

# Skin Peptides in *Xenopus laevis*: Morphological Requirements for Precursor Processing in Developing and Regenerating Granular Skin Glands

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**Abstract.** The biosynthesis of the peptides caerulein and PGL<sup>a</sup> in granular skin glands of *Xenopus laevis* proceeds through a pathway that involves discrete morphological rearrangements of the entire secretory compartment. Immunocytochemical localization of these peptides during gland development indicates that biosynthetic precursors are synthesized in intact secretory cells, whereas posttranslational processing requires morphological reorganization to a vacuolated stage. The bulk of the processed secretory material is then stored in vacuolae-derived storage granules. In the mature gland, storage granules are still formed at a low level. However, in this case processing takes place in a distinct cytoplasmic structure, the multicored body, which we suggest to be functionally equivalent

to vacuolae.

When granular glands regenerate after having lost all their storage granules upon strong stimuli, another morphological pathway is used. 2 wk after gland depletion, secretory cells become arranged in a monolayer that covers the luminal surface of the gland. Storage granules are formed continuously within these intact secretory cells. Here, precursor processing does not require a vacuolated stage as in newly generated glands but occurs in multicored bodies. Most storage granules seem to be formed in the third week of regeneration. The high biosynthetic activity is also reflected by the high activity of the putative processing enzyme dipeptidyl aminopeptidase during this period of regeneration.

**S**KIN glands of amphibians, in particular those of anurans, contain large quantities of biologically active peptides and of biogenic amines (11). These compounds are either identical or highly homologous to hormones or neurotransmitters of the diffuse endocrine system of higher vertebrates (12). Some peptides that have originally been isolated from amphibian skin were subsequently found in the mammalian central nervous system and/or the gastrointestinal tract, and vice versa (13, 14). The active peptides thus far isolated from *Xenopus laevis* skin secretion are caerulein, thyrotropin-releasing hormone, xenopsin, PGL<sup>a</sup> (2–4), and several others (16).

The decapeptide caerulein was first isolated from the skin of *Hyla caerulea* (1). This peptide bears a close resemblance to gastrin and cholecystokinin in both its primary structure and in its biological activity (7). Hoffmann et al. (18) found cDNA clones that code for a hitherto unknown peptide. Assuming that all established processing reactions had occurred, it was suggested that this new peptide would consist of 24 residues with an amino-terminal tyrosine and a carboxy-terminal leucine amide. It was therefore designated PYL<sup>a</sup>, according to the nomenclature of Tatemoto and Mutt (37). However, another proteolytic cleavage, in addition to

the predicted processing events, seems to occur in vivo. Cleavage at a single arginine residue yields a peptide that is shorter than PYL<sup>a</sup> by three amino acids from the amino terminus. This peptide, PGL<sup>a</sup>, has been isolated from the skin secretion and was characterized by HPLC and mass spectroscopic analysis (3, 16).

Secretory peptides from frog skin are formed in dermatus granular glands (6, 10). Mature granular glands in *X. laevis* skin consist of a syncytial secretory compartment filled with spheroidal “granules.” These granules have been referred to in the literature as “secretory granules.” However, this term implies a direct descent from the Golgi apparatus and the liberation of secretory products by exocytosis. This though does not reflect the situation in *X. laevis* skin glands where the mature granules have exclusively an extracellular storage function. To distinguish these granules from membrane-bounded intracellular secretory granules we will be using the term “storage granules.” The fine structure of the mature granular gland has been described (10, 19). Initial formation of granular glands takes place during metamorphosis (39); however, a steady de novo formation of new glands occurs in adult animals as well. During the development of immature granular glands, a stage is reached where cell mem-

branes of secretory cells disintegrate but where no mature granules have as yet been formed. Based on the foamy appearance of such glands in light microscopic preparations, Spannhof (35) named this stage "Schaumstadium;" here it will be referred to as "vacuolated stage."

Considering these different morphological stages of the developing gland we were interested to see if these reflect distinct biochemical processes in the biosynthesis of secretory peptides. However, granular glands formed by de novo synthesis are unsuitable for the study of specific biochemical parameters, such as processing enzymes. Therefore, we also examined the granular gland during regeneration.

After experimentally stimulated discharge of the secretory products (10) glands require a period of several weeks before comparable quantities of secretion can again be released (5). We were interested to see if regenerating glands undergo similar morphological changes as newly forming glands in the process of their maturation and on what time scale this process occurs. Furthermore, by using a biochemical marker we hoped that in the regenerating gland we could more precisely identify the morphological stage in which precursor processing occurs. For this purpose, the putative processing enzyme dipeptidyl aminopeptidase (DAP)<sup>1</sup> has been chosen. This enzyme can easily be identified and isolated from skin secretion (25). The morphology of the regenerating granular gland, the immunoreactivity towards the secretory skin peptide caerulein, and the enzymatic activity of DAP were then studied.

The data presented here show that de novo formation and regeneration of granular glands proceed through two different pathways. Whereas biosynthesis, processing, and storage are confined to distinct, consecutive developmental stages in the newly forming gland, these processes occur simultaneously during regeneration within intact secretory cells and do not require the formation of a syncytially organized secretory compartment. Thus skin glands of *X. laevis* offer intriguing possibilities to study the relationship between biochemical processes and morphological structures in the development of a secretory system.

## Materials and Methods

### Production of Antisera

**Antigens.** Caerulein-8-conjugate was generously supplied by K. Richter (Institute for Molecular Biology, Salzburg, Austria); PYL<sup>a</sup> was synthesized by Dr. Leban (Biogen, Basel, Switzerland). PYL<sup>a</sup> was adsorbed on KAl(SO<sub>4</sub>)<sub>2</sub> according to the method of Bradley et al. (8).

**Immunization.** A group of four rabbits (mixed breed) was immunized with either antigen. Caerulein-8-conjugate (800 µg/animal) was diluted in 0.5 ml 0.01 M phosphate-buffered 0.15 M saline (PBS), pH 7.5 and suspended in an equal volume of complete Freund's adjuvant and then administered by subcutaneous injection at multiple sites along the spine. PYL<sup>a</sup> (50 µg/animal) was adsorbed to KAl(SO<sub>4</sub>)<sub>2</sub>, suspended with incomplete Freund's adjuvant, and injected in the axillary lymph node (32). Booster immunizations were performed at intervals of ~1 mo by intravenous and subcutaneous injections. The rabbits were bled from the lateral ear vein 6–12 d after every booster immunization. All sera were lyophilized and screened for anti-caerulein or anti-PYL<sup>a</sup> antibodies, respectively, with the peroxidase-anti-peroxidase (PAP) method and the dot-blot test (36, 38).

**Antisera.** For the experiments reported here, anti-caerulein antiserum, anti-CL, and the anti-PYL<sup>a</sup> antiserum, PYL<sup>a</sup>-V/2, were used. Optimal di-

lutions for both antisera were found to be 1:1,000 for the PAP method, and between 1:500 and 1:1,000 for the immunogold staining (IGS) technique.

### Specificity Test

To establish method specificity of the applied immunocytochemical methods, various controls were performed. These included using increasing dilutions of the primary antiserum, omitting it, or replacing it by preimmune serum or unrelated antisera (15, 40). To show serum specificity the following tests were carried out.

**Absorption Test.** The specific antisera were incubated with increasing concentrations of the respective antigen as well as unrelated antigens such as other *Xenopus laevis* skin peptides (29).

Peptides used in specificity tests were: caerulein (Montedison Farmaceutica, Freiburg, FRG), h-gastrin 1-17 (UCB-Bioproducts S. A., Bruxelles, Belgium), penta-gastrin (generously supplied by K. Richter; Institute for Molecular Biology, Salzburg, Austria), PGL<sup>a</sup> (isolated from frog skin secretion), xenopsin (Peninsula Laboratories Inc., Merseyside, England), vasopressin (Sigma Chemical Co., St. Louis, MO) and BSA (Serva Feinbiochemica GmbH & Co., Heidelberg, FRG).

### Tissue Preparation

Adult *Xenopus laevis* were anesthetized with MS 222 (Sandoz Ltd., Basel, Switzerland). Samples of dorsal skin were fixed in Bouin's solution, dehydrated, and embedded in paraplast for histological staining (hematoxylin-eosin, azocarmine-aniline blue)(31) and for immunocytochemistry at the light microscopic level. Alternatively, small pieces of skin were quick-frozen in liquid propane and freeze-dried at -40°C before fixation in formaldehyde vapors (33). For immunocytochemistry at the electron microscopic level, fixation in Karnovsky's solution or, alternatively, in buffered 4% paraformaldehyde and 0.25% glutaraldehyde was chosen. After three washes in Millonig's buffer (pH 7.3; containing 3% sucrose) the samples were usually postfixed in 1% osmium tetroxide and then embedded in Epon 812 (Serva Feinbiochemica GmbH & Co.) or LR-White (London Resin Comp., Basingstoke, England). Alternatively, some specimens, not treated with osmium tetroxide, were subjected to 0.5 mg/ml potassium borohydride in PBS for three times 15 min and embedded in 20% polyvinyl-alcohol, 10,000 mol wt (Janssen Pharmaceutica, Beerse, Belgium) according to the method of Small (34).

### Isolation of Skin Secretion

Discharge of secretory granules from the granular skin glands of *Xenopus laevis* was stimulated either by adrenaline injection (100 µg/kg) into the dorsal lymph sac (10) or by mild electric shock (15 V). After this initial stimulation frogs were killed at weekly intervals with MS 222 (Sandoz), and samples of dorsal skin were then processed for light or electron microscopic studies. Alternatively, frogs were again stimulated 1, 2, 3, 4, or 8 wk after initial treatment and their secretion collected in 0.9% NaCl for enzymatic studies.

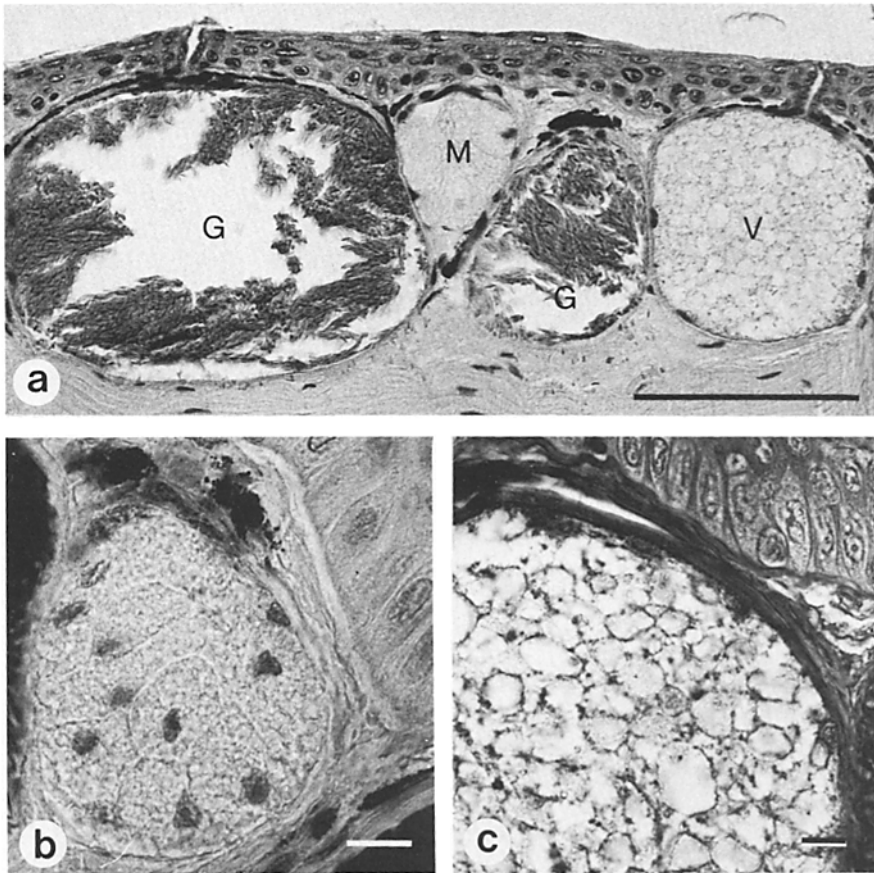
### Protein-Chemical Procedures

Storage granules were isolated from the secretion by centrifugation at 6,000 rpm for 30 min. The granules were then lysed by adding 0.05 M ammonium acetate, pH 7.0. Subsequent precipitation of proteins between 65 and 90% ammonium sulfate yields a partially purified DAP. Alanyl-prolyl-*p*-nitro-anilide (Ala-Pro-pNA) (Bachem, Bubendorf, Switzerland) was used as substrate and the liberation of free pNA measured at 410 nm. Proteins were separated on 10% SDS PAGE (23).

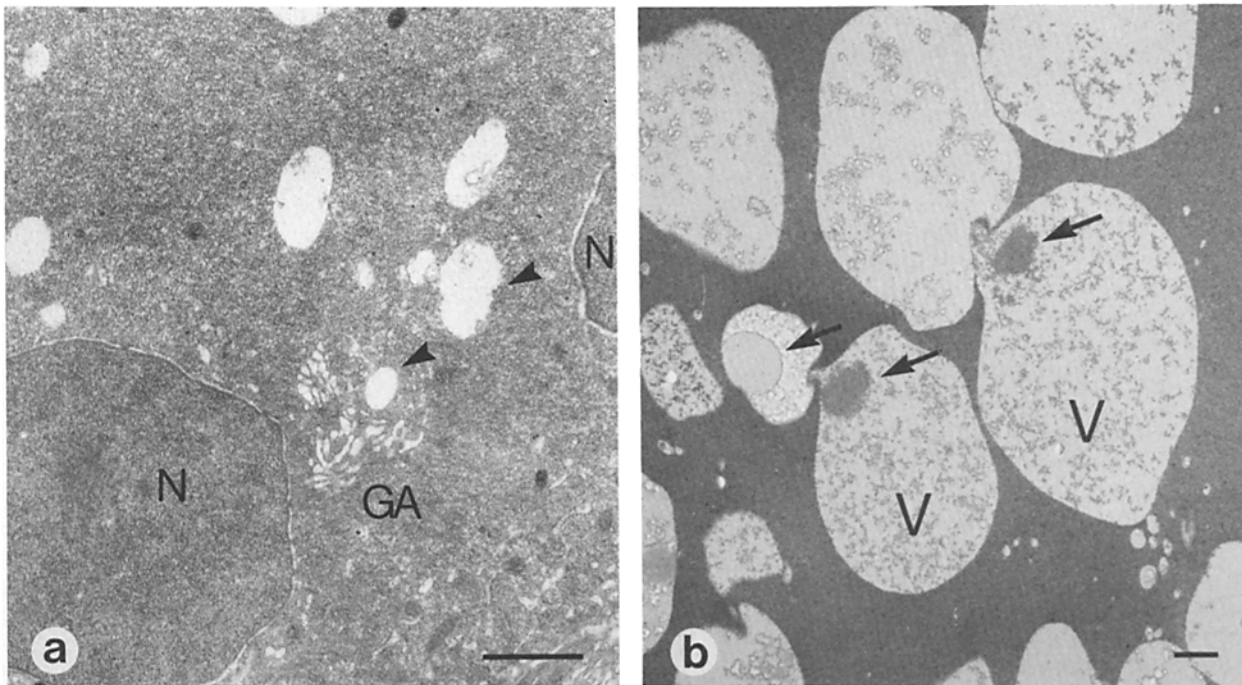
### PAP Method

Tissue sections (6 µm) which were mounted on glass slides were submerged in xylene to remove paraffin, rehydrated, and washed in PBS (pH 7.5) and then treated with pepsin (4 mg/ml 0.2 N HCl) for 20 min at 37°C (30). The proteolytic reaction was stopped by washing thoroughly in ice-cold PBS. Before the primary antiserum, 5% normal goat serum was applied to the sections. The sections were then incubated with anti-CL or anti-PYL<sup>a</sup>-V/2 for 40–44 h at 4°C in a humid chamber. After washing the samples with PBS they were exposed to goat anti-rabbit gamma globulin (Behring GmbH, Frankfurt, FRG) at a dilution of 1:50, washed, and incubated in PAP complex (DAKO, Copenhagen, Denmark) at a dilution of 1:100 at room temperature and washed again. Subsequently, the sections were transferred to a solution of 75 µg diaminobenzidine and 10 µl H<sub>2</sub>O<sub>2</sub> in 100 ml PBS for

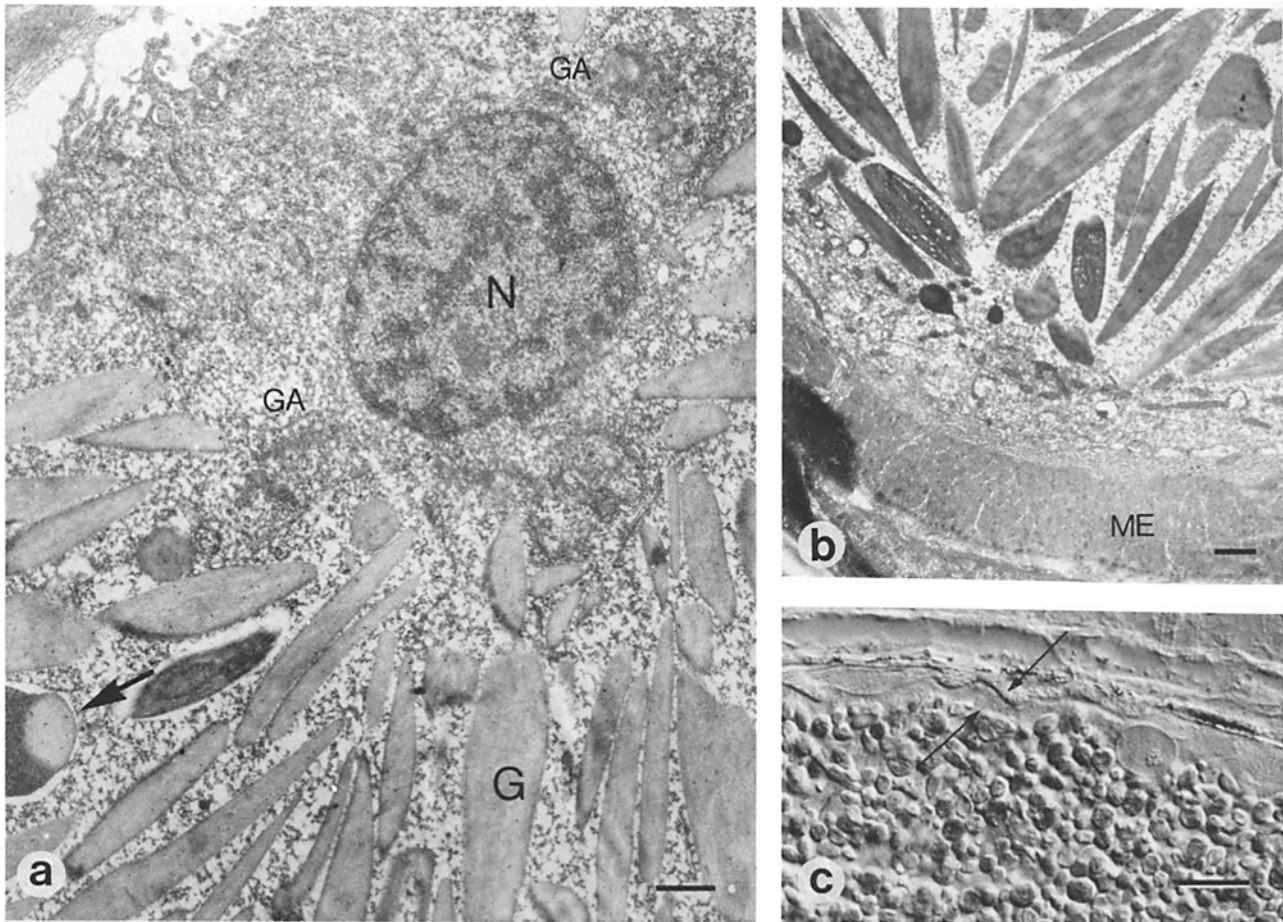
1. Abbreviations used in this paper: Ala-Pro-pNA, alanyl-prolyl-*p*-nitro-anilide; DAP, dipeptidyl aminopeptidase; IGS, immunogold staining; PAP, peroxidase-anti-peroxidase.



**Figure 1.** Light micrographs of the skin of adult *Xenopus laevis*. (a) Overall view of Bouin-fixed and azocarmine-aniline blue stained sections show mature granular glands (G), mucous glands (M), and the vacuolated stage (V) of premature granular glands. The glands lie in the dermis and open towards the surface via ducts. (b) Early stage in granular gland development where the cell membranes of the secretory cells are still intact. (c) Detail of the vacuolated stage. Cell membranes have now disappeared and the nuclei are situated at the periphery of the acinus, however, storage granules are still absent at this stage. (a) Bar, 100 µm. (b) Bar, 10 µm. (c) Bar, 10 µm.



**Figure 2.** Electron micrographs of the vacuolated stage of a premature granular gland. The tissue was fixed in Karnovsky's solution and 1% OsO<sub>4</sub>. (a) Residual cell organelles are confined to areas close to the nucleus (N) at the periphery of the acinus. Near the Golgi apparatus (GA) vesicular structures can be observed (arrowheads). (b) The interior of the gland is filled with vacuolated material. The vacuoles (V) contain droplets and electron-opaque cores (arrows), the sites where immunoreactive material first becomes aggregated during granular gland development. (a) Bar, 1 µm. (b) Bar, 1 µm.



**Figure 3.** (a and b) Electron micrographs of granular skin glands in adult *Xenopus laevis*. The tissue was fixed in Karnovsky's solution and 1% OsO<sub>4</sub>. Cytoplasmic organelles, such as Golgi apparatus (GA) and multicored bodies (arrow), are confined to the area around the nucleus (N) at the periphery of the gland. The interior of the acinus is filled with spheroid storage granules (G). The storage granules in (b) show sites of immunoreactivity after incubation with anti-caerulein antiserum and gold-labeled goat anti-rabbit IgG (gold particle size, 10 nm). The granular gland is surrounded by a myoepithelial cell layer (ME). (c) Light micrograph of freeze-dried tissue. Arrows indicate the narrow cytoplasmic area between the myoepithelial cells and the storage granules. (a) Bar, 1  $\mu$ m. (b) Bar, 1  $\mu$ m. (c) Bar, 10  $\mu$ m.

4 min. Finally, they were rinsed in distilled water and dehydrated before a coverslip was placed on top of the specimen.

### Immunogold Staining Technique

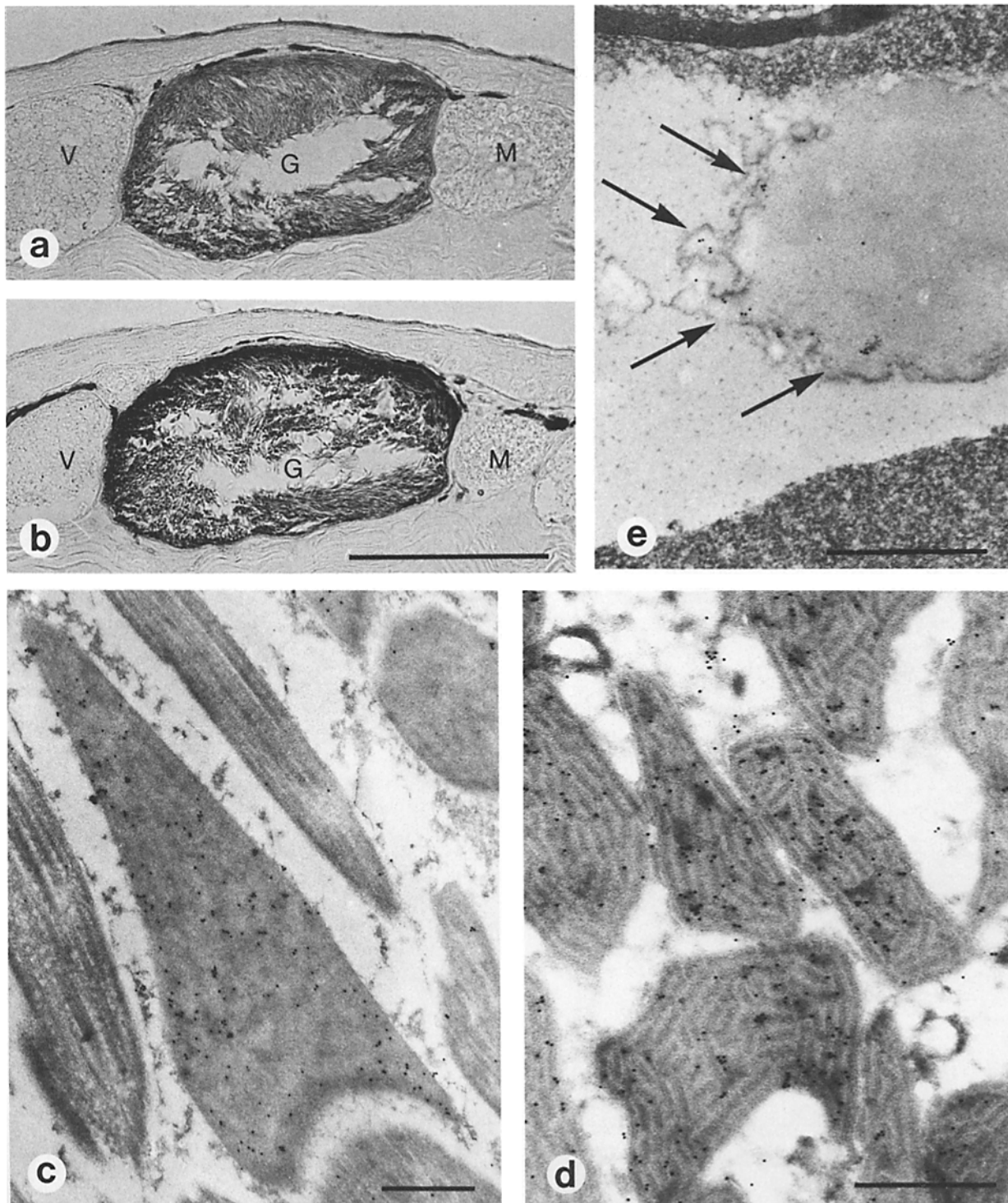
Ultra-thin sections (80 nm) were mounted on formvar-coated nickel or gold grids. For immunolabeling a modified IGS technique according to De Mey (9) was performed. After washing the sections thoroughly, they were etched with 10% H<sub>2</sub>O<sub>2</sub> for 10 min, washed, and incubated in 5% normal goat serum diluted in 20 mM Tris-buffered 0.15 M saline (TBS; pH 8.2, containing 0.1% BSA) for 20 min. The grids were exposed to the primary antiserum for 2 h, washed in TBS for 20 min, and incubated with gold-labeled goat anti-rabbit (GAR G-10 or GAR G 20, EM-Grade; Janssen Pharmaceutica; Beerse, Belgium) at dilutions of 1:10 to 1:20 for 1 h. Subsequently the sections were washed in TBS and PBS and then fixed in 2% glutaraldehyde for 10 min. Incubation of all antisera was performed at room temperature. In the washing steps, the grids were allowed to float on re-distilled water or on buffer in 24-well culture-plates which were placed on a rocking table. Glass beads were put into the wells to obtain better circulation of the fluid. All buffers were filtered under sterile conditions before use.

## Results

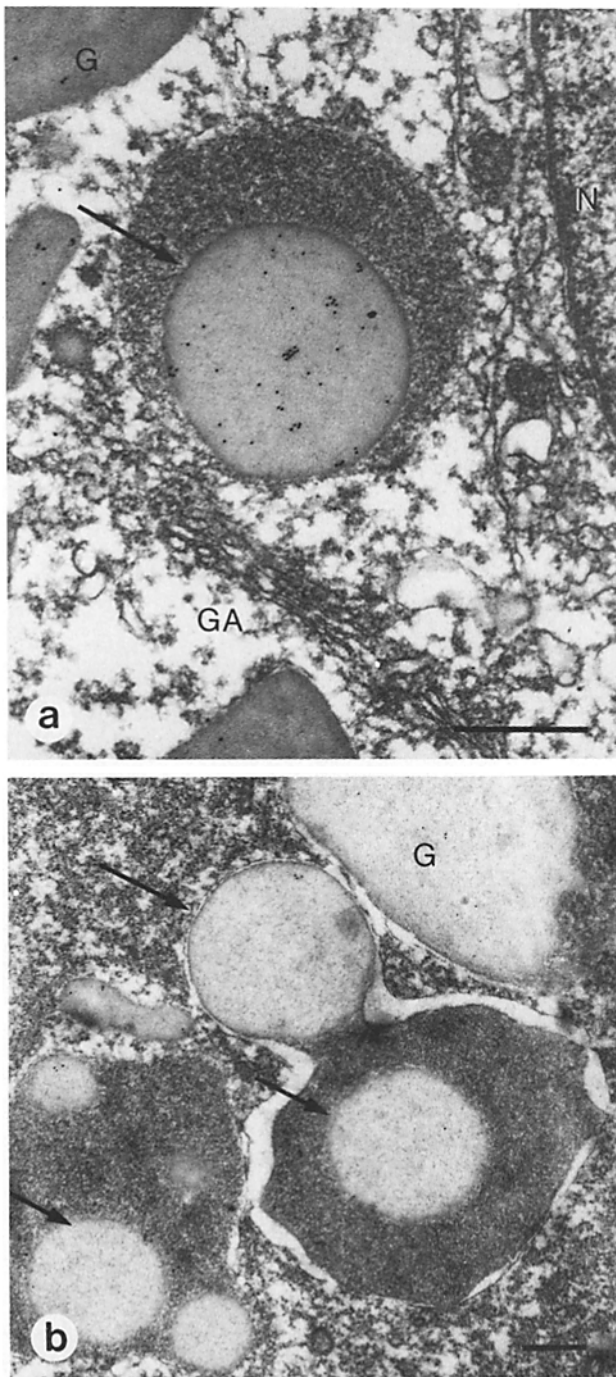
### De Novo Formation of Granular Glands

In the skin of *Xenopus laevis*, one can observe mature mucous glands, granular glands, and various immature glandu-

lar structures (Fig. 1 a). A steady formation of new glands occurs in the skin of adult animals. The secretory compartment of immature granular glands consists of intact cells that fill the whole acinus (Fig. 1 b). When developing granular glands have nearly reached the size of mature glands, a dramatic reorganization occurs. The membranes of the secretory cells begin to disintegrate and the cytoplasmic material forms a uniform syncytium. In light microscopic preparations the content of such glands appears "foamy" (Fig. 1, a and c). Electron microscopy reveals large electron-translucent vacuoles within a homogeneous electron-dense matrix (Fig. 2, a and b). This stage will therefore be referred to here as the "vacuolated stage." Storage granules could not be observed at this stage in either light or electron microscopic preparations. However, some of the vacuoles contain small droplets of dense material and/or electron-opaque cores, which we suggest to be the sites of emerging granules (Figs. 2, a and b and 4 e). Nuclei and other cellular organelles are located at the periphery of the acinus but are absent from the interior of the gland (Fig. 2, a and b). Only some vesicular structures that are often associated with Golgi complexes near the nuclei occur within the matrix. However, for a secretory system of this size conspicuously



**Figure 4.** Caerulein and PYL<sup>a</sup>-immunocytochemistry. (a and b) Colocalization of caerulein- and PYL<sup>a</sup>-immunoreactive material in the granular gland. 6- $\mu$ m sections of Bouin-fixed tissue were treated with anti-caerulein (a) or anti-PYL<sup>a</sup> antisera (b), followed by a routine PAP staining method (for details see Materials and Methods). Caerulein- and PYL<sup>a</sup>-immunoreactive material could be demonstrated within the same granular gland (G) in serial sections, whereas the vacuolated stage (V) and the mucous gland (M) showed no immunoreactivity. (c-e) Electron micrographs of immunolabeled storage granules. Ultrathin sections of 4% paraformaldehyde/0.25% glutaraldehyde (c and d) or Karnovsky-fixed (e) tissue were subsequently incubated with anti-caerulein (c) or anti-PYL<sup>a</sup> antisera (d and e) and gold-labeled goat anti-rabbit IgG (gold particle size, 10 nm). Embedding media: (c) LR-White; (d) polyvinyl-alcohol. (c and d) Both, caerulein- and PYL<sup>a</sup>-immunoreactive material is located within the storage granules. (e) In the vacuolated stage immunoreactive material is localized in the electron-opaque cores within the vacuoles (arrows). Here, the secretory peptides can be demonstrated first during granular gland development. (a and b) Bar, 100  $\mu$ m. (c) Bar, 0.5  $\mu$ m. (d) Bar, 0.5  $\mu$ m. (e) Bar, 0.5  $\mu$ m.



**Figure 5.** Electron micrographs of multicored bodies in mature granular glands. *Xenopus laevis* skin was fixed in Karnovsky's solution (a) or 4% paraformaldehyde/0.25% glutaraldehyde (b) and 1% OsO<sub>4</sub>. Ultrathin sections were then incubated with anti-caerulein (a) or anti-PYL<sup>a</sup> antisera and gold-labeled goat anti-rabbit IgG (gold particle size, 20 nm [a] and 10 nm [b]). (a) The multicored bodies are located near the Golgi apparatus (GA). Immunoreactive material seems to become concentrated in a granular-like dense core (arrow), which finally buds off from the organelle (b); nucleus (N), storage granule (G). (a) Bar, 0.5 μm. (b) Bar, 0.5 μm.

little rough endoplasmic reticulum is found in such glands (Fig. 2, a and b). Furthermore, this rough endoplasmic reticulum is present exclusively in the vicinity of the nuclei.

The secretory compartment of the mature granular gland

is a syncytium with nuclei located at the periphery of the gland and an acinus that is densely packed with granules (Figs. 1 a and 3 c). These are either spheroidal, amorphous, or of rod-like substructure (Fig. 3, a and b). However, our immunocytochemical data do not allow one to distinguish between different types of storage granules (Fig. 4, c and d). In the mature gland, cytoplasmic organelles are also confined to areas around the nuclei (Fig. 4 a). In addition, distinct cytoplasmic structures that we call "multicored bodies" can be observed in the mature gland, where they seem to be proximal to the Golgi apparatus (Fig. 5, a and b).

**Immunocytochemistry.** Caerulein and PYL<sup>a</sup> have been chosen to study the localization and distribution of *X. laevis* skin peptides during gland development with immunocytochemical methods. Specificity tests showed that the antisera anti-CL and anti-PYL<sup>a</sup>-V/2 used for this study recognize their respective antigen and highly homologous peptides but do not cross-react with other *X. laevis* skin peptides. Anti-CL is specific for the carboxy-terminus of caerulein and can therefore not distinguish among caerulein, gastrin-like, or cholecystokinin-like peptides. Anti-PYL<sup>a</sup>-V/2 showed no difference in its reactivity with PYL<sup>a</sup> and the natural derivative PGL<sup>a</sup>. Furthermore, adsorption tests and other controls were performed (see Materials and Methods), which proved that caerulein and PYL<sup>a</sup> immunoreactivity in *X. laevis* granular glands is due to specific antibodies and not to nonspecific reactions. However, the antibody against PYL<sup>a</sup> is not suitable to identify pre-pro-PGL<sup>a</sup> or pro-PGL<sup>a</sup>. Only 5% of such molecules derived from a coupled transcriptional-translational system are recognized by the antibody and only when they are denatured (Zimmermann, R., personal communication).

Immunocytochemistry at the light microscopic level revealed both caerulein and PYL<sup>a</sup> immunoreactivity exclusively in the mature granular glands (Fig. 4, a and b). No specific staining could be observed in premature glands, mucous glands, or any other cells in the skin of *X. laevis*. The IGS technique applied on ultrathin sections confirmed the presence of caerulein- and PYL<sup>a</sup>-immunoreactive material within the storage granules (Fig. 4, c and d). However, the high sensitivity of this method at the electron microscopic level disclosed additional sites of caerulein and PYL<sup>a</sup> immunoreactivity. Apart from secretory granules, immunoreactive material is also contained in multicored bodies (Fig. 5 a).

Furthermore, with the IGS technique it was possible to demonstrate caerulein and PYL<sup>a</sup> immunoreactivity in the vacuolated stage. There it is contained in small droplets and dense cores within the vacuoles where the immunoreactive peptides seem to aggregate (Fig. 4 a). However, even this highly sensitive method failed to detect caerulein- and PYL<sup>a</sup>-immunoreactive material in earlier stages of developing granular glands. This is remarkably different from the finding that during gland regeneration (see below), immunoreactive peptides can be detected in intact secretory cells even at the light microscopic level.

In summary, the data thus show that the earliest point in granular gland development where caerulein and PYL<sup>a</sup> immunoreactivity can be observed is the vacuolated stage. However, such glands lack rough endoplasmic reticulum within the interior of the acinus. This implies that peptide precursors are synthesized at an earlier stage of granular gland development.

## The Regenerating Gland

1 wk after stimulation the myoepithelial layer is still contracted, no storage granules can be observed and the gland appears shrivelled. Cells from the apical region of the gland proliferate and begin to invade the acinus (Fig. 6 *a*). At this stage one cannot detect any anti-caerulein immunoreactivity. After 2 wk of regeneration, cells begin to fuse at several sites within the acinus, thus forming small syncytial units (Fig. 6 *b*). Here, weak anti-caerulein immunoreactivity can be observed (Fig. 6 *b'*). Electron microscopy of such glands reveals that the cells are densely packed with rough endoplasmic reticulum, indicating high biosynthetic activity (Fig. 7 *a*). Furthermore, there are some multicored bodies within the polynucleated secretory compartments. Finally, it is at this time of gland regeneration that the first storage granules are formed. As time proceeds, the secretory cells become organized, so that after 3 wk of regeneration a monolayer of secretory cells can be observed to cover the inner surface of the acinus. Also, the interior of the gland is now densely packed with storage granules (Fig. 6 *c*). Immunoreactive caerulein can be shown to exist both in mature granules as well as within the secretory cells (Fig. 6 *c'*). This is fundamentally different from the situation in developing glands, where no immunoreactivity towards processed peptides is detectable in secretory cells, but only in the vacuolae of the vacuolated stage and in mature granules. Furthermore, at this stage of regeneration, cells not only contain extended rough endoplasmic reticulum but also numerous multicored bodies (Fig. 7 *b*). Storage granules are formed continuously until the gland has reached its original size. This process requires 4–6 wk for completion. From the fourth week onward, the cellular monolayer slowly disintegrates starting at the basal side of the gland (Fig. 6, *c* and *c'*). The mature gland thus formed is morphologically indistinguishable from glands generated by the regular developmental pathway.

Supplementary to the structural studies on regenerating skin glands, we used secretion collected from glands at different times during regeneration to determine the activity of DAP, a putative processing enzyme. Storage granules were collected from the secretion and their protein content was isolated and analyzed on PAGE (Fig. 8, lanes 1–5). Different time courses of production can be observed for several protein bands. DAP then was partially purified from these samples, and comparable aliquots were again analyzed by PAGE (Fig. 8, lanes 6–10). Secretion from frogs that had not been stimulated for more than 8 wk contain a basal level of DAP activity. However, it could be shown by measuring the enzymatic activity with Ala-Pro-pNA as substrate that the enzymatic activity increases significantly during regeneration (Table I). The enzyme is most active 3 wk after initial discharge of granules and levels off again in the fourth week. These data correlate with the observation that massive formation of granules occurs in this regeneration period and that secretory cells contain numerous multicored bodies.

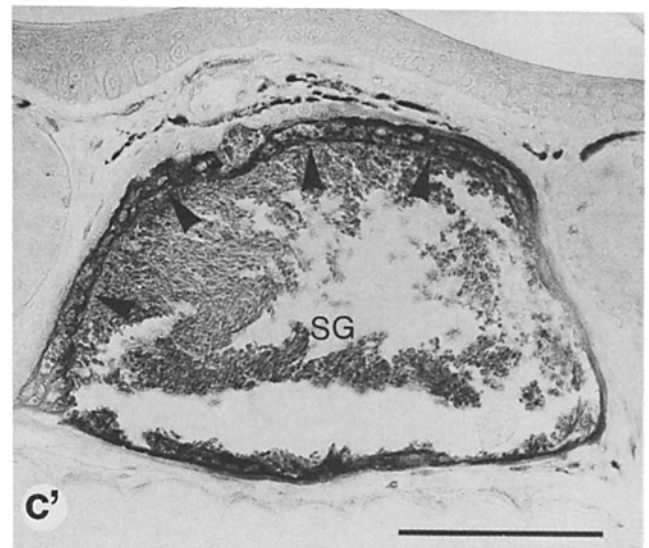
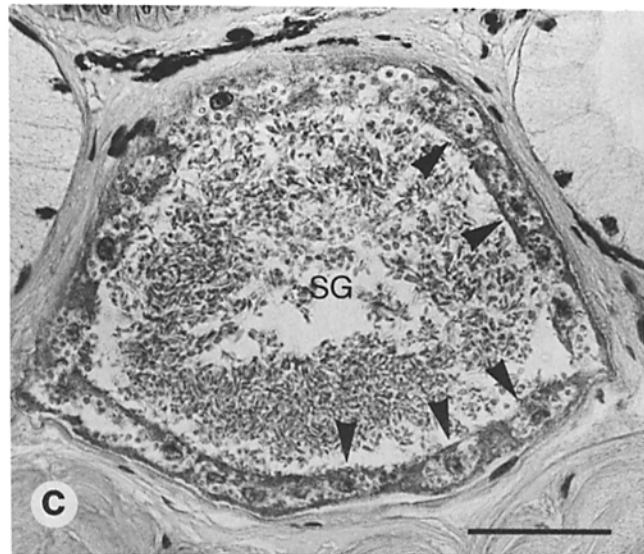
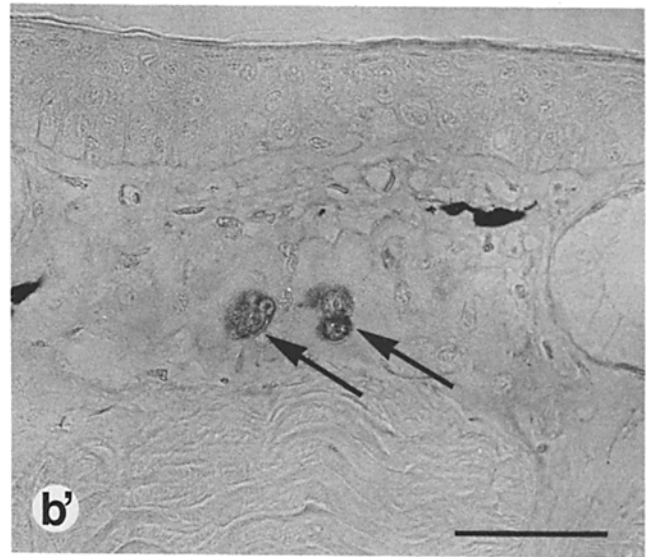
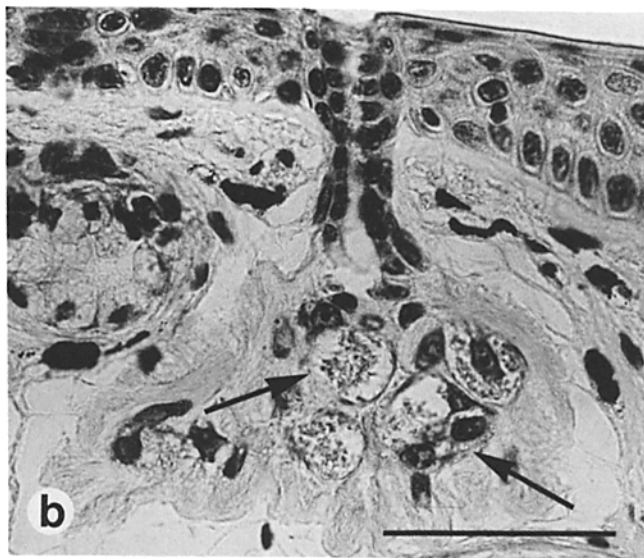
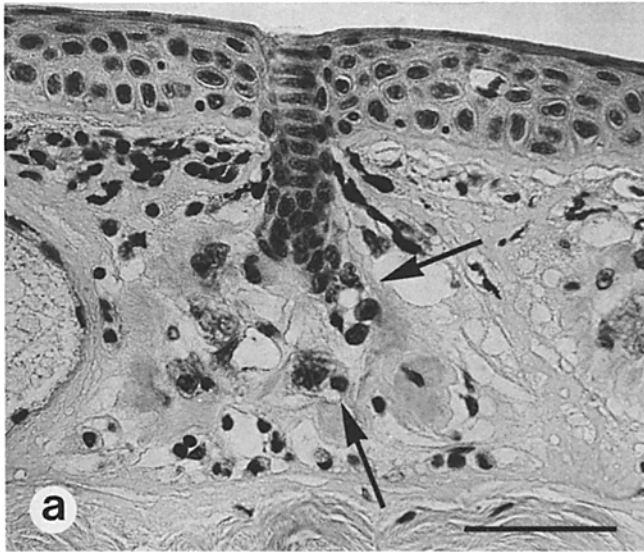
## Discussion

It is well established that the granular skin glands of *Xenopus laevis* expel upon stimulation large quantities of various peptides including caerulein, PGL<sup>a</sup>, and other biologically active peptides (5, 10, 16). In adult animals the development of granular glands proceeds through morphologically distinct

stages (35). Antibodies raised against caerulein and PYL<sup>a</sup>, the immediate biosynthetic precursor of PGL<sup>a</sup>, were used to identify the stage at which these peptides first occur in an immunoreactive form.

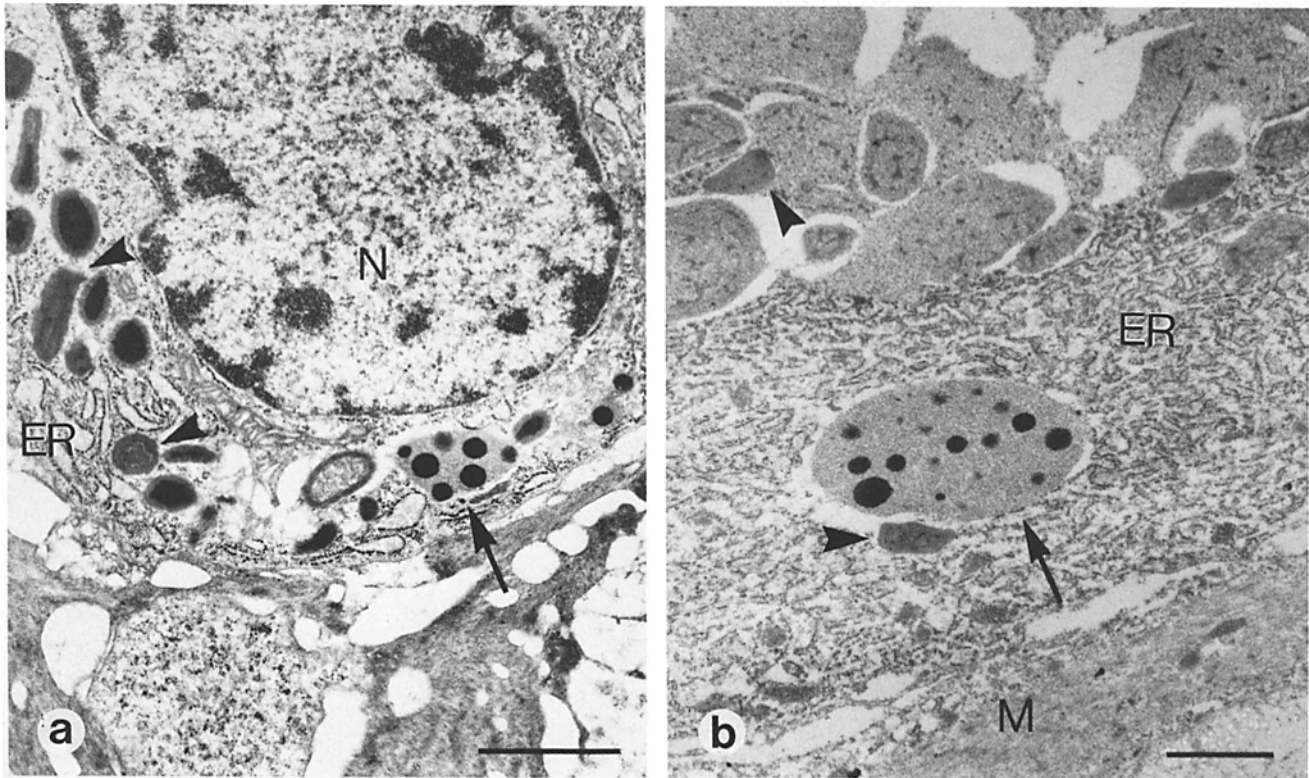
In the case of newly formed glands, both caerulein- and PYL<sup>a</sup>-immunoreactive material could be demonstrated at the light microscopic level only in the storage granules of the mature glands. The uniform distribution of these peptides as well as their coexistence with other constituents of the secretion such as thyrotropin releasing hormone, xenopsin, and serotonin (6; Flucher, B. E., unpublished results) suggest that all peptides are synthesized by the same cells and are stored in the same granules. Higher resolution at the electron microscopic level, however, shows that caerulein- and PYL<sup>a</sup>-immunoreactive material is present not only in the storage granules but also in the multicored bodies of the mature gland. Furthermore, these peptides can be identified within the vacuoles of the vacuolated stage. Thus, it is in this stage of granular gland development that peptides occur first in a processed form. Not even the highly sensitive IGS method has revealed any immunoreactivity in earlier developmental stages of granular glands. However, in the vacuolated stage cell membranes have already disappeared and only little rough endoplasmic reticulum exists in the vicinity of the peripheral nuclei, indicating that protein synthesis has practically ceased. The lack of the cellular machinery that is required for protein biosynthesis thus allows one to infer that the precursors of secretory peptides must have been synthesized at an earlier stage in the granular gland development. So it seems that in the case of skin gland formation in *X. laevis* synthesis and processing of peptide precursors are each confined to particular developmental stages of one secretory compartment. This interpretation is based on the following three facts: (a) extended rough endoplasmic reticulum as would be required for massive synthesis of secretory peptides is not seen in the vacuolated stage of newly forming glands; (b) the immunoreactivity detected in vacuolae must be directed against mature peptides, as the employed antibody cannot recognize propeptides; and (c) our techniques have proven to be sufficiently sensitive to detect immunoreactive peptides within intact secretory cells of regenerating glands. Thus, the absence of immunoreactive labeling in the cells of newly generated glands can be taken as negative proof. However, to positively identify the site of peptide biosynthesis in the developmental stages of granular glands we are currently attempting to establish *in situ* hybridization techniques that allow to localize respective mRNAs.

Our observations also pose an interesting question, namely, how processing enzymes are kept from acting before the vacuolated stage is reached. After all, these enzymes also have to be synthesized in the still intact cells of the premature gland. Two different ways to solve this problem can be considered: One would be that the enzymes and the peptide precursors are compartmentalized in different secretory vesicles which fuse during vacuolae formation. Alternatively, a change in the microenvironment of the secretory compartment, e.g., pH, may cause activation of enzymes or proenzymes. Spannhof (35) actually described a dramatic change in the histochemical reactivity of the secretory material immediately before the vacuolated stage is reached, that is when cell membranes begin to disintegrate. One of the above-mentioned mechanisms, or both, could occur at this point of gland development and initiate the processing of



**Figure 6.** Appearance of the regenerating gland after experimental discharge of storage granules at different times of regeneration. *Xenopus laevis* skin sections (5  $\mu$ m, Bouin-fixed and embedded in paraffin): (a) In glands regenerating for 1 wk, the myoepithelial cells are still contracted. Cells in the interior of the gland proliferate (arrows). No storage granules can be seen at this stage. (b) 2 wk after stimulation,





**Figure 7.** Electron micrographs of secretory compartments of regenerating granular glands: the tissue was fixed in a solution of 4% paraformaldehyde and 0.25% glutaraldehyde and (a) was postfixed in OsO<sub>4</sub>. (a) Syncytial secretory compartment in a 2 wk regenerating granular gland contains extended rough endoplasmic reticulum (ER), multicored bodies (arrows), and some mature storage granules (arrowheads). (b) The secretory epithelium of a gland 3 wk after extrusion of secretory granules is packed with rough endoplasmic reticulum (ER) and contains large multicored bodies (arrows) as well as mature storage granules (arrowheads). Nucleus (N); myoepithelial cells (M). (a) Bar, 1  $\mu$ m. (b) Bar, 1  $\mu$ m.

peptide precursors and granula formation. Processing enzymes have actually been shown to exist in storage granules isolated from *X. laevis* skin secretion (24). However, no information is available on their location or activity in different stages of granular gland development.

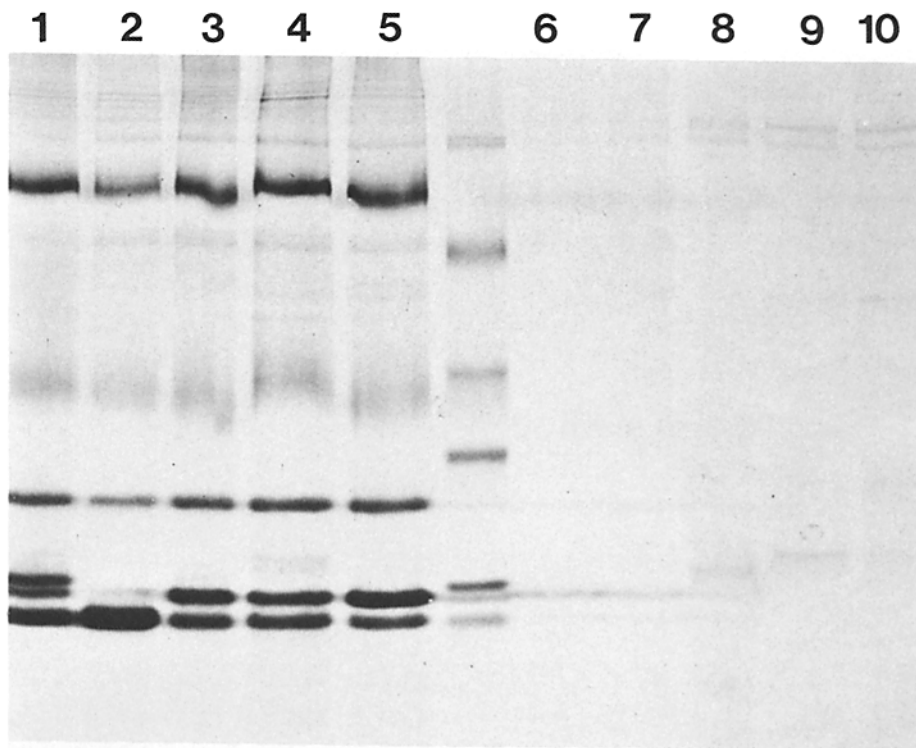
Granula formation in frog skin glands has not yet been described. These structures do not bud off from the membranes of the Golgi apparatus. They seem to be generated in the vacuoles of the vacuolated stage during gland development. Furthermore, granula formation seems to occur concomitantly for all secretory products and to be a spontaneous event since only few glands could be observed in which vacuolae and granulae coexist. Yet, granula formation has not totally ceased in mature granular glands (19). It could be shown that immunoreactive peptides are located in multicored bodies and that mature granules bud off from these organelles. This would indicate that the products of a basal, low-level protein synthesis are processed in the multicored bodies, which thus may be considered to be functionally equivalent to the vacuolae. An observation made on speci-

mens from young animals actually might substantiate such an interpretation. There, the gland increases in size as the animal grows, and considerably more multicored bodies are found in otherwise apparently mature glands. So it may in fact be the pathway via multicored bodies that also provides secretory products during the growth of the gland. Moreover, these organelles are also found in great number within regenerating glands (see below).

Recently, it has been reported that in the B cells of the pancreatic islets, proinsulin maturation occurs not in the Golgi stacks but in *trans* Golgi derived coated vesicles (27, 28). Here again, we show that processing of peptide precursors occurs in structures that are distinct from the Golgi apparatus. The developing granular gland, furthermore, might be unique in that the respective, specialized environments required for synthesis and processing of peptide precursors as well as the storage of the products seem to be fulfilled only when the entire secretory compartment goes through distinct morphological stages.

In contrast, in regenerating glands peptide biosynthesis

the secretory cells begin to fuse and form small syncytial compartments (arrows). (c) After 3 wk, the lumen is filled with mature storage granules (SG) and the gland has formed a cellular secretory epithelium. (b' and c') Immunocytochemistry in regenerating granular skin glands. Sections were incubated with anti-caerulein antibody and processed with a routine PAP staining technique. (b') 2 wk after stimulation immunoreactive caerulein can first be observed; (c') the secretory epithelium within the 3 wk regenerating gland reveals intensive immunoreactivity. (a) Bar, 50  $\mu$ m. (b) Bar, 50  $\mu$ m. (c) Bar, 50  $\mu$ m. (b') Bar, 50  $\mu$ m. (c') Bar, 100  $\mu$ m.



**Figure 8.** SDS PAGE of total protein content and partially purified DAP isolated from granules derived from regenerating glands. Lanes 1-5, proteins in storage granules expelled by glands that were allowed to regenerate for (1) 8, (2) 1, (3) 2, (4) 3, and (5) 4 wk. Lanes 6-10, DAP partially purified from secretory granules isolated from glands aged (6) 8, (7) 1, (8) 2, (9) 3, and (10) 4 wk. The band previously identified as DAP (reference 9) is indicated by a dot.

requires a different structural organization. During regeneration, secretory cells become organized in a monolayer that covers the inner surface of the glandular compartment. These cells contain extended rough endoplasmic reticulum and large multicore bodies. Furthermore, it could be shown by immunocytochemical techniques that the secretory epithelium contains immunoreactive caerulein. Thus, both biosynthesis and processing of peptide precursors seem to occur simultaneously in these cells. This finding is basically different from the development of newly generated glands. Also, as opposed to the situation in the developing gland, granules are formed continuously during regeneration as soon as the secretory cells become biosynthetically active in the second week of regeneration. Finally, the mature gland is again densely packed with these storage granules within a period of 4-6 wk.

Granules from *X. laevis* skin glands are highly unstable structures without membranes that can therefore not be isolated quantitatively. Nevertheless, it could be demonstrated that granules contain biologically active peptides (5, 10) and enzymes that are required to generate those peptides from their respective biosynthetic precursors (26). However, we have noted previously that frogs that have not been stimulated for a considerable time expel very low concentrations of processing enzymes. This indicated that protein synthesis and processing do not occur continuously at high rates and that processing enzymes are likely to be degraded in mature glands. Since we are currently studying such enzymes, it was of practical interest to see at what time after stimulation skin glands would contain high levels of processing activity.

DAPs are known to be involved in the processing of promelittin to melittin in bee venom glands (21) and in the maturation of alpha-factor in yeast (20). The characteristic feature of this type of enzyme is that it recognizes only every

**Table I.** Specific Activity of DAP at Different Times of Regeneration after Initial Discharge of Secretory Granules\*

Regeneration period	Specific activity
wk	$\mu\text{mol}/\text{mg per min}$
1	1.5
2	1.6
3	2.0
4	1.4
8	0.4

\* Specific activity of DAP is expressed in micromoles Ala-Pro-pNA cleaved per milligram protein per minute.

other amino acid from the amino-terminal side of a protein, whereby a particular DAP can cleave exclusively after a few, specific residues (22). The caerulein sequence is contained in a precursor sequence in which it is flanked by pairs of basic amino acids (17). After excision at these sites, the peptide still has an additional amino-terminal fragment. To remove this extra sequence a DAP is suitable and, elsewhere, we have reported the purification of such an enzyme of appropriate specificity from skin secretion of *Xenopus laevis* (25). We now have found that high levels of this enzyme are present in storage granules when the morphological appearance of the regenerating gland suggests high processing activity. These data strengthen the role of DAP as processing enzyme in *X. laevis* and suggest, furthermore, that granular glands must be depleted every 3-4 wk if one wants to isolate processing enzymes.

In summary then, the data show that at least final processing of precursor proteins is blocked in intact secretory cells of developing glands, whereas this process is completed in

the secretory cells of the regenerating skin glands. This observation thus seems to indicate that the granular gland in the skin of *X. laevis* might represent a system in which a particular cell type can be subjected to two distinctly different developmental programs. It remains to be elucidated which factors commit the cell to either pathway.

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