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Localization of xenopsin and xenopsin precursor fragment immunoreactivities in the skin and gastrointestinal tract of *Xenopus laevis*

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Summary. Xenopsin (Xp) and xenopsin precursor fragment (XPF) are bioactive peptides derived from a single precursor molecule; both were isolated previously from extracts of *Xenopus laevis* skin. The present immunohistochemical study was undertaken to determine the specific cellular localization of these two peptides in the skin and also in the gastrointestinal tract of adult *Xenopus*. We report here that Xp-like and XPF-like immunoreactivities co-exist in the granular glands of the skin and specific granular cells in the lower esophagus and stomach. However, only Xp-like immunoreactivity, not XPF-like immunoreactivity, was detected in tall, thin cells of the duodenum and in club-shaped cells of the large intestine. The immunochemical co-localization of the two peptides in specific cells of the skin, lower esophagus and stomach suggests that the same gene is expressed in each of these cells, and that the precursor molecule undergoes similar post-translational processing. In contrast, the observation that certain cells of the duodenum and large intestine display only one peptide immunoreactivity suggests an alternative phenomenon, possibly involving selective peptide accumulation or expression of a different gene.

Key words: Xenopsin – Xenopsin precursor fragment – Immunohistochemistry – Skin – Gastrointestinal tract – *Xenopus laevis* (Anura)

The skin of many species of frogs contains high concentrations of a wide variety of bioactive compounds (for a recent review, see Bevins and Zasloff 1990). Many of these compounds are analogues of mammalian peptide hormones located in the brain, gut and other neuroendocrine cells (Erspamer and Melchiorri 1980; Goedert et al. 1984; Bevins and Zasloff 1990). One such peptide found in high levels in *Xenopus* skin is xenopsin (Xp), pyrGlu – Gly – Lys – Arg – Pro – Trp – Ile – Leu – OH (Araki et al. 1973, 1975). Four of the five amino acids at the carboxyl terminus of this octapeptide are identical to the carboxyterminal amino acids of the mammalian hormone neurotensin (NT) (Araki et al. 1975; Carraway et al. 1982). Moreover, both peptides have similar biological activities in mammals, e.g., causing contraction of strips of rat fundic stomach, as well as inducing endocrine and exocrine pancreatic secretion, hyperglycemia, and cyanosis, and inhibiting gastric acid secretion (Araki et al. 1979; Brown and Miller 1982; Carraway et al. 1982; Feurle et al. 1982; Zinner et al. 1982; Huidobro-Toro and Kullak 1985). In fact Xp was more potent than NT in some assays, such as stimulating vascular permeability (Araki et al. 1979) and inducing motor hypoactivity (Meisenberg and Simmons 1985). On the other hand, Carraway et al. (1982) demonstrated that Xp is not simply the amphibian analog of NT and that both peptides exist in amphibian tissues; this suggests that the two may have different physiological roles. Radioimmunoassay (RIA) revealed that, of the amphibian species tested, immunoreactive Xp (iXp) is detectable in highest concentrations in *Xenopus* tissues; the highest levels were found in skin, followed by pancreas, intestine and stomach, with relatively moderate to low levels in brain (Carraway et al. 1982). However, the cellular localization and a physiological role of Xp in *Xenopus* remain to be clearly defined.

In addition to the peptides with hormonal activity, another family of frog skin peptides, the magainins, exhibit broad-spectrum, potent antibiotic activity. These peptides are similar in size (23–25 kDa), charge (cationic) and amphipathic secondary structure (Gibson et al. 1986; Giovannini et al. 1987; Zasloff 1987; Soravia et al. 1988; Zasloff et al. 1988). The putative precursor peptides have nearly identical signal sequences and most contain a hormone-like molecule in addition to the antimicrobial peptide (Hoffmann et al. 1983; Sures and Crippa 1984; Wakabayashi et al. 1985; Richter et al. 1986; Zasloff 1987; Hunt and Barker 1988; Terry et al. 1988; Bevins and Zasloff 1990). One antibiotic peptide

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in this family is a product of the precursor that contains Xp (Sures and Crippa 1984). This 25 amino-acid antimicrobial peptide has therefore been termed xenopsin precursor fragment (XPF) (Gibson et al. 1986; Soravia et al. 1988). It has been isolated from both *Xenopus* skin (Gibson et al. 1986) and stomach (Moore et al. 1991).

The present immunohistochemical study was undertaken to determine if iXp and iXPF are co-localized in specific cells of the skin and gastrointestinal (GI) tract of *Xenopus*. Evidence of co-localization would support the hypothesis that the same gene is expressed and that the Xp-XPF precursor is processed in a similar way in the skin and GI tract, thereby producing detectable steady-state levels of both peptides. This observation would support the hypothesis that these molecules are serving similar physiological functions in these two anatomical sites. On the other hand, a different cellular distribution of iXp and iXPF may reflect tissue-specific precursor processing, selective peptide accumulation or degradation, or expression of alternative genes, as well as supporting alternative hypotheses as to physiological roles.

Materials and methods

Tissue preparation

Adult *Xenopus laevis* (Nasco Biologicals, Fort Atkinson, Wis.) were killed in 5% urethane (Sigma Chemicals, St. Louis, Mo.) prior to dissection. Samples of dorsal skin, esophagus, stomach, duodenum and large intestine were dissected and fixed in chilled 4% paraformaldehde in 50 mM phosphate-buffered saline, pH 7.0 (PBS), kept overnight at room temperature, and stored from 1–10 days at 4° C. They were dehydrated in a series of graded ethanol, cleared in xylene and embedded in paraffin. Serial longitudinal sections of 4–7 μm were cut on a rotary microtome and mounted on slides coated with poly-L-lysine (MW 420000; Sigma Chemicals). All immunohistochemical reactions were repeated on tissues from at least three different frogs.

Antisera

Dilutions of all antisera were made in 1.5% normal goat serum (Vector Laboratories, Burlingham, Calif.) in PBS. The antibody dilutions were chosen to yield roughly equivalent protein concentrations in each sample (as determined by absorbance at 280 nm in a Gibson 202 spectrophotometer). An IgG fraction was isolated from anti-XPF immune and preimmune sera using recombinant protein A (Beckman Instruments, Sommerset, NJ.) as described (Moore et al. 1992). An IgG fraction of pooled normal rabbit sera (Vector Laboratories) was also isolated. Commercial antisera were used without further purification.

Anti-XPF (aXPF). Polyclonal antibodies were raised in rabbits against synthetic XPF as previously described (Moore et al. 1992). Briefly, synthetic XPF (generous gift from Dr. Michael Zasloff, Children's Hospital of Philadelphia) was covalently coupled to keyhole limpet hemocyanin (KLH; Sigma Chemicals) with carbodiimide and subcutaneously injected into rabbits. Serum was obtained every four weeks. Each sample was assayed by ELISA to determine samples with optimal antibody titers. Serum from one rabbit was used at dilution of $1:10^4$, and that from a second rabbit was used at a dilution of $1:3\times10^4$. The specificity of the antisera has been described elsewhere (Moore et al. 1992).

Anti-xenopsin (aXp). Lyophilized polyclonal antiserum raised in rabbits to synthetic Xp was purchased from Cambridge Research Biochemicals, Inc. (Wilmington, Del.). Anti-xenopsin (aXp) was used at a dilution of $1:4\times10^3$.

Anti-KLH. Affinity purified rabbit polyclonal serum against KLH was purchased from Sigma Chemicals and used at a dilution of $1:5\times10^2$.

Controls

Normal rabbit serum and pre-immune serum were purified with the Beckman rProtein A method and used as control sera in place of primary antibody in the immunohistochemical experiments. Other controls for both iXp and iXPF included the omission of each of the following: primary antibody, secondary antibody (biotinylated goat anti-rabbit), avidin:biotin complex (ABC), or peroxidase substrate. In addition, the intensity of the final DAB signal was analyzed as a function of increasing dilutions of aXPF ($1:10^4$ to $1:4\times10^4$) and aXp ($1:4\times10^3$ to $1:4\times10^4$).

aXPF Controls. Two methods of absorbing the XPF specific antibodies from aXPF were used to generate controls for aXPF:

1. The IgG fraction of aXPF serum was passed over an XPF affinity column (25 mg synthetic XPF coupled to 3 mls Affigel; Bio-Rad Laboratories, Richman, Calif.). The flow-through was collected and re-applied to the column five times. The fifth flow through was collected and used at a dilution of $1:8\times10^4$, containing equivalent protein concentration as the original serum.
2. The XPF-specific antibodies were preabsorbed by incubation of the serum at working dilution with 80 μg/ml of synthetic peptide prior to incubation with the tissue.

To determine the specificity of aXPF, diluted serum ranging from $1:10^4$ to $1:2.5\times10^4$ was incubated with either synthetic magainin, PGLa, caerulein precursor fragment (CPF), or levitide precursor fragment (LPF) at concentrations ranging from 10 μg/ml to 200 μg/ml. Another control included incubation of aXPF (dilution of $1:10^4$) with 400 μg/ml KLH. All peptides were a generous gift from Dr. Michael Zasloff. All incubations were carried out for 1 h at room temperature, or overnight at 4° C. The time and temperature of antigen-antibody incubation did not significantly alter results over this time period.

aXp Controls. Working dilution aXp was pre-absorbed with 4 μg synthetic Xp (Sigma Chemicals) per ml of aXp as described above.

All controls described above were carried out on skin sections; in addition, one or more were used with sections of the gastrointestinal tract.

Immunohistochemistry

The presence of immunoreactive antigens was visualized by the avidin-biotin-peroxidase complex method (ABC) (Hsu et al. 1981), as described previously (Moore et al. 1992). Briefly, tissue sections were deparaffinized in xylene and then rinsed in absolute ethanol. Endogenous peroxidase activity was quenched by incubating the sections in 0.30% H_2O_2/methanol for 5–10 min. The sections were then hydrated to water, non-specific protein binding sites were blocked with a 1.5% normal goat serum in PBS, and sections were incubated with the primary antibody 16–22 h in a humidity chamber at 4° C. The sections were treated by the ABC method from the Vectastain ABC peroxidase kit (Vector Laboratories) ac-

Table 1. General localization of iXp and iXPF in the skin and gastrointestinal tract of *Xenopus*

Peptide	Tissue/cell type				
	Skin[a] Granular glands	Esophagus[a] Granular cells in lower esophagus[b]	Stomach[a] Granular cells[b]	Duodenum[a] Thin columnar cells	Large intestine[a] Club-shaped cells
iXp	+	+	+	+	+
iXPF	+	+	+	−	−

[a] All other cells, not indicated, were negative
[b] Very similar to granular multi-nucleated cells as described by Moore et al. (1992)

cording to manufacturer's protocol. This included two incubations, one with biotinylated goat anti-rabbit serum and the other with the avidin-biotin complex (ABC). Then the sections were incubated for 8 min in a peroxidase substrate solution (1 mg/ml diaminobenzidine-hydrochloride (DAB); Sigma Chemicals) in 50 mM TRIS, pH 7.4, with 0.003% H_2O_2. In order to enhance the DAB signal, the sections were exposed to vapors from 0.1% OsO_4. The sections were then counterstained with light green yellowish (Aldrich Chemicals, Milwaukee, Wis.), dehydrated in a graded ethanol series and cleared with xylene. Coverslips were mounted with Permount (Fisher Scientific, Inc.; Pittsburgh, Pa.).

Black and white micrographs were made with a Zeiss Axiophot inverted microscope and the use of Kodak Technical Pan film.

Results

Comparison of immunohistochemical results on adjacent sections of *Xenopus* tissues (Table 1) indicated that iXp and iXPF are co-localized in the granular glands in the skin and in specific epithelial granule-containing cells in the lower esophagus and stomach. However, tall, slender cells in the duodenal epithelium and club-shaped epithelial cells in the large intestine contain iXp, but not iXPF. All other cells in the skin and gastrointestinal tract were negative for both peptides.

Skin

Immunoreactivities of Xp and XPF are evident in the syncytial lining of the granular glands and in the elliptical storage granules packed in the lumen; neither peptide was detected in the epidermis, mucous glands or connective tissue (Fig. 1a, b). All control experiments (see below) were negative (e.g., Fig. 1c). Authenticity of Xp- and XPF-immunoreactivities is supported by the isolation of both peptides from *Xenopus* skin secretions (Gibson et al. 1986).

Lower esophagus and stomach

The cellular morphology at the junction of the esophagus and stomach includes many mucus-producing and ciliated cells lining the lumen. In addition, this anatomical region has large granule containing cells that often are seen to come in direct contact with the lumen. These cells display iXp and iXPF colocalization (Fig. 2a, b). No immunoreactivity was detected in the upper esophagus (data not shown), in other cells at the esophageal-stomach junction, or in control sections.

The body and distal regions of the stomach contain immunoreactive granular cells at the periphery of the mucous neck cells of the gastric glands (Fig. 3a, b, c). They are especially numerous in the body of the stomach and in a few sections appear to empty into the gastric pits. A cell with a very similar morphology was recently designated as a granular multi-nucleated cell (GMC), and its striking similarities, at the electron-microscopic level, to the syncytial cutaneous granular glands has been described (Moore et al. 1992). In cross sections of the gastric glands, such cells are crescent-shaped. iXp and iXPF typically are co-localized in these cells (Fig. 3a, b, c). Neither iXp or iXPF was found in any other cells of the stomach, or in any of the control sections. Authenticity of the XPF-immunoreactivity is supported by the isolation of this peptide from *Xenopus* stomach extracts (Moore et al. 1991).

Intestine

The intestinal epithelium contains two types of cells which contain iXp – one type located in the small intestine and the other in the large intestine. A thin columnar cell, extending from the basement membrane to the duodenal lumen, contains only iXp (Fig. 4a, b), not iXPF (Fig. 4c). In addition, in the large intestine, a club-shaped columnar cell with a thin neck extending to the intestinal lumen, stains positively for iXp (Fig. 5a, b) but not iXPF (Fig. 5c). No immunoreactivity was detected in any other intestinal cells, or in any of the control sections.

Controls

The following types of control experiments were completely negative: 1) preabsorbtion with the peptide to which the antiserum was raised (80 µg/ml XPF, 4 µg/ml Xp); 2) replacement of the primary antiserum with nor-

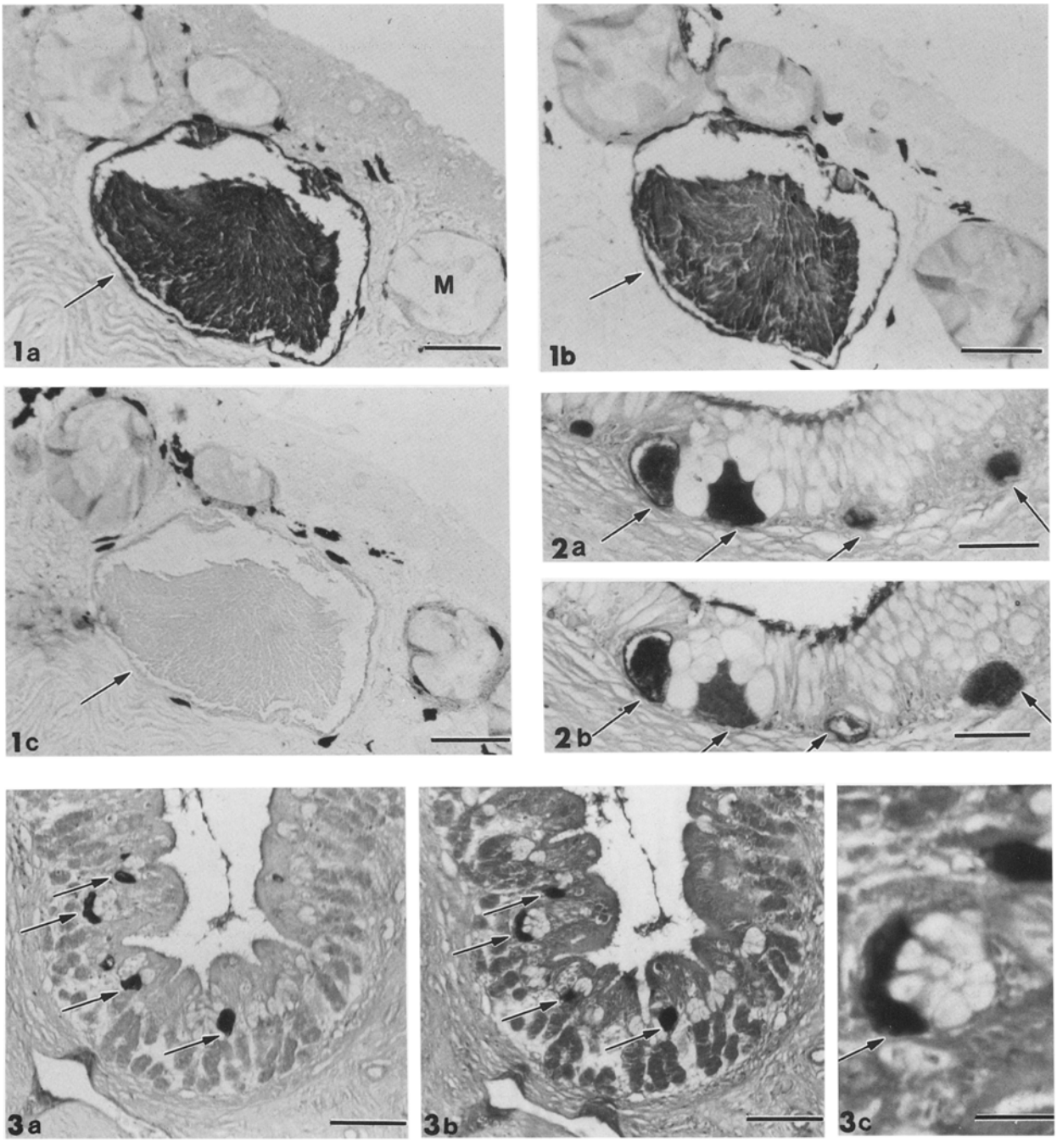
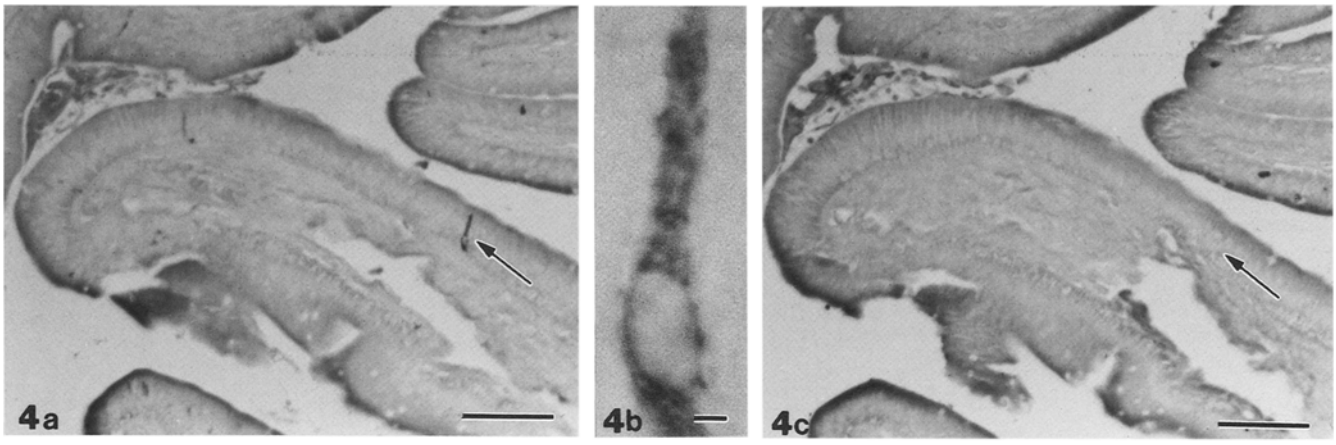


Fig. 1. Serial sections of dorsal skin from adult *Xenopus laevis*, demonstrating colocalization of iXp (**a**) and iXPF (**b**) in granular glands (*arrows*). (**c**) Control: Xp antiserum preabsorbed with Xp. *Bars:* 80 μm (×150)

Fig. 2. Serial sections of lower esophagus demonstrating colocalization of iXp (**a**) and iXPF (**b**) in specific cells (*arrows*). *Bars:* 80 μm (×150)

Fig. 3. Serial sections of stomach demonstrating colocalization of iXp (**a**) and iXPF (**b, c**) in specific cells (*arrows*) in necks of gastric glands. (**c**) Higher-power view of iXPF-positive crescent-shaped cell shown in **b** (*arrow*). *Bars* in (**a**) and (**b**): 80 μm (×150); *bar* in (**c**): 20 μm (×600)

mal rabbit serum or pre-immune serum; 3) omission of primary or secondary antiserum, or omission of the avidin-biotin complex, DAB, or peroxide. With high dilutions of primary antisera, the intensity of the signal decreased, and it was undetectable at dilutions over $1:4 \times 10^4$. Specificity of the aXPF serum has been previously documented (Moore et al. 1992). The specification sheet included with the aXp antiserum indicated that no cross reactivity with neurotensin or neuromedin was observed. Furthermore, preabsorbtion of the aXPF serum with other antimicrobial peptides (magainin 1 and 2, PGLa, CPF) at concentrations of 200 μg/ml did not significantly decrease the immunoreactive signal. The one exception was LPF, a peptide with a primary sequence very



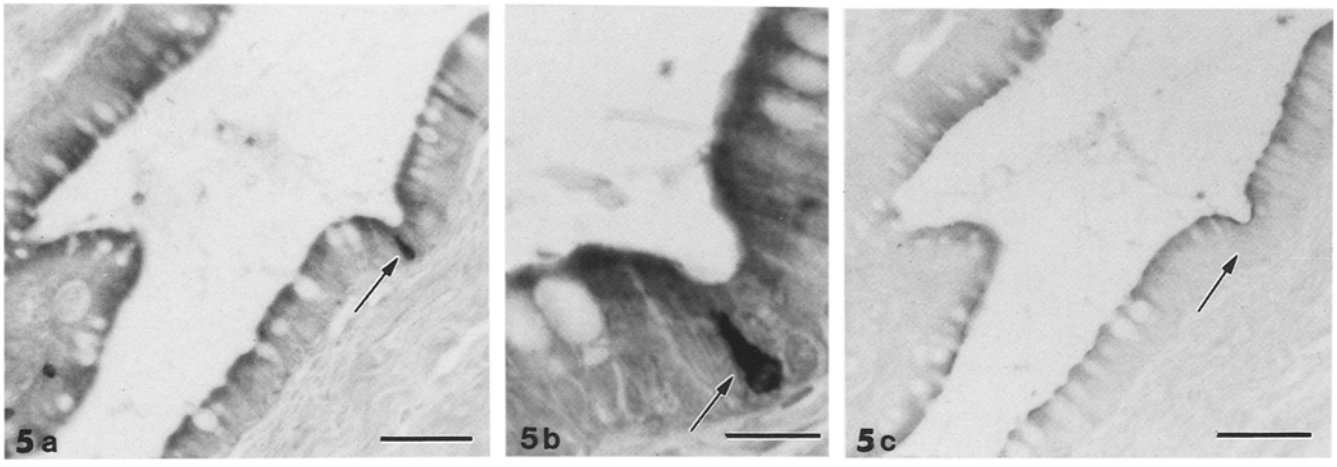


Fig. 4. Serial sections of duodenum demonstrating iXp (**a, b**), but not iXPF (**c**) in tall, thin mucosal cells (*arrows*). (**b**) Higher-power view of iXp positive tall, thin cell in **a**. *Bars* in (**a**) and (**c**): 80 μm (×150); *bar* in (**b**): 2.4 μm (×1700)

Fig. 5. Serial sections of large intestine demonstrating iXp (**a, b**) but not iXPF (**c**), in club-shaped mucosal cells (*arrows*). (**b**) Higher-power view of iXp positive club-shaped cell in **a** (*arrow*). *Bars* in (**a**) and (**c**): 60 μm (×200); *bar* in (**b**): 15 μm (×800)

similar to XPF (Poulter et al. 1988), which as anticipated was successful in absorbing aXPF at concentrations lower than any other peptide in the magainin family (80 μg/ml). KLH, the carrier protein for immunization did not effectively compete for aXPF at a concentration of 400 μg/ml.

Discussion

The present immunohistochemical results are the first to demonstrate the cellular localization of both iXp and iXPF in the skin and representative regions of the gastrointestinal tract of *Xenopus laevis*. Evidence from reactions on serial sections indicated co-localization of these two bioactive peptides in the granular glands of the skin and in specific granular cells in the lower esophagus and stomach. However, iXp, but not iXPF, was also detected in two other specific cell types in the epithelium of the duodenum and large intestine.

Amphibian skin contains a host of bioactive peptides, including Xp and XPF (review: Bevins and Zasloff 1990). The localization of some of these peptides, e.g., caerulein, has been studied (Dockray and Hopkins 1975; Flucher et al. 1986). However, to our knowledge the present study is the first to show the precise localization of both Xp and XPF within the skin. Their co-existence within the granular glands is perhaps not surprising, since they are derived from the same precursor (Sures and Crippa 1984), and since many other hormonal and antimicrobial peptides are present in these glands (Bevins and Zasloff 1990).

Some of the iXP- and iXPF-positive granular cells in sections of the lower esophagus and stomach seem to be in contact with the lumen, which suggests the possibility of release of secretion onto the mucosal surface (Moore et al. 1992). Moreover, the positive cells in the stomach are associated with mucous cells of the gastric glands and appear to be identical with the large, granular multinucleated cells (GMC) described by Moore et al. (1992). Such cells, although smaller, are similar to the syncytial cutaneous glands with respect to their content of granules and multiple peptides, including such antimicrobial peptides as XPF (Moore et al. 1992). Moreover, the crescent shape of the GMC in cross-section is similar to that of amphibian gastric cells reported to contain NT/Xp immunoreactivity (Flucher et al. 1988) and reminiscent of mammalian gastric G cells in which

both Xp and gastrin have been demonstrated (Rix et al. 1986).

Differences in morphology of the gastric cells with both iXp and iXPF and intestinal cells with only iXp suggest that co-existence of the two peptides is cell specific. The tall thin shape of the duodenal cells containing iXp and not iXPF (Fig. 4) is similar to that of *Xenopus* intestinal cells reported earlier to contain NT/Xp-like and gastrin/caerulein-like substances (Flucher et al. 1988).

Possible mechanisms for such cell-specific expression should be considered. The cells which contain both iXp and iXPF likely express the previously cloned xenopsin gene (Kuchler et al. 1989) and undergo similar posttranslational processing. This gene encodes a precursor that contains the Xp molecule at its carboxyl terminus and the XPF molecule in the putative propeptide region (Sures and Crippa 1984). However, in cells which show iXp but no iXPF, a different gene from that previously described may be expressed. On the other hand, the same gene might be expressed in cells containing immunoreactivities for both Xp and XPF and for those with only Xp immunoreactivity. In such a case alternative RNA splicing or perhaps differences in posttranslational precursor processing could explain the selective detection of Xp. Characterization of processing enzymes, as well as molecular biological studies beyond the scope of this investigation will be important in exploring these possibilities.

Although we have localized iXp in *Xenopus* skin and GI tract, and others have shown this peptide to be biologically active in mammals, its functions in *Xenopus* are not established. In many amphibian species granular gland secretions are of great importance in defense and are discharged in time of stress. Certain alkaloids and peptides which they contain are toxic to predators; e.g., in one behavioral study, secretions from *Xenopus* glands were shown to inhibit mouth closure in snakes, thus facilitating escape of the frog (Barthalmus and Zielinski 1988). Electrolyte and water regulation (Grimm-Jørgensen and Voûte 1979), even species recognition for mating or for maintaining territorial boundaries (L. Kaplan, personal communication) are considered among possible functions of various skin peptides. On the other hand, XPF and other members of the magainin families of peptides may provide *Xenopus* with protection from microorganisms in the surrounding water (Zasloff 1987), as well as in the lumen of the GI tract (Moore et al. 1991; Moore et al. 1992). The specific roles of peptides in cutaneous glands and within specific cells in the lining of the GI tract are in need of further clarification.

In conclusion, the immunohistochemical evidence indicates that Xp and XPF co-exist in specific cells of the skin and gastrointestinal tract, but that certain intestinal cells contain only Xp and not XPF. This suggests that regulation of gene expression at the level of transcription or posttranslational processing is cell specific.

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