The dimension of these vesicles increased toward the tip of the beak where cells became fully differentiated as vacuolated cells (Figs. 1G, 3E). Tonofilaments of these cells, initially sparse throughout the cytoplasm, were progressively concentrated along the plasma membrane and eventually aggregated to form a corneous wall. In the final stage of maturation, most of the cytoplasm had disappeared, and in a few cells, a coagulated mass of cytoplasm remained between the 0.5 μm thick corneous wall of these cells. Only limited electron-dense regions within the less-electron-dense corneous material of the wall of most of the distal corneocytes of the oral sheath showed a density and keratin pattern similar to those of mature cone cells.

The differentiation of cells of the labial sheath occurred with the formation of much-smaller and less-numerous vesicles. The numerous keratin filaments within pre-corneous cells began to aggregate in transitional cells where filaments became indistinct, and an amorphous type of keratin mass became evident (Figs. 4A, B). The ribosomes of differentiating corneocytes decreased in number and became aggregated among the amorphous keratin material. The plasma membrane formed an electron-dense corneous envelope while desmosomes remained as the only extracellular plaque. As corneocytes matured in the more-external and -distal layers of the sheath, the amorphous keratin material occupied most of these cells, and intermediate keratin filaments were no longer seen. (Figs. 4B, C). Only a few cytoplasmic areas contained ribosomes, some vesicles, and remnants of the Golgi apparatus which were sandwiched among a thick and electron-dense corneous material. The latter represented most of the final material comprising these corneocytes, with a similar density and keratin pattern as in mature cone cells.

The protein patterns of the horny beak and normal epidermis mainly showed protein spots in the keratin range (45-63 kDa, Fig. 5). The horny beak analysis revealed diffuse spots in the range of 45-63 kDa with isoelectric point (pI) values of 4.5-6.3. Among these, 2 spots at 50 kDa and 2 spots at 55 kDa with pI values of 4.9 and 5.0 were detected (Fig 5A). Other faint spots were detected at 60-62 kDa with pI values of 6.6, 6.8, 7.0, 7.4, and 7.8 (Fig. 5A). In the horny beak, however, a light spot of 30-31 kDa with a pI of 9.5 was seen (arrowhead in Fig. 5A). The analysis of horny beak proteins, which were concentrated by chromatographic tools to better visualize proteins with molecular weights of < 35 kDa, revealed more-diffuse spots in the range of 15-35 kDa. The latter separation revealed 2 main spots with pIs of 9.5 and 9.2-9.8, and molecular weights of 30-31 (double arrowhead in Fig. 5B) and 16-17 kDa (arrowhead in Fig. 5B).

The epidermis separation revealed spots at 46-56 kDa with pI values ranging 4.0-6.3. The immunoblot analysis of the epidermis showed 2 bands at 44 and 55 kDa when stained by the

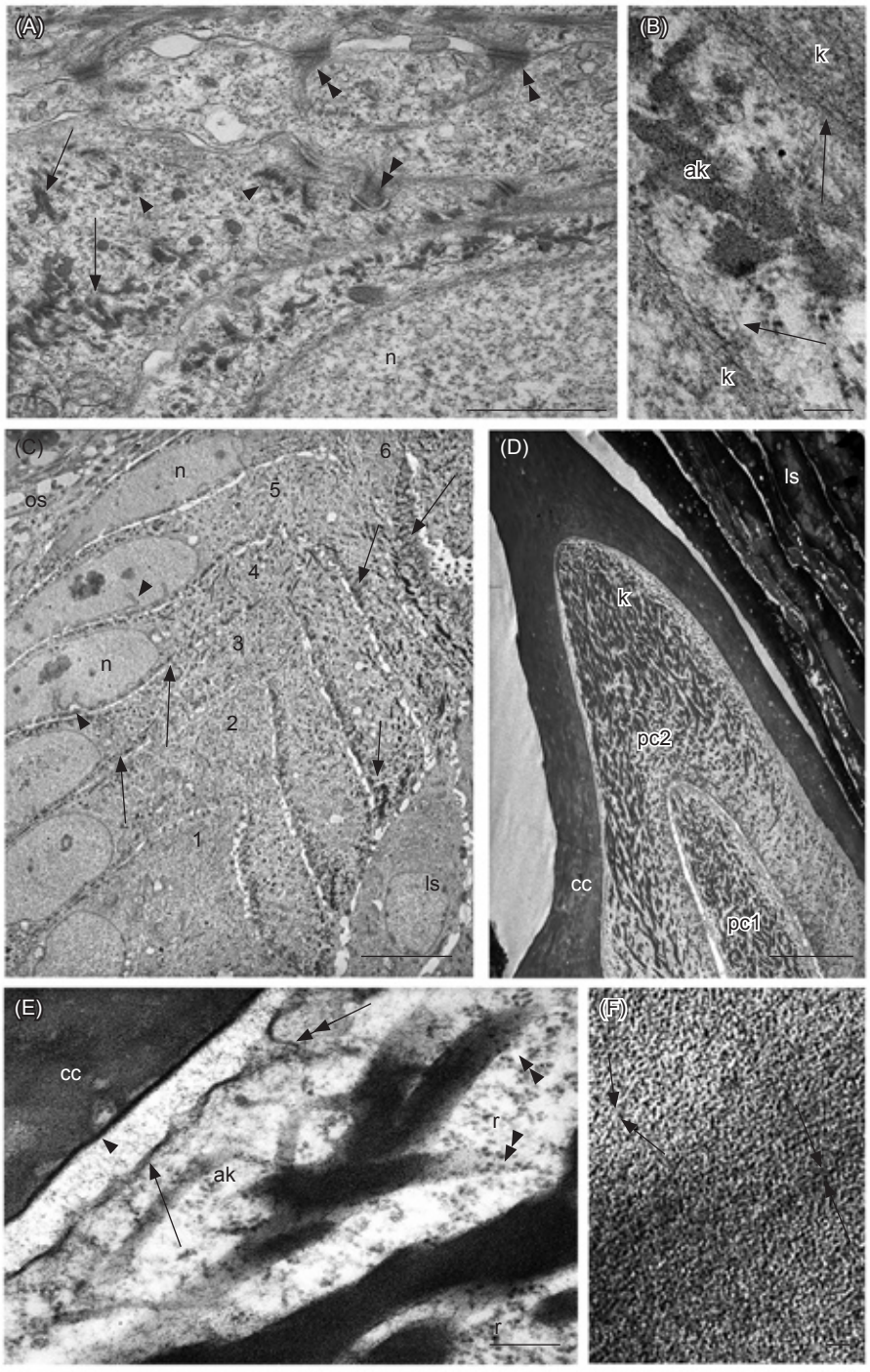

Fig. 2. Ultrastructural features of differentiating pre-cone and cone cells. (A) Early differentiating cone cell showing numerous, short keratin bundles (arrowheads) among the numerous free ribosomes. Long amorphous keratin bundles (arrows) are less frequent, while numerous desmosomes (double arrowheads) keep the cells united. (B) High-magnification detail of early deposited amorphous keratin in comparison to a tonofilament with visible intermediate keratin filaments (arrows). (C) Sequence of wedge-piled pre-cone cells contacting flat cells of the oral and labial sheaths. Note the lateral nuclei and the forming, distal spine that give a wedge-like appearance to pre-cone cells. Arrows indicate the larger, peripheral amorphous keratin bundles. Arrowheads point to nuclear infoldings. (D) Detail of 2 piled pre-cone cells (1, 2) filled with merging dense keratin filaments in contact with a distal fully cornified cone cell. (E) Detail of amorphous keratin bundles in a pre-cone cell with associated ribosomes (double arrowhead). The double arrow indicates an exocytotic vesicle. The arrow indicates the cornified cell membrane of pre-cone cells, while the arrowhead shows the corneous cell membrane of a cone cell. (F) High-magnification details of the keratin pattern of the cone cell (between arrows are the single keratin filaments). ak, amorphous keratin bundles; cc, cone cell; k, keratin bundles; ls, labial (external) sheath; n, nucleus; os, oral (internal) sheath; pc, pre-cone cell; r, ribosomes. Scale bars: A = 1 μm; B = 100 nm; C, D = 5 μm; E = Bar, 200 nm; F = 25 nm.

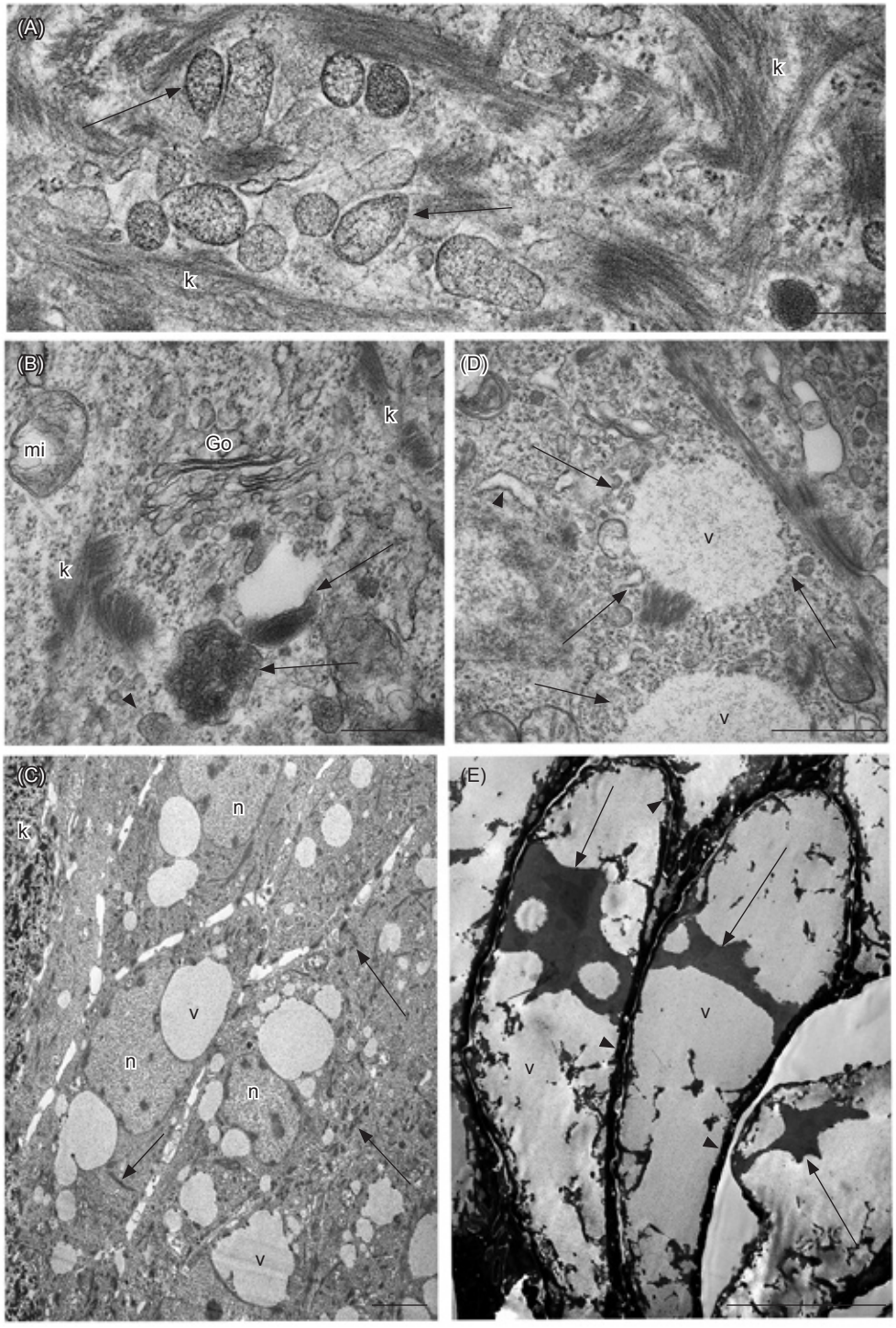

Fig. 3. Ultrastructure of cells forming the sheath. (A) Detail of an early differentiating labial cell showing the fibrous tonofilaments (k) and vesicular bodies (arrows). (B) Detail of an early differentiating labial cell with sparse and short keratin bundles, and large (arrows) and small (arrowhead) mucus granules. (C) Spindle-shaped cells of the oral sheath (see arrow in Fig. 1 B) with large pale vesicles and sparse, short keratin bundles (arrows). Arrowheads indicate nuclear folds. (D) Detail of a small vesicle containing secreted material derived from the enlargement of the endoplasmic reticulum (arrows) in an oral sheath cell. The arrowhead indicates short cisternae of the endoplasmic reticulum. (E) Mature corneous cells of the oral sheath with vacuolated cytoplasm, some cytoplasmic remnants (arrows), and cornified cell membranes (arrowheads). k, keratin bundle; Go, Golgi apparatus; mi, mitochondrion; n, nucleus; v, enlarged vesicle. Scale bars: A = 250 nm; B = 250 nm; C = 2.5 μm; D = 0.5 μm; E = 5 μm.

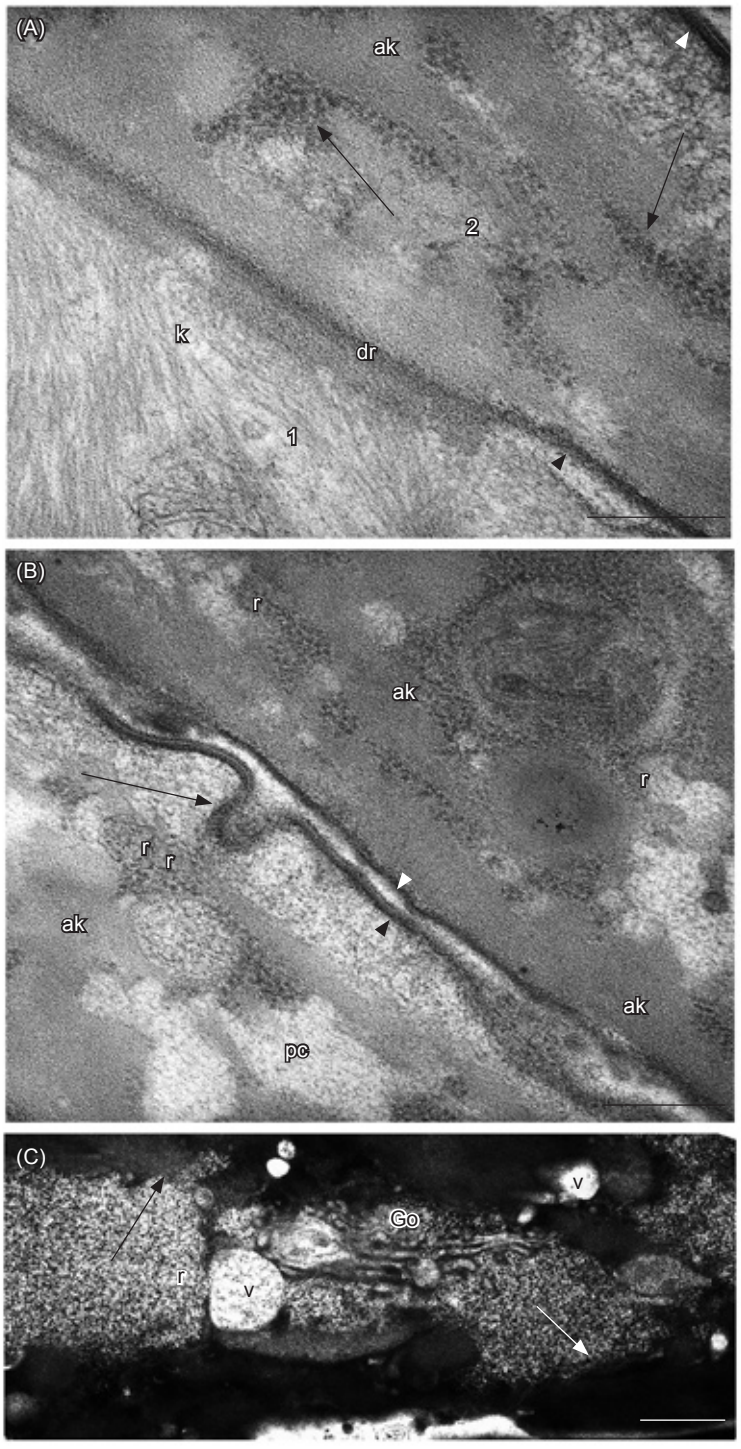

Fig. 4. Ultrastructure of cells forming the labial (external) sheath. (A) Detail of pre-corneous cells (1) rich in keratin filaments in contact with a transitional cell (2) where keratin filaments have largely disappeared and ribosomes (arrows) are grouped into nests among amorphous keratin material. Arrowheads indicate the dense and thickened cell corneous membrane. (B) Detail of more-external cells than those of the previous figure where few ribosomes nests remain among amorphous keratin. Arrowheads indicate the corneous cell membrane that occasionally shows exocytotic-like vesicles (arrow). (C) Electron-dense and flat corneocytes in the mature sheath, in which the central cytoplasm contains ribosomes and some vesicles are formed from the Golgi apparatus. The external cell surface has formed a thickened corneous layer (arrows). ak, amorphous keratin; dr, desmosome remnant; Go, Golgi apparatus; k, keratin filaments; pc, pale (amorphous) cytoplasm; r, ribosomes; v, vesicle. Scale bars: A, B = 200 nm; C = 0.5 μm.

pan-cytokeratin antibody (Fig. 5D3). Three bands at 41, 45, and 50 kDa were observed after immunoblotting and reaction for K10 (Fig. 5D5), and 3 bands at were observed at 41, 46, and 65 kDa when stained by the loricrin antibody (Fig. 5D7). The immunoblot analysis of the horny beak revealed the presence of 3 bands at 43-44, 50, and 54-55 kDa when stained with the pan-cytokeratin antibody (Fig. 5D4). Three bands at 43-44, 50, and 54-55 kDa were seen when proteins were immunostained stained for K10 (Fig. 5D6), and a main band at 41 kDa was observed after immunostaining for loricrin detection (Fig. 5D8).

DISCUSSION

Cornification process

It is believed that mucus and glycoproteins are the 1st inter-keratin matrix molecules in evolving non-terrestrial vertebrates (Mittal and Banerjee 1977, Matoltsy 1987, Alibardi 2003a 2006).

While normal, mucogenic epidermis shows numerous mucus granules and relatively scarce tonofilaments, the opposite is true for heavy corneous derivatives such as anuran beaks (Luckenbill 1965, Mittal and Banerjee 1977, Marinelli and Vagnetti 1988), claws (Maddin et al. 2007, Alibardi, unpubl. observ.), and nuptial pads (Forbes et al. 1975). The latter authors showed the main histological, histochemical, and ultrastructural characteristics of the beak in anuran tadpoles. In the epidermis, most mucus is extruded outside mature keratinocytes, while matrix proteins remain intracellular. Therefore KAPs are not stored in specific granules for exportation, but like other internal proteins, they are produced in ribosomes and rapidly aggregate onto a framework of specialized, probably glycine-serine-rich intermediate keratin filaments (Alibardi 2006).

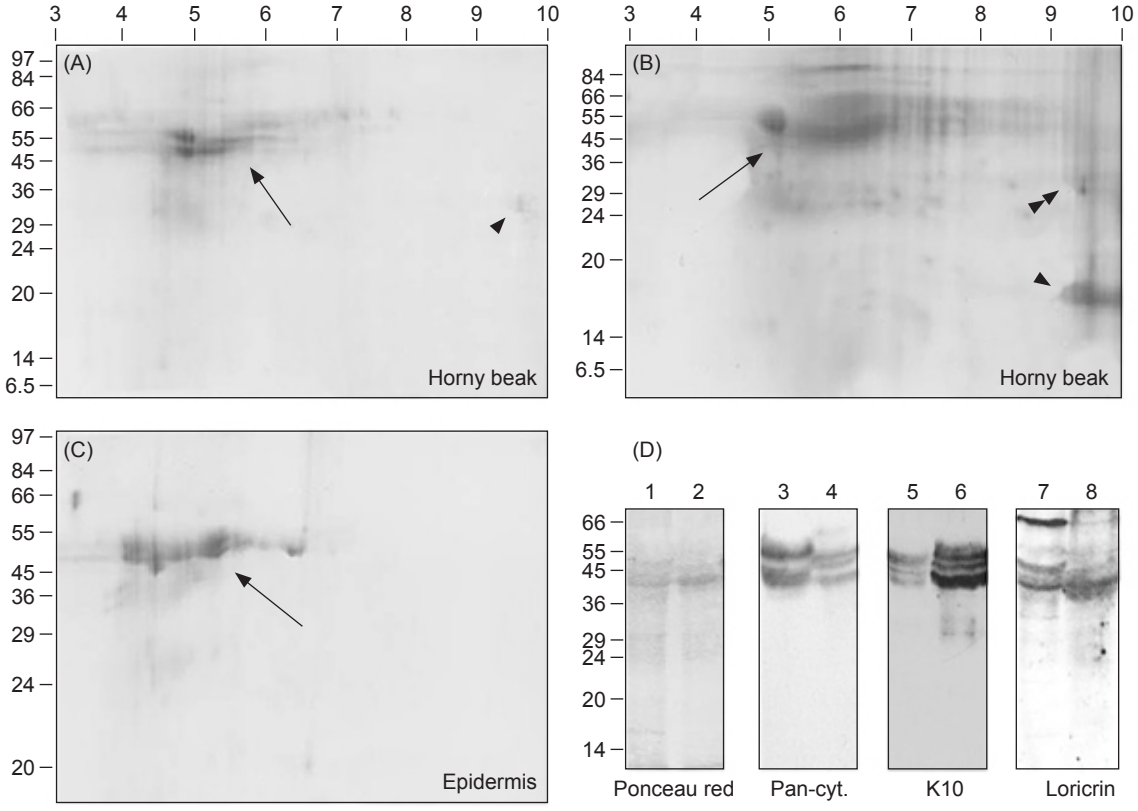

Fig. 5. Electrophoretic pattern of proteins extracted from the horny beak (A) after chromatographic concentration (B), and compared to general epidermal proteins (C). (D) 1 dimensional analysis of proteins extracted from the epidermis (lanes 1, 3, 5, and 7) and the horny beak (lanes 2, 4, 6, and 8). Proteins were stained by Ponceau red (lanes 1 and 2), and immunostained by pan-cytokeratin (lanes 3 and 4), K10 (lanes 5 and 6), and loricrin (lanes 7 and 8) antibodies (see text for further details).

The present study confirms the general histological (Beaumont and Deunff 1959, Kaung 1975) and ultrastructural characteristics of horny beaks in other anuran species (Luckenbill 1965, Marinelli and Vagnetti 1988), but it was mainly focused on the cornification process, and on the possible nature of the dense, amorphous keratin (or corneous) material of the mature beak. As for claws (Maddin et al. 2007), horny beaks are also supported by the growth of oral cartilage. Possible effects of the growing cartilage on the differentiation of the associated epidermal derivative are not known.

Three main types of cornification occur in cells of the beak: a hard type in cone cells, a softer type in labial sheath cells, and a soft type with vacuolization in the oral sheath. All these types of differentiation are derived from the rapid synthesis of hard, electron-dense matrix material, produced in the 3 corneous regions. The ultrastructural study showed that in the central row of pre-cone cells, ribosomes synthesize KAPs which are rapidly deposited as a dense and amorphous corneous material onto the few corneous intermediate filaments of keratin. Otherwise a process of degradation of intermediate keratin filaments should be invoked to explain the disappearance of fibers in early differentiating pre-cone cells. Cone cells have denser corneous material, but why these cells are shaped like wedge-shaped piled cells remains unexplained (Luckenbill 1965).

Both ultrastructural and immunocytochemical information suggests that a large part of the corneous material of sheath cells is made up of keratins, and the formation of empty spaces gives rise to a corneous material that is somehow softer than that of cone cells. The presence of a harder horny material in cone cells versus the corneous material of sheath cells suggests that sheath cells are worn more rapidly than cone cells, and that this external wearing leaves a constantly sharp central (cone) portion. The faster cell turnover rate of cells of the sheath regions (Luckenbill 1965, Kaung and Kollros 1977) can explain why sheath cells proliferate and move toward their final position at a higher rate than cone cells, as they have to replace desquamated cells more frequently than central, more-slowly differentiating cone cells.

Cells of the sheath may also incorporate a certain amount of mucus or lipoproteins produced in the rough endoplasmic reticulum and Golgi apparatus, and perhaps these lipids are condensed with proteins of the corneous material as in the normal epidermis (Spearman 1968). Previous histochemical studies however were negative for either mucus or lipids (Luckenbill 1965), so the nature of the substances that later disappear in mature hollowed oral sheath cells remains unknown. Eventually, cells of the sheath are densely cornified or possess an empty core space with reinforced corneous walls in mature oral sheath cells. Therefore, while the beak tip is formed by hard corneous cells for tearing aquatic plant material which is utilized as food, both labial and oral sheets of the beak may also be coated by a flaking or wearing material that makes the surface slippery, along which food particles can move.

Information on keratins and other corneous proteins of the normal epidermis, claws, and beak can shed important light on infections from the chytrid fungus *Batrachochytrium dendrobatidis*, a parasite largely responsible of the worldwide decline of amphibian species (Rachowicz and Vredenburg 2004, Berger et al. 2005). In fact, it appears that after infection of the epidermis by chytridiomycetes, keratinocytes lose their diffuse keratin network, and prematurely form the dense corneous material of mature cells of the stratum corneum. This premature cornification may limit the amount of mucus eliminated in the replacement and corneous layers producing lethal effects in the functionality of the epidermis. The possibility that this fungus may feed on keratins should be clarified. The fungus also affects the “pigmentation” of the horny beak and teeth creating different patterns of de-pigmentation. Often the infection causes beak-loss. Since the color of the beak, like the claw, is not due to melanosomes but to the deposition of darker proteins over keratins (Maddin et al. 2007, Alibardi, pers. observ.), it is possible that the fungus may actually affect the process of darkening and cornification. Studies in this direction may shed new information on the pathogenic process.

Cornification proteins

The present results strongly support the hypothesis that non-keratin proteins, here indicated as amphibian (a)KAPs, are present and contribute to the hardness of the 3 components of the horny beak (cones, and labial and oral sheaths).

The lack of immunoreactivity of mature cone cells to anti-keratin antibodies (K10 and pan-cytokeratin) and to the anti-loricrine antibody suggests masking of keratin and loricrine-like epitopes after aKAPs have been deposited over

the keratin framework. A similar lack of keratin immunoreactivity was also observed in the hard horny teeth of the lamprey (Zaccone et al. 1995). The immunoreactivity of these proteins is better seen in immunoblots after keratins and associated proteins have been extracted and have migrated in the electrophoretic gel. These procedures can probably unmask epitopes that are somehow hidden in fixed tissues.

The increase in sulfhydryl and disulphide groups in horny beak cells of anuran tadpoles from the beginning of formation to the more-external regions of the beak was previously reported (Beaumont and Deunff 1959). The general histochemical reaction indicated by those authors as cone cells matured was an increase in the content of keratins containing numerous sulfhydryl groups (SH). This previous datum and the present electrophoretic and immunocytochemical observations on the dense amorphous material indicate that keratins constitute most of the corneous mass of the beak, but that they become cross-linked and their immunoreactive epitopes become masked or altered as the corneocytes mature. In addition, our new data also suggest for the 1st time that other non-keratin proteins indicated as sulfur-rich aKAPs are especially produced in maturing cone cells. The latter cells probably contain aKAPs, and keratins are masked by the deposition of non-keratin proteins. This is also indicated by the lack of a typical alpha-keratin pattern of 8-10 nm in the dense horny material, replaced by a pattern of 3-5 nm filaments. The latter was indicated as the “beta-pattern” for keratins, and was so far detected only in sauropsids (Baden and Maderson 1970, Gregg and Rogers 1986). The pattern found in the hard corneous material of the beak, however, does not reflect the presence of beta-keratins in amphibian tissues, but of other unknown proteins rich in sulfur.

In contrast to cone cells, in lateral cells of both the oral and labial sheaths, the process of aKAP deposition is less marked, and initially many keratin bundles (tonofilaments) are produced in these cells. The deposition of aKAPs is indicated by the association of ribosomes with keratin filaments, and by the aggregation of ribosomes among amorphous keratin filaments at advanced stages of keratinization. Also, differently from cone cells, lipid vesicles or even lipoproteins are present in sheath cells, especially those of the oral sheath.

Data from the present study suggest that aKAPs are at least partially represented by proteins spots of 16-18 kDa and basic pI values (9.0-9.5). The present study shows that most components of the beak are represented by acidic to neutral keratins, as in the epidermis. Single keratin spots were not specifically resolved as it was not the main purpose of the present study, but most beak keratins were in the range of 45-57 kDa, very similar to those of the non-modified amphibian epidermis. These proteins possess common epitopes for keratins identified by the pan-cytokeratin antibody (mixtures of 4, 5, 6, 8, 10, 13, and 18 human cytokeratins) and in particular K10, a marker of pre-corneous and corneous layers of the mammalian epidermis (O’Guin et al. 1987). Also, loricrine immunoreactivity suggests the presence of a loricrin-like epitope or of proteins very rich in glycine sequences, as is known to be the case for loricrin (Resing and Dale 1991). The indicated molecular weights of loricrin-like immunoreactive bands, of around 40, 48, and 65 kDa, were previously reported for the epidermis of other amphibian species (Alibardi and Toni 2004).

After concentrating beak proteins, our 2D electrophoretic analysis showed that 2 or 3 main spots at 16-18 and another at 30 kDa were present, with pI values of 9.0-9.5. The sequencing of proteins within these ranges will be possible in future analysis of the amino acid sequence of these spots. KAPs of similar molecular weights were also found in the claw of *Xenopus laevis*, although the main components have pI values of 7.0-7.3, and minor components have pI values of 9.0-9.5 (Alibardi, unpubl. observ.).

KAPs determine the hardness of corneous tissues in mammalian hairs, nails, claws, and horns where 3 main types of KAPs are known, referred to as high-glycine tyrosine, high-sulfur, and ultrahigh-sulfur proteins which constitute the hard matrix (Gillespie 1991, Powell and Rogers 1994, Rogers et al. 2006). The deposition of sulfur-rich KAPs in mammalian corneous tissues is responsible for the electron-dense nature of this material. In particular, cysteine and cystine react with the osmium and appear as the electron-dense inter-keratin or matrix material (Filshie and Rogers 1962, Fraser et al. 1972, Orwin 1979, Alibardi 2008). The above studies also indicated that lead salts utilized to stain the thin sections for the ultrastructural observations are mainly adsorbed in the matrix components of the corneous material of hairs and nails.

The increase in the electron-density observed in beak, claws, and nuptial pads of amphibian skin is likely derived from a similar affinity to osmium

and lead utilized for post-fixation and staining and to matrix proteins rich in cysteine or cystine (-S-S-). It is also likely that in hard epidermal derivatives of fish, the increase of electron-density is derived from deposition of cysteine-rich KAPs among the keratins. These derivatives are represented by breeding tubercles of many teleostean families (Collette 1977, Mittal and Whitear 1979), adhesive organs (Das and Nag 2005), and horny beaks of lamprey (Uehara et al. 1983, Zaccone et al. 1995). Future proteome studies can also clarify this hypothesis on hard skin derivatives of fish.

In conclusion, the present study indicates that KAPs are present in the epidermis and skin derivatives of amphibians, especially in hard epidermal derivatives. Future proteomic studies are needed to determine the amino acid sequences of these basic proteins in order to clarify their molecular characteristics, and their organization and interaction with keratins for the production of hard corneous material of epidermal derivatives.

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