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Wound keratins involved in mucous granule extrusion during differentiation of amphibian keratinocytes

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ABSTRACT. The synthesis of specific keratins in differentiating amphibian epidermis has been studied by autoradiography after tritiated histidine injection, immunocytochemistry and immunoblotting. Most labeling is present in upper spinosus and corneous layers suggesting higher protein synthesis in these differentiating keratinocytes. In the epidermis of the toad *Bufo viridis* and of the newt *Triturus vulgaris* most of the synthesized epidermal proteins are keratins of acidic-neutral type, including K6, K16, and K17-like keratins of 45-60kDa. Proteins of lower molecular weight and with basic pI are present in very low amounts or are absent. Ultrastructural immunolabeling for K6 and K16 keratins shows that they are associated with dense material among keratin filaments and with dense mucous granules, but not with tonofilament bundles of differentiating keratinocytes. After the release of mucous granules in upper keratinocytes of the intermediate layer and in pre-corneous keratinocytes, the immunolabeling for the above keratins is localized along the plasma membrane of maturing keratinocytes. This distribution is similar to that of actin, and suggests that actin together with K6, K16, and K17-like keratins may be involved in the process of extrusion of mucous-containing glycoproteins from differentiating amphibian keratinocytes.

KEY WORDS : Amphibian epidermis; Mucous granules; Ultrastructure; Immunocytochemistry; Wound keratins.

INTRODUCTION

The adult amphibian epidermis comprises a basal layer, 3-5 intermediate cell layers, and a stratum corneum that generally consists of a single layer of dead keratinocytes (Fox, 1994; Warburg et al., 1994; Alibardi, 2001). The limited cornification of superficial cells in amphibian epidermis allows for cutaneous respiration before the corneous layer is replaced (Budz, 1977). These studies have shown two types of submicroscopic granules, one type of 0.4-0.9µm diameter and another type of 0.1-0.2µm diameter, in differentiating keratinocytes (Bani, 1966; Ceresa-Castellani, 1969; Lodi & Bani, 1971; Lavker, 1972; 1974).

The smaller granules probably contain mainly mucous or glycoproteins while some of the larger ones contain glycoproteins and perhaps other proteins involved in the process of keratinization and coating of the cell membrane of superficial keratinocytes (Bueno et al., 1981; Navas et al., 1987). The latter ultrastructural studies coupled to lectin histochemistry have indicated that at least some small mucous granules may represent the equivalent of the "membrane coating granules" or multilamellar bodies present in mammalian keratinocytes (Menon & Norlen, 2002).

Mucous and sparse lipid-like bodies are either extruded extracellularly (small granules) or dispersed intracellularly (some of the large granules). Lipids contribute to limiting water-loss from the epidermis, especially in more terrestrial amphibians such as hylids and bufonids (Lavker, 1972; 1974; Lillywhite & Maderson, 1982; Toledo & Jared, 1993). Glycoproteins coat the plasma membrane of maturing keratinocytes of the pre-corneous or replacement layer and of the corneous layer, like lipids coat the cornified cell envelope of mammalian corneocytes. High amounts of glycoproteins are produced in the epidermis and they are secreted extracellularly. The extrusion of mucous granules probably requires the production of specific cytoskeletal proteins.

The large granules may also release, apart from glycoproteins, some matrix proteins that contribute to the process of maturation of the corneous layer (Lavker, 1972; 1974; Fox, 1994; Alibardi, 2001). The molecular nature of inter-keratin or matrix material in amphibian keratinocytes, however, remains unsolved. Matrix proteins in mammalian epidermis, including the histidine-rich filaggrins, are essential for the formation of the corneous material present within mature keratinocytes and along cornified cell membranes (Resing & Dale, 1991; Kalinin et al., 2002).

Previous studies have shown that tritiated histidine is incorporated in amphibian epidermis, from newt, frog and toad (Alibardi, 2002; 2003; Alibardi et al., 2003). These studies have indicated that keratins (alpha- or intermediate filament keratins) and a small amount of proteins incorporating histidine are synthesized in the upper cells of the intermediate layer and of the replacement layers in amphibian epidermis. Other studies have shown that a slightly basic keratin of 63kDa is a major protein of adult epidermis in *Xenopus laevis* (Nishikawa et al., 1992). Immunocytochemical studies have, however, shown that in adult epidermis more acidic than basic keratins are present (Alibardi, 2001; 2002) but the specific types are not known.

Among acidic keratins, mammalian K16 and K17 are known to be upregulated during epidermal wounding and regeneration (McGowan & Coulombe, 1998a;b). This is also likely for reptilian epidermis covering the amputated tail or limbs (Alibardi & Toni, 2005). As amphibian epidermis is capable of extensive regeneration to cover

amputated limbs or broad areas of injured skin, it would be interesting to evaluate the presence of K16 and K17 acidic keratins, and of K6, a basic keratin also upregulated in regenerating epidermis.

In the present study, the specific nature of some of the keratins produced during epidermal differentiation has been analyzed using autoradiography, immunocytochemistry, and immunoblotting for wound keratins (K6, K16, and K17) and for actin, a ubiquitous cytoskeletal protein associated with cell motility and cytoskeletal re-arrangement.

MATERIALS AND METHODS

Animals and experiments

Adult specimens of toad (*Bufo viridis*; n=9) were injected with 3-4μCi/g body weight of tritiated histidine (L-2,5-3H-Histidine, specific activity 53.0Ci/mmol, Ge Healthcare, UK) diluted in saline. Adult specimens of newt (*Triturus vulgaris*; n=17) received 6-8μCi/g body weight of tritiated histidine as previously reported (ALIBARDI, 2003). This allowed evaluation of protein synthesis in the main epidermal layers.

Five newts received 2-3μCi/g body weight of tritiated thymidine (3H-TdR, Amesham, specific activity 70-90Ci/mM). This treatment allowed evaluation of the sites of cell proliferation and time of migration through the epidermal layers.

After sacrifice of the animals by decapitation, the skin from ventral trunk, tail and digits areas in both species was collected at 4 and 8 hours post-injection, and prepared for autoradiography. From the specimens that received tritiated thymidine, samples were collected at 4-5 hours (n=5), 4 days (n=2), and 6-7 days (n=3) post injection.

Fixation, immunocytochemistry and autoradiography

Skin fragments of 2 by 4mm were fixed for 3-5 hours in 4% paraformaldehyde in 0.1M phosphate buffer at pH 7.4, dehydrated, and embedded in Durcupan or Bioacryl resins (SCALA et al., 1992) as previously detailed (ALIBARDI, 2001; 2002). Other tissues were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer for 5 hours, post-fixed in 2% osmium tetroxide, dehydrated and embedded in Durcupan resin. The latter tissues were studied under the electron microscope for routine ultrastructural analysis of the epidermis.

Tissues were sectioned with an ultramicrotome in order to obtain sections of 2-5μm in thickness. Sections from Araldite- or Bioacryl-embedded tissues were stained with 0.5% toluidine blue and studied under the light microscope. Sections from Bioacryl-embedded tissues were collected over chromoalume-albumin coated slides for the following application of immunocytochemical reactions.

Immunocytochemistry was performed using the anti-cytokeratin antibodies to K6, K16 and K17 (generous gift from Dr. P. Coulombe, Johns Hopkins University, USA). These are rabbit polyclonal antibodies that recognize specific epitopes of mouse keratins 6 and 16 (data in TAKAHASHI et al., 1994; MCGOWAN & COULOMBE, 1998a;b). The anti-total actin here employed is a general antibody against actin produced in rabbit.

Semithin sections were pre-incubated for 30 minutes at room temperature with 2% BSA in 0.05M Tris/HCl buffer at pH 7.6 containing 5% normal goat serum. The sections were later incubated overnight at 0-4°C in the primary antibody diluted in the Tris buffer (1:200 for the K6, K16, or K17 antibodies and actin antibody). In controls, the primary antibody was omitted. Sections were rinsed in buffer and incubated with secondary HRP-conjugated antibody (SIGMA, anti-rabbit for K6, K16, K17-incubated tissues) at 1:50 dilution. Detection was performed using a DAB-hydrogen peroxide reaction.

Other sections of 40-90nm thickness were collected with the ultramicrotome on nickel grids, and incubated in the primary antibody medium with Tris buffer and 1% Cold Water Fish Gelatin as reported above (no primary antibodies were used in controls). The dilution for K6, K16 and anti-actin was 1:200. After being rinsed in the buffer, sections were incubated for 1 hour at room temperature in the secondary antibody (anti-mouse IgG anti-rabbit, conjugated with 10nn gold particles to be visualized under the electron beam) diluted 1:40 in the Tris buffer. After rinsing, sections on grids were stained for 5 min in 2% uranyl acetate, and then observed under a Philips CM-100 electron microscope.

For light and ultrastructural autoradiography (see details in ALIBARDI, 2003), sections of 2-5μm thickness were attached to glass slides and then coated with Nuclear emulsion for light microscopic autoradiography (Ilford K5), developed and fixed 1-2 months after exposure. Thin sections (40-90nm thick) were coated with Nuclear emulsion for electron microscopic autoradiography (Ilford L4), developed and fixed after 3-4 months of exposure. Thin sections were lightly stained with uranyl acetate and lead citrate, and observed under a CM 100 Philips electron microscope operating at 60-80kV.

Electrophoresis, autoradiography and immunoblotting

The remaining skin from the whole body of injected animals was frozen in liquid nitrogen and stored at -80°C before protein analysis (SYBERT et al., 1985). Tissues were incubated in 5mM EDTA in phosphate buffered saline (PBS) for 5min at 50°C and 4min in cold PBS, then the epidermis was separated from the dermis by dissection under the stereomicroscope. The epidermis was homogenized in 8M urea/50mM Tris-HCl (pH 7.6)/0.1M 2-mercaptoethanol/1mM dithiothreithol/1mM phenylmethylsulphonyl fluoride. The particulate matter was removed by centrifugation at 10,000g for 10min. Protein concentration was assayed by the Lowry method before electrophoresis.

For monodimensional electrophoretic analysis, proteins were denatured by boiling the extracted solution in the Sample Buffer for 5min. Then, 50-100μg of protein was loaded in each lane and separated in 10% or 12% SDS-polyacrylamide gels (SDS-PAGE) according to LAEMMLI (1970). For two-dimensional electrophoretic analysis, the Ettan IPGphor III IEF System (Ge Health-

care, U.K.) was used for the isoelectrofocusing (IEF). An 80µg protein sample (containing 2% CHAPS (Sigma, USA) and 1% carrier ampholyte mixture, pH 3,5-10 (Ge Healthcare, U.K.)) was loaded onto a 7cm (pH 3-10) strip (Ge Healthcare, U.K.). Application of the strips and the running procedure were carried out as described by the manufacturer. The following protocol was used. Rehydratation was performed for 12h at room temperature and was followed by the IEF, step by step, from 0.5h 500V, 0.5h 1000V, 0.5h 5000 gradually, and for 1h at 5000V. Strips were kept at 50V until loaded on the second dimension. Before starting the second dimension, the strips were equilibrated for 10min in 6M urea, 30% glycerol, 50mM Tris, pH 6.8, and 2% DTT. Afterward, strips were briefly rinsed with double distilled water and equilibrated in 6M urea, 30% glycerol, 50mM Tris, pH 6.8, and 2.5% iodoacetamide for an additional 10min. The second dimension was carried out in 12% SDS-polyacrylammide gels by using the MiniProtean III electrophoretic apparatus (Bio-Rad). After electrophoresis gels were immunoblotted on nitrocellulose paper or exposed for autoradiography.

For autoradiography experiments, gels were fixed in 2-propanol:distilled water:acetic acid (25:65:10) solution for 30 minutes. The gels were incubated in Coomassie blue, a staining solution designed for the detection of proteins separated in polyacrylamide gels. Afterwards, the gels were incubated in Amplify solution (Ge Healthcare, UK) for 15min and dried with a gel dryer (BioRad, USA) for about two hours at 80°C. Finally, gels were exposed to X-ray film (X-Omat LS, Kodak, USA) at –80°C for 3-4 weeks, in autoradiographic boxes equipped with intensifying screens, and films were developed to obtain final fluorographs.

For western blotting, the proteins separated in SDS-PAGE were transferred to nitrocellulose paper. Ponceau red, a reversible staining solution designed for rapid (5 minutes) staining of protein bands on nitrocellulose membranes, was used after western blot to verify the protein transfer. The membranes were then incubated with primary antibodies directed against keratins K6 (diluted 1:1000), K16 (diluted 1:2000) and K17 (diluted 1:5000). Detection was performed using the enhanced chemioluminescence procedure developed by Amersham (ECL, Amersham, UK). In electrophoresis experiments, Wide Range (MW 6500-20 500) molecular weight markers (Sigma, USA) were used.

RESULTS

Autoradiography and immunocytochemistry

Thymidine-labeled nuclei in the epidermis of *T. vulgaris* were mainly present in sparse cells of the basal and sometimes also in the first suprabasal layers at 4 hours post-injection (Fig. 1A). Numerous silver grains were present over the nuclei of these cells (data not shown). At four days post-injection, sparse cells with labeled nuclei, with variable numbers of silver grains per cell, were still present in the basal layer and in suprabasal layers but not in the corneous layer (Fig. 1B). At six days post-injection, cells in suprabasal layers and some in the corneous layer were seen (Fig. 1C). In both species, the corneous layer appeared as a thin superficial coat of the epidermal surface (Figs 1C-D). This was confirmed by the electron microscopic analysis on the epidermis of *T. vulgaris*, which showed a very thin corneous layer above a stratified epithelium (Fig. 1E). Detailed observation of the pre-corneous (replacement) layer in the epidermis of both the newt *T. vulgaris* and toad *B. bufo* disclosed large amounts of mucous-like granules, many of which appeared to be releasing material into the extracellular space facing the corneous layer (Fig. 1F). The latter was denser than the inner epidermal layers and contained a thick meshwork of keratin filaments and a dense cornified cell membrane.

Autoradiographic analysis of the epidermis 4 and 8 hours after histidine injection in *T. vulgaris* and in *B. viridis* showed that silver grains were mainly localized over the cytoplasm of pre-corneous layers (Figs 2A-C). Grains were more frequently observed in the cytoplasm of these cells, often associated with denser areas among keratin filaments (Fig. 2D). Silver grains were also present in dense areas among packed keratin filaments in cells forming the corneous layer and in dense material associated with the plasma membrane (Fig. 2D).

The immunolabeling for cytokeratins K6 and K16 was weak in the epidermis of *T. vulgaris* using light microscopic immunocytochemistry, but immunoreactive sites were only detected in upper pre-corneous and corneous layers (Fig. 3A). Under the electron microscope, K6 and K16 antibodies applied to the epidermis of *T. vulgaris* showed a similar pattern of labeling. They showed a scarce or diffuse immunolabeling over tonofilaments of cells of the upper intermediate and pre-corneous keratinocytes. Most of the labeling was instead associated with mucous and dense granules, or with a dense material not limited by a membrane, that was present in localized areas of the cytoplasm of differentiating and pre-corneous keratinocytes (Figs 3B-C). Gold labeling was also more intense along the plasma membrane and the periphery of desmosomes in pre-corneous and corneous cells (Figs 3C-D), where dense material resembling that of mucous granules was present (Fig. 3E). No significant labeling was seen over tonofilament bundles of desmosomes or among the packed keratin filaments of the corneous layer.

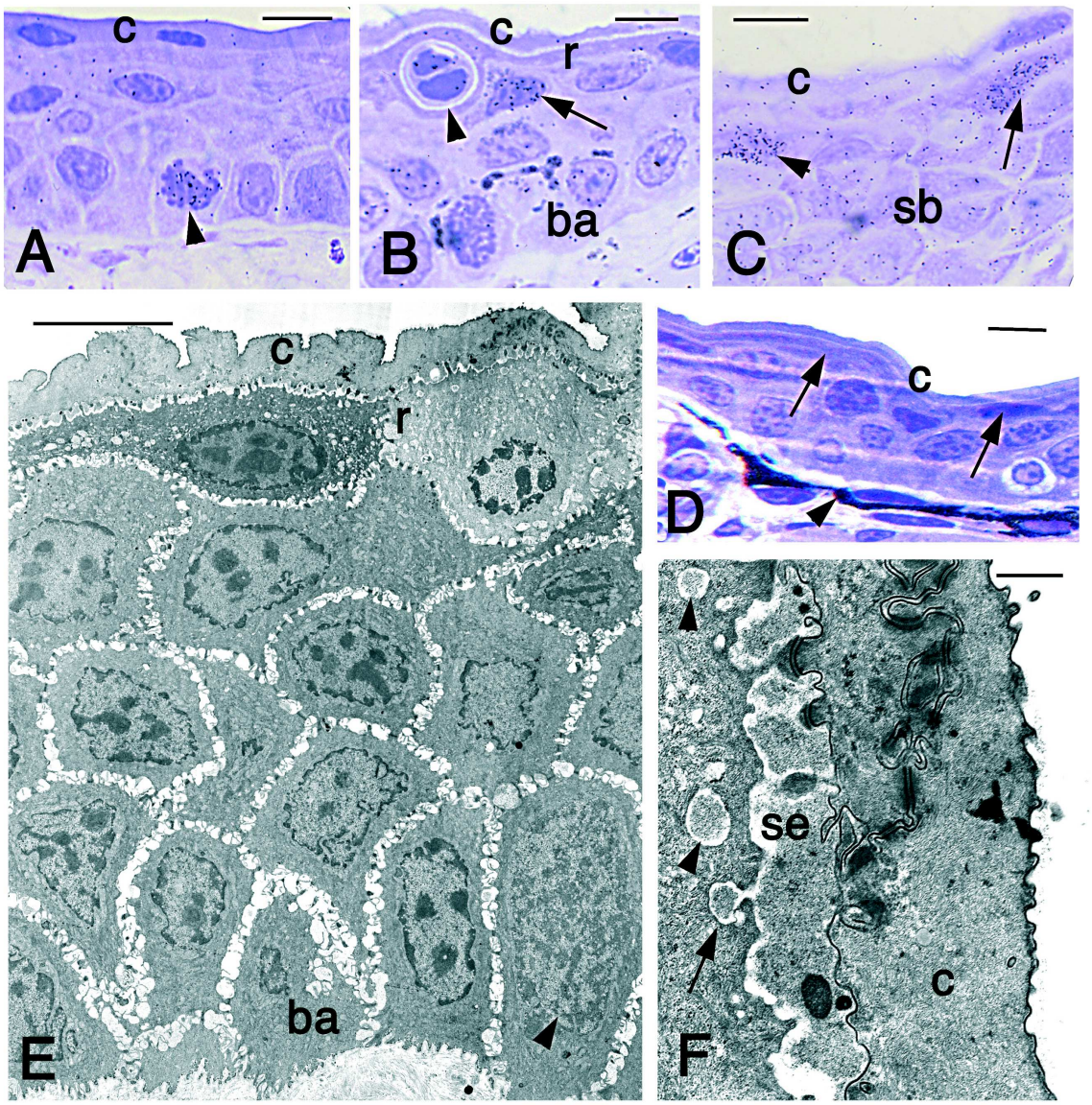

Fig. 1. – Light autoradiography for thymidine in the tail epidermis of *T. vulgaris* (**A-C**), for the digit epidermis of *B. viridis* (**D**) and ultrastructure of the tail epidermis of *T. vulgaris* (**E-F**). **A**, mitosis in still-labeled basal cell (arrowhead) at 4 days post-injection. Bar 10µm. **B**, labeled cell (arrow) in upper spinosus layer 4 days post-injection. The arrowhead points to a likely phagocyte. Bar 10µm. **C**, labeled cells in upper (arrow) and pre-corneous layer (arrowhead) 4 days post-injection. Bar 10µm. **D**, epidermis of the toad *B. viridis* with indicated the corneous layer (arrows). The arrowhead points to melanophores. Bar 10µm. **E**, general view of newt tail epidermis with polygonal basal cells (the arrowhead points to a mitotic cell) and forming precorneous (replacement) layer. Bar 10µm. **F**, detail on pale mucous granules (arrowheads) in pre-corneous layer. The arrow indicates a granule discharging into the extracellular space where mucous-like material is present. Bar 0.5µm. **Legends**: ba, basal layer; c, corneous layer; r, replacement (pre-corneous) layer; sb, supra-basal layers; se, secreted material

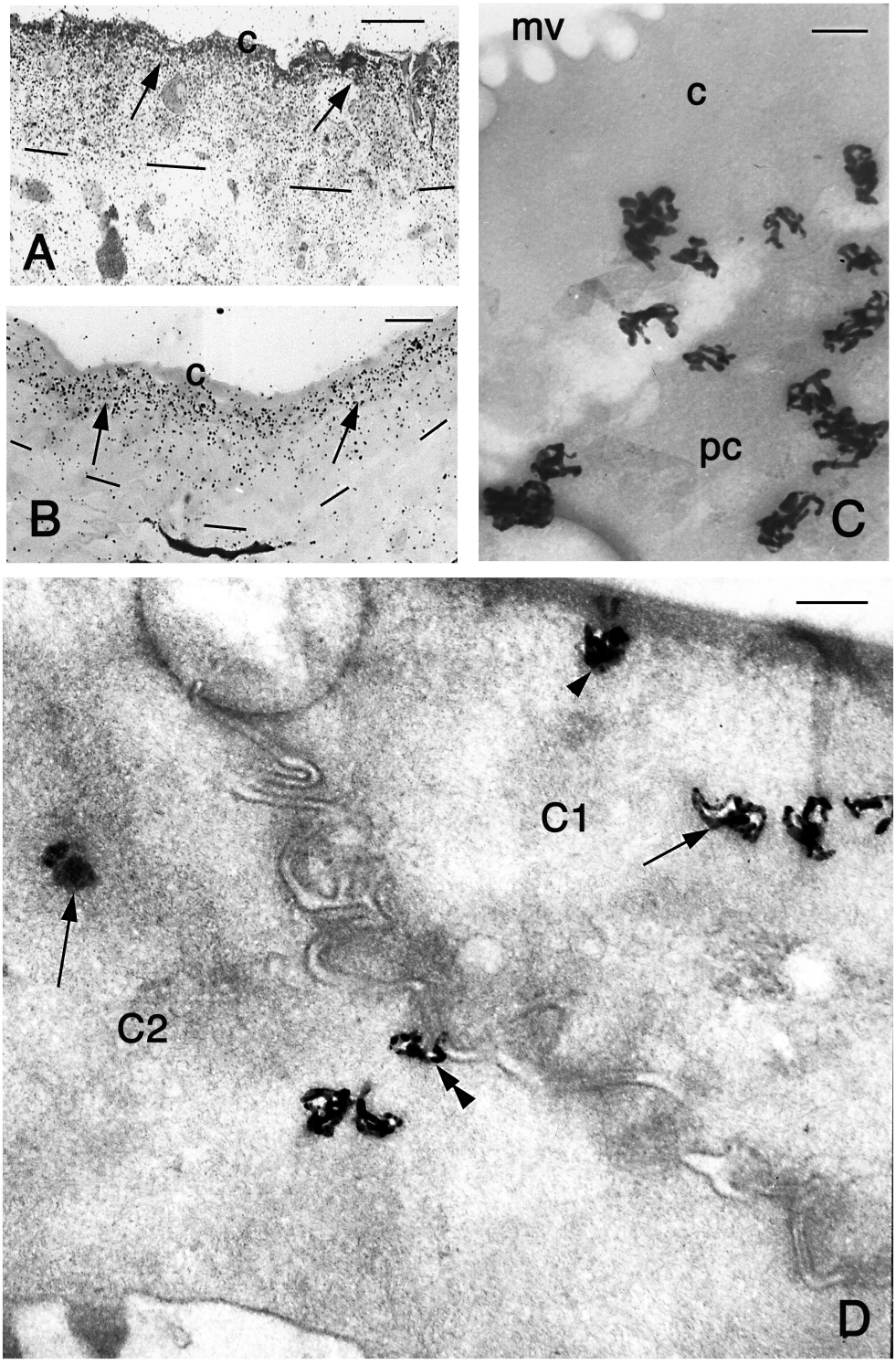

Fig. 2. – Light (**A-B**) and electron-microscopic (**C-D**) autoradiography of the epidermis 4 hours post-injection of tritiated histidine. **A**, most silver grains are localized toward the corneous layer (arrows) of the tail epidermis of *T. vulgaris*. Bar 10µm. **B**, silver grains mainly present over the pre-corneous layer of the ventral epidermis of the toad *B. viridis* (arrows). Bar 10µm. **C**, detail of trace (silver) grains over most of the pre-corneous layer of newt digit epidermis. Bar 200nm. **D**, detail of trace grains associated with denser areas (arrows), the surface (arrowhead), or near desmosomal junction (double arrowhead) in two differentiating corneous cells (C1 and C2) of digit epidermis in toad *B. viridis*. Bar 200nm. Dashes underlie the basal layer of the epidermis. **Legends**: c, corneous layer; pc, pre-corneous layer.

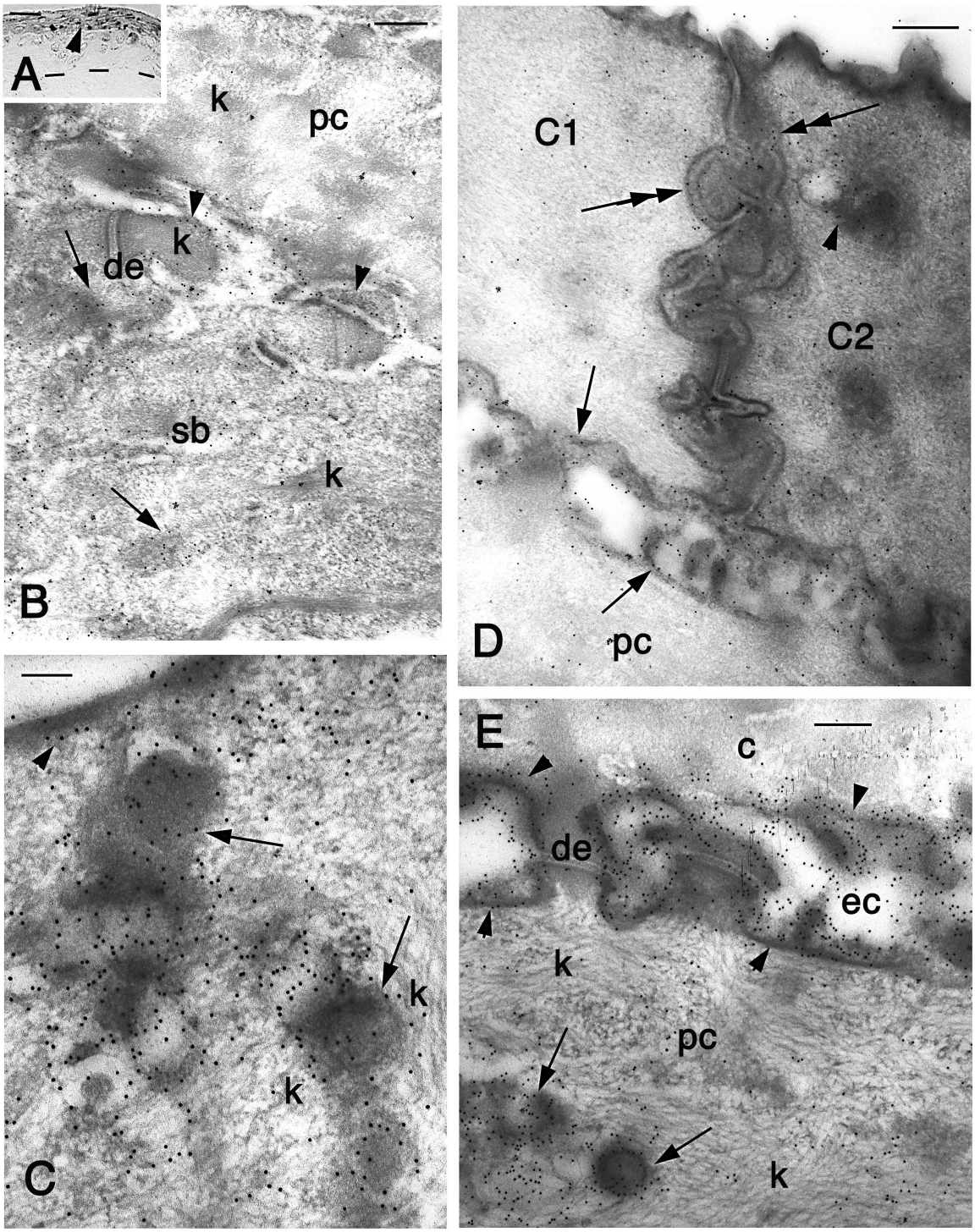

Fig. 3. – Immunolabeling with K16 of the epidermis of *T. vulgaris*. **A**, light microscopy shows a weak but positive reaction in upper layers of the digit epidermis (arrowhead). Dashes underlie the basal layer of the epidermis. Bar 20µm. **B**, diffuse gold labeling of upper spinosus cell of digit epidermis. More concentrated labeling is seen on denser areas (arrows) and along the plasma membrane (arrowheads). Keratin bundles are not or little labeled. Bar 250nm. **C**, detail of dense areas associated with granules (arrows) or with the plasma membrane (arrowhead) in pre-corneous cell of tail epidermis. Keratin filaments are not labeled. Bar 100nm. **D**, labeling along the cell surface of pre-corneous and corneous cells (arrows) of tail epidermis, along the junctions between two corneocytes (C1 and C2, double arrows), and in a dense material within the corneous material (arrowhead). Bar 200nm. **E**, detail on the intense labeling (arrowheads) present on the surface of a pre-corneous and a corneous cell of digit epidermis. The arrows indicate labeling on dense granules but not over keratin filaments. **Legends**: de, desmosome; ec, extracellular space; k, keratin bundles; pc, precorneous cell; sb, suprabasal cell.

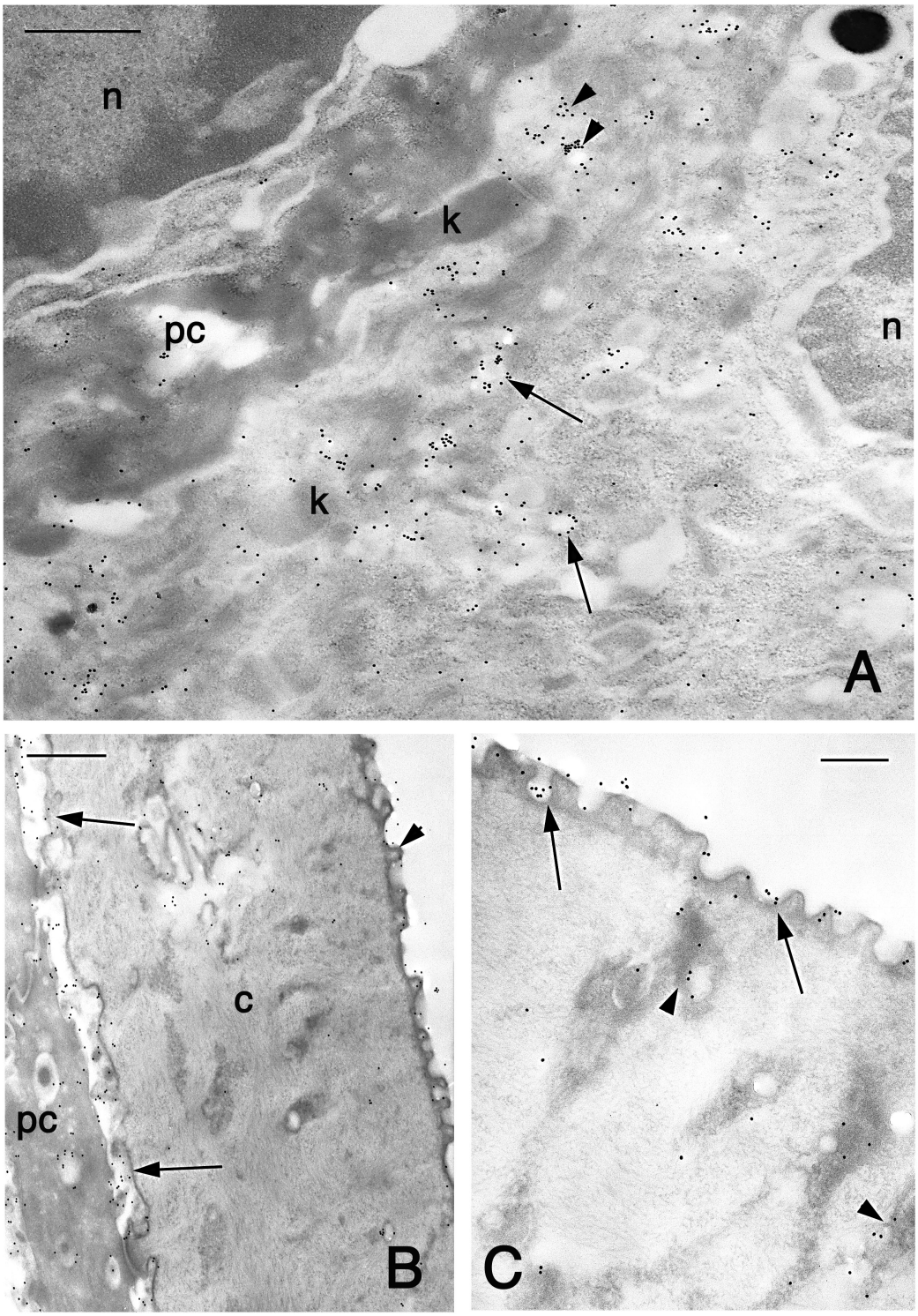

Fig. 4. – Ultrastructural immunolocalization of actin in the digit epidermis of the newt *T. vulgaris*. **A**, upper spinosus cell showing gold labeling mainly associated with pale mucous granules (arrows), some in phase of discharging their amorphous content into the extracellular space (arrowheads). Bar 0.5µm. **B**, immunolabeling mainly associated with the inner surface (arrows) and the outer surface with microvilli (arrowhead) of the corneous layer. Bar 250nm. **C**, detail of the labeling (arrows) on the microvillar surface of the stratum corneum. Other labeling is present over denser areas among the pale keratinized material (arrowheads). Bar 150nm. **Legends**: c, stratum corneum; k, keratin bundles; n, nucleus; pc, stratum precorneum.

The ultrastructural immunolabeling with the actin antibody in the epidermis of *T. vulgaris* showed most of the labeling in upper intermediate and pre-corneous cells, especially associated with pale vesicles or, less commonly, with denser areas, but no gold particles were associated with keratin bundles (Fig. 4A). Some of these vesicles appeared in a stage of discharging their material into the extracellular space. In pre-corneous cells, most gold particles were associated with the peripheral cytoplasm of the plasma membrane but not with desmosomes (Fig. 4B). In the corneous layer, gold particles were seen in more electron-dense areas along the inner and outer rim of cytoplasm, and the plasma membrane (Fig. 4C).

Electrophoresis, autoradiography and immunoblotting

The protein pattern of epidermal proteins (stained with Coomassie blue) showed that essentially most of the proteins were represented by alpha-keratins in both newt (*T. vulgaris*, Fig. 5A) and toad (*B. viridis*, Fig. 5B).

In the newt *T. vulgaris* one-dimensional electrophoresis showed most bands concentrated at 45-64kDa, and less intense bands at 33-35, and 18kDa (Fig. 5A1). In two-dimensional electrophoresis gels (Fig. 5A2), some protein spots were seen at 48-50kDa with pI at 5.2-5.5, 6.2 and 7.0. Other spots at 55-57kDa showed pI at 5.2, 5.6-6.2, and 6.8. Finally, spots of 65kDa showed pI at 5.3-5.7, 5.9-6.2, 6.8-7, and 7.2. The band at 18kDa that was present in one-dimensional electrophoresis was not clearly seen in two-dimensional-gels. The one-dimensional autoradiographic examination showed main labeled bands at 42-45 and 55-60kDa, and a much weaker one around 30kDa (Fig. 5A3). The 2D-autoradiographic examination showed reactive spots around 45kDa with pI at 5, 6 and 6.8, and another, diffuse spot at 62-65kDa with pI at 5.0-5.5 (Fig. 5A4). Very small to undetectable spots were seen around 30kDa.

Similarly, in the toad *B. viridis*, most of the proteins were not well resolved in mono- and two-dimensional gels, and were concentrated in the alpha-keratin range, at 44-60kDa (Figs 5B1-2). Main spots were seen at 44kDa with pI at 5.0-5.5, at 50-52kDa with pI at 4.8-5.2 and 5.5, at 55kDa with pI around 6, at 60-64kDa with pI at 5-6.2, and 7.0-7.7. The autoradiographic analysis in one-dimensional gels showed main bands at 30 and 50-52kDa (Fig. 5B3). In two-dimensional gels only, we observed positive spots at 45-47kDa with pI at 5.0-5.6 and 6, and at 55-57kDa with pI at 6.2-6.5 and 7.0-7.2 (Fig. 5B4). A very weak and diffuse spot around 20kDa with pI 8 was seen.

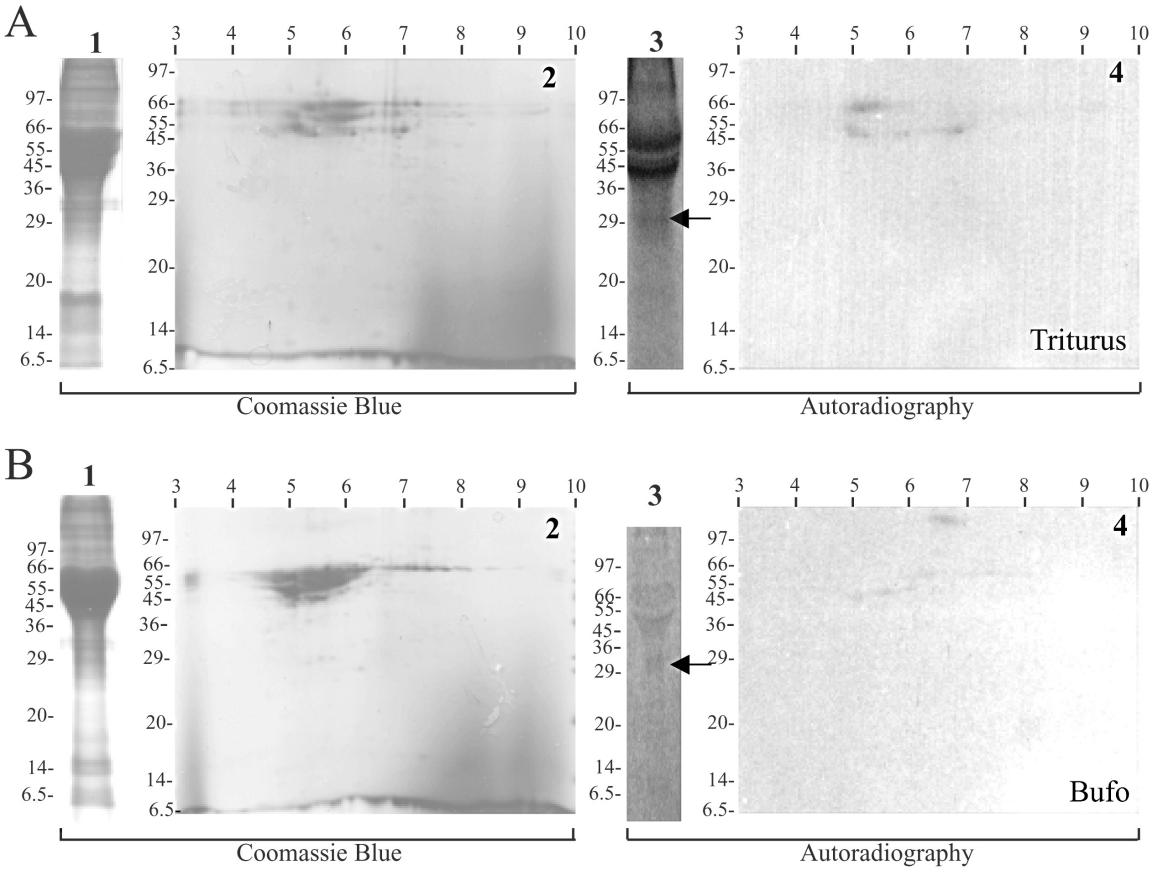

Fig. 5. – One-dimensional (A1, 3 and B1, 3) and two-dimensional (A2, 4 and B2, 4) electrophoretic patterns of epidermal proteins. Coomassie pattern (A1-2) and relative autoradiography (A3-4) in *T. vulgaris*. Coomassie blue (B1-2) and relative autoradiography (B3-4) in *B. viridis* (see text for details). The arrows indicate a weakly labeled band at 30kDa. Numbers in abscissa indicate pH, those in ordinate indicate molecular weight.

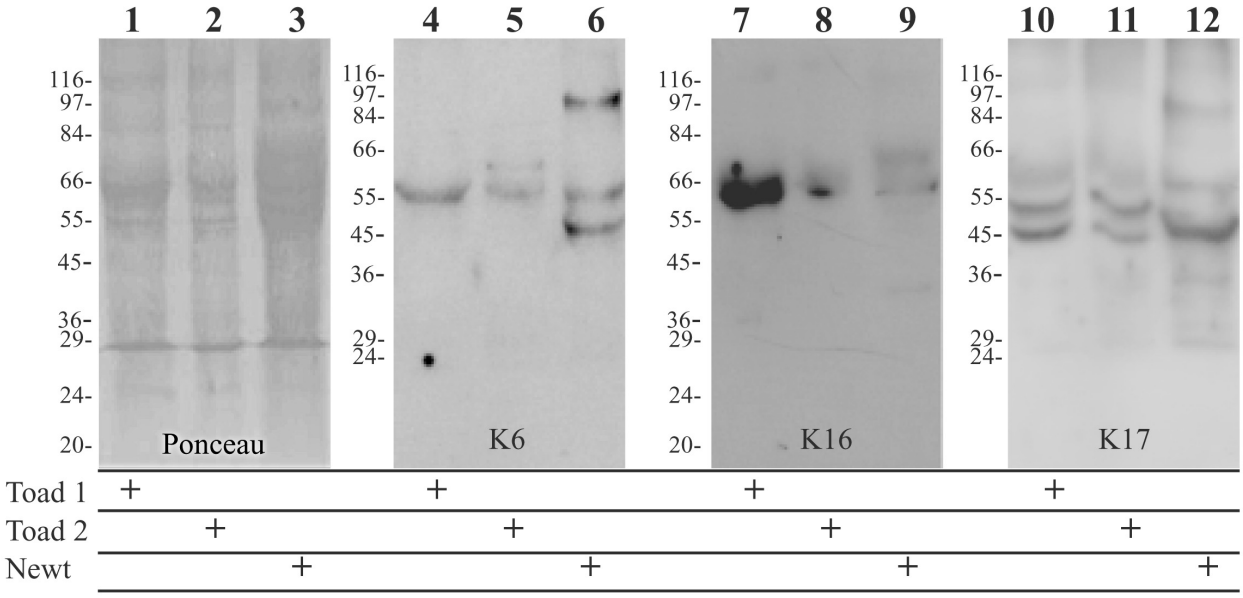

Fig. 6. – One-dimensional protein patterns stained by using Ponceau red (1-3) or K6 (4-6), K16 (7-9), and K17 (10-12) antibodies on *B. viridis* and *T. vulgaris* epidermal proteins (see text for further details).

The presence of protein bands that cross-react with K6, K16, and K17 antibodies was revealed by one-dimensional immunoblotting of epidermal proteins (Fig. 6). In samples from the epidermis of *B. viridis* (Fig. 6, lanes 1 & 2) K6 bands were seen at 55kDa (Fig. 6, lanes 4 & 5). K6 reactive bands appeared at 45, 55, and above 80kDa in the newt (Fig. 6, lane 6).

With the K16 antibody, positive bands were seen at 60-65kDa for the toad *B. viridis* (Fig. 6, lines 7 & 8), and weak bands were seen at 65-70kDa for the newt *T. vulgaris* (Fig. 6, lane 9). Finally, the K17 antibody showed bands at 45, 53 and a weaker band at 60kDa for the toad (Fig. 6, lanes 10 & 11), and at 45, 57 and a weak band above 80kDa for the newt (Fig. 6, lane 12).

DISCUSSION

Previous and the present studies show that in amphibian epidermis proliferation occurs in the basal and also suprabasal layers (Luckenbill, 1965; Fox, 1994; Alibardi, 2002). Therefore early differentiating cells of the intermediate layers can represent a population of expanding (transiently expanding) keratinocytes in amphibians. In normal conditions, with an active shedding (Budz, 1977), the time of migration of undifferentiated cells from the basal to the corneous layer occurs between 8 and 14 days. Main proteins synthesized from migrating keratinocytes are keratins and mucous with its specific glycoproteins.

Previous research on *Xenopus laevis*, a model amphibian, has indicated that cells of the upper stratum spinosus and pre-corneous layer synthesize numerous and specific proteins in preparation for cornification, including a type II, basic keratin of 63kDa (Ellison et al., 1985; Nishikawa et al., 1992). That particular keratin appears as one of the main components of the pre-corneous and corneous layers of the post-metamorphosis epidermis of *X. laevis*. Other studies showed that the main keratins found in adult amphibian epidermis comprise acidic, type I keratins, of 45-55kDa and pI 5-5.5 (Hoffmann et al., 1985). At least three spots of type II keratins of 64-66kDa were also found in high amounts in the adult epidermis of *X. laevis*. The latter possess a slightly basic pI at 7-7.4, and were characterized in their nucleotide and amino acid sequence showing extensive glycine-rich regions in the variable regions (Hoffmann et al., 1985).

Proteins with similar values of MW and pI have been reported in the present study on the newt *T. vulgaris* and the toad *B. bufo*. The present study indicates that the main keratins produced in the epidermis of these amphibian species (as labeled with tritiated histidine) are acidic or neutral (pI at 5-7), especially in the newt, which is a more aquatic species than the toad. In the latter some more basic keratins of 62-66kDa and pI at 7.0-7.7 are present in the epidermis, but in much smaller amounts than the acidic keratins.

The silver labeling after histidine injection has indicated an association mainly with dense areas among keratin filaments in differentiating keratinocytes and in those of the stratum corneum (Alibardi, 2003; Alibardi et al., 2003). The study has also indicated that some of the synthesized proteins (mucins, glycoproteins, or inter-keratin proteins) are associated with dense material present on the surface of pre-corneous and corneous cells, and along the cell junctions. These sites are the same as where most of the immunoreactivity for keratins K6, K16 and for actin is found, suggesting that high amounts of these proteins are also actively synthesized during keratinocyte differentiation in amphibian epidermis. We could not observe any specific pattern in the localization of the above keratins. Therefore these keratins appear associated with a general process of extrusion of glycoproteins, but not with the specific secretion of different glycoconiu-

gates responsible for their different localization in amphibian epidermis (ZACCONE et al., 1987). The increased immunolabeling for actin in upper and pre-corneous keratinocytes resembles a similar pattern observed in superficial keratinocytes in fish where the microfilament (actin) cytoskeleton sustains the formation of the dynamic superficial microridges (WHITEAR, 1977).

The immunoultrastructural study has shown that K6- and K16-like proteins are associated with dense granules or matrix material produced amongst the usual network of keratin filaments. K6-K16-like proteins do not make long bundles as do the other cytokeratins. The latter are responsible for the formation of the typical bundles of keratin filaments (tonofilaments) in basal, intermediate and pre-corneous cells, but the specific keratins composing the long bundles are not known in the two species analyzed in the present study.

The immunoreactive bands for K6, K16 and K17 fall within the range of more abundant types of keratins reported for the epidermis of *X. laevis*, such as the 49, 53, 56 and 63-64kDa (HOFFMANN & FRANZ, 1984; ELLISON et al., 1985; HOFFMANN et al., 1985; NISHIKAWA et al., 1992). An explanation for this result is that the specific epitopes present in mammalian K6, K16, and K17, against which the antibodies were produced (in TAKAHASHI et al., 1994; MCGOWAN & COULOMBE, 1998a;b), can be present in non-orthologous keratins of amphibians. However, the lack of any labeling in long bundles of filaments also suggests that the observed immunolabeling is specifically directed to keratins that are absent from tonofilaments but are localized in a more diffuse network connected to the dense material or mucous granules present in differentiating amphibian keratinocytes.

In mammalian keratinocytes, numerous alpha-keratins of type I (basic, K1-K8) and type II (acidic, K9-K19) are present (MOLL et al., 1982; O'GUIN et al., 1987; COULOMBE & OMARY, 2002). One type I and one type II keratin form a base pair that gives origin to single alpha-keratin filaments, and then to keratin bundles called tonofilaments. Among keratins, K6, K16 and K17 form a special group whose members do not form typical tonofilaments but remain separated or incorporated into small bundles, typical for wound keratinocytes (MCGOWAN & COULOMBE, 1998a;b; FREEDBERG et al., 2001). This is also the case for reptilian wound keratinocytes that cover the amputated limb or tail stumps (ALIBARDI & TONI, 2005). These keratins probably have elastic properties, and are upregulated during wound healing of mammalian wounds where they mainly replace keratins of tonofilaments. The association with actin may therefore determine the formation of an elastic cytoskeletal network within amphibian keratinocytes.

In *T. vulgaris* keratinocytes, K6/K16-immunoreactive-like keratins surround secretory granules, especially those containing mucous, that are later secreted extracellularly among upper spinosus keratinocytes, and that participate in the formation of the coat membranes of pre-corneous and corneous cells. Therefore some of the keratins produced in newt epidermis are used for the extrusion of mucous and glycoproteins to coat the cell surface of pre-corneous or corneous cells. Among these glycoproteins that contain N-acetyl-glucosammine and galactose, a protein at 52 and others at 110-150kDa have been found in the adult epidermis (VILLALBA et al., 1992).

The presence of wound-like keratins (K6, K16 and K17) or of keratins containing characteristics epitopes for these keratins in normal epidermis of the newt *T. vulgaris* suggests that these or cross-reactive keratins are constitutive cytoskeletal proteins in amphibian keratinocytes. Therefore the production of these proteins does not require a long lag phase after wounding of the epidermis in amphibians, and their synthesis is probably quickly upregulated during epidermal regeneration. The activated keratinocytes are capable of covering the entire amputated surface of a limb in 16-18 hours, a process that is very rapid and efficient in amphibians with respect to other vertebrates. It is well known that amphibian skin produces a specific epidermis, the apical wound epithelium, necessary for the regeneration of the limb, which contains specific keratins (GERAUDIE & FERRETTI, 1998).

The mucous production, the ability to divide in suprabasal layers, and the synthesis of wound-type keratins that do not form long bundles, are characteristics of embryonic or poorly specialized keratinocytes. Besides, K6, K16 and most of all K17 keratins have elastic properties, and their association with actin may indicate that in the normal epidermis these proteins participate in the cytoskeletal mechanism of movement of organelles toward the plasma membrane for extrusion of their secretion. It is known that mucous contains anti-microbial substances, which help the innate protective immunity of the skin (FOX, 1994). Destruction of the dynamic keratin network from parasitism may therefore impair the resistance of the epidermis to infections. In fact, it appears that after infection of the epidermis by chytridiomycetes, keratinocytes lose their typical, diffuse keratin network, and prematurely form the dense corneous material of mature cells of the stratum corneum (BERGER et al., 1998; 2005). This premature cornification may limit the amount of mucous eliminated in the replacement and corneous layers, but further study is required on this point.

Within the limitation of our techniques, the present study suggests that non-keratin proteins or those in the range of 20-30kDa with basic pI are low to absent in amphibian epidermis. Only in *B. viridis*, the more terrestrial species, is a possible diffuse spot of protein present at 20kDa with pI around 8. This very low amount suggests that possibly, basic proteins in this range are very scarce in normal epidermis. It may be that specialized keratins and higher amounts of "keratin associated proteins" (KAPs) are present in tissues with a higher degree of cornification than the epidermis, such as the claws of the frog *Xenopus laevis* (MADDIN et al., 2007) or the beak of frog tadpoles (LUCKENBILL, 1965). The presence of KAPs in amphibian epidermis remains to be studied using more sensitive methods, but it is likely that amphibian KAPs may be present in regions where an intense cornification takes place, such as claws and the larval beak (FOX, 1994; WARBURG et al., 1994).

CONCLUSIONS

In conclusion, the present study indicates that mainly acidic-neutral keratins of 55 and 65kDa are synthesized in adult epidermis, among which are elastic types of keratins (K6,16,17-like wound keratins). The latter may work together with actin to permit the movement of mucous granules toward the cell surface and their secretion to coat the plasma membrane of maturing keratinocytes.

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